Oligodendrocytes in the periaqueductal gray matter and the corpus callosum in adult male and female domestic sheep

Agata Wawrzyniak, Krzysztof Balawender, Roman Lalak, Rafał Staszkiewicz, Dariusz Boron, Beniamin Oskar Grabarek

Abstract

There is little information about oligodendrocytes (OLGs) in the Periaqueductal Gray matter (PAG). The literature has not provided data on the number, morphology, or quantification of the expression of the OLG protein yet. Myelin Basic Protein (MBP) in this region of the Central Nervous system (CNS). The study aimed was to perform a comparative analysis: the location and morphology of OLGs, the cellular and regional distribution of iron, and the number of OLGs in PAG and corpus callosum (CC) of adult 16 male and 16 female sheep.

To determine the location of the OLG of PAG and CC, the method of impregnation of the neuroglia with silver salts was applied. In turn, the Nissl method was used to determine the location of the brain structure and to analyse the number of OLG. The performed analysis showed that PAG, OLGs are located singly or in pairs in blood vessels and neurons, while in CC they are arranged in characteristic rows and accompany both nerve fibres and blood vessels. Immunofluorescent staining for the presence of MBP confirmed the location of OLGs in male and female sheep. Morphometric analysis showed the importance of these glial cells in OLG-myelin fibres, correlation in adults regardless of sex even after the creation of the completion of myelin. The results obtained indicate that the functions of OLGs are not only confined to myelination in young individuals, but also play a crucial role in the brain of adults. Our observations seem to be useful to better understanding OLGs biology.

1. Introduction

Glial cells are one of the two components of nervous tissue, the other being neurons. Glial cells constitute most cells in the nervous system. Despite their number and role during development, their active participation in brain physiology and the consequences of their dysfunction on the pathology of the nervous system have been stressed in recent years. The characterization of the main types of glial cells was the result of microscopic studies developed by Ramon y Cajal and Rio Hortega. Ramony Cajal identified the astrocyte and Rio Hortega found two other cell types - the oligodendrocytes (OLGs) and microglia. Glial cells are necessary for correct neuronal development and the functions of mature neurons (Bradl and Lassmann, 2010). More and more evidence suggest that neuroglia play an active role in brain functions. It seems that OLGs have functions other than those related only to myelin formation and maintenance. The number of glial cells increases during evolution; glial cells constitute 90% in the human brain, 65% in rodents, and about 25% of the total cells in Drosophila (Baumann and Pham-Dinh, 2001; Simons and Nave, 2016). OLGs, present in the white and gray matter of the Central Nervous system (CNS), are round or oval cells with poorly branched processes. There are also satellite OLGs that may not be directly connected to the myelin sheath but may serve to regulate the microenvironment around neurons (Bradl and Lassmann, 2010). The OLGs are small, they have a density of both the cytoplasm and nucleus.

* Corresponding author.

E-mail addresses: a17041962@gmail.com (A. Wawrzyniak), krzysztofbalawender2@gmail.com (K. Balawender), roman.lalak@up.lublin.pl (R. Lalak), rafalstaszkiewicz830@gmail.com (R. Staszkiewicz), dariusz@boron.pl (D. Boron), bgabarek7@gmail.com (B.O. Grabarek).

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they lack intermediate filaments and glycogen in the cytoplasm, and they have the presence of many microtubules in their processes (Thomson et al., 2020). Based on their morphological features and size at the light microscopic level, Del Rio-Hortega classified OLs into four categories in relation to the number of their processes. The I-st and II-nd categories have a small body and from 5 to 10 processes. Type III cells have larger bodies and fewer processes, and type IV cells are the largest and have no processes (Baumann and Pham-Dinh, 2001). OLs are located mainly near nerve fibres and capillaries, which accompany neurons and other glial cells. OLs are believed to perform myelinating functions mainly in the CNS. They are also involved in regulating the balance of water and electrolytes in the brain, forming a scaffold for neurons, and are the main iron-accumulating cells in the CNS (Brasl and Lassmann, 2010). At the electron microscopic level, oligodendrocytes have a spectrum of morphological variations involving their cytoplasmic densities and the clumping of nuclear chromatin. Mori and Leblond distinguish three types of OLs: light, medium, and dark. The dark type has the densest cytoplasm. OLs, the myelinating cells of the central nervous system, have specialized morphologies that serve their function (Tanaka et al., 2021; Danu et al., 2021). Qualitative studies suggest that OLs in different brain regions can differ in their morphological characteristics, including the number of branches, internodes, and internode length (Simons and Nave, 2016). OLs such as neurons are highly sensitive to injury by oxidative stress, excitatory amino acids, deprivation of trophic factors, and activation of apoptotic pathways. In addition, oxidative stress and excitotoxic injury contribute to rapid oligodendrocyte death (French et al., 2009). These cells have a complex cytoarchitecture taking the form of a network of microtubules that perform important functions during the transport of myelin proteins. The specificity of this cytoskeleton determines the integrity of OLs and is an important factor for their survival (Richter-Landsberg, 2001). OLs are a very important element of white matter and comprise approximately the foetus and neonate, but their number rapidly decreases as myelination progresses and thus with age (Moore et al., 2020; Salas et al., 2020; Slade et al., 2020). Structural changes in OL involve the loss or degeneration of myelin proteins and those specific to OLs, such as MBP, myelin oligodendrocyte glycoprotein (MOG) and myelin-associated glycoprotein (MAG). Studies of OLs in the CNS have focused mainly on myelinating cells, while little is known about their morphology and functions after myelination is complete. Histological identification of OLs in the white matter tracts using light microscopy is relatively simple due to their characteristic morphology and organization of ‘string of pearls’; however, it is much more challenging to identify oligodendrocytes in Gray matter. Several antibodies to the cellular components of OL have been used in animal models. These include antibodies to myelin proteins, such as MBP, proteolipid protein (PLP) or carbonic anhydrase (2). OLs are rich in microtubules, whose function is to transport essential myelin proteins to their processes. OLs contain the microtubule-associated protein, tau, and rapid alterations in its immunoreactivity provide evidence that OLs are acutely sensitive to damage (Richter-Landsberg, 2001). The corpus callosum (CC) is the main white matter tract in the brain and is involved in interhemispheric communication. The development of CC is not homogeneous through its three main regions: the genu, body, and splenium. This commissure is made up of axons and glial cells, while neuronal somata make up <1 %. After postnatal day 5 glial cells predominate, accounting for 99 % of all cells (Aboitiz and Montiel, 2003). The size, myelination, and density of fibres in callosal subregions are related to the regions of the brain they connect (Güntrü¨kkin et al., 2020; Belcher et al., 2021). Thus, thin unmyelinated fibres are the densest in the genu and splenium (anterior and posterior CC), where they connect association and prefrontal areas, while large diameter myelinated fibres are concentrated in the midbody of the CC, where they connect primary and secondary sensory and motor areas (Aboitiz and Montiel, 2003). Analysis under the electron microscope revealed that the number of callosal axons increases early after birth and is maintained until the adult stage in individuals (Luders et al., 2010). Myelination in the CC starts with non-compacted cytoplasmic myelin sheaths, and each OL sheath has an average of 13 axons. When compact myelin is formed, a single OL sheath, on average, has 15 axons (Simons and Nave, 2016). The CC regions might differ in the density of the glial cells. Interestingly, there are no data on whether the number and density of glial cells increase or remain constant throughout development. The PAG is a well-myelinated region of grey matter in the CNS. This region is involved in emotional and defensive reactions, memorization, urination, vocalization, and feelings of fear and anxiety. The PAG is a midline structure that encircles the mesencephalic aqueduct. Although appearing homogeneous by some neuroanatomical criteria, the authors have suggested the existence of four subdivisions in the PAG with different neuronal diameters and neuronal packing densities (Lavezzi and Melbøø, 2021; Vázquez-León et al., 2021; Eidson and Murphy, 2019). The PAG receives neural pathways and sends nerve fibres myelinated by OLs to numerous areas of the brain. These areas also have differential connectivity’s with the hypothalamic, cortical, limbic, medullary, and spinal afferents and reciprocal efferent connections, as well as populations of interneurons. The PAG integrates behavioural responses to threats and stress stimuli (Benarroch et al., 2010; Averitt et al., 2019). It represents a crucial relay station in the vocalization control system and is integrative. In addition, it is a relay centre of the brain that is important in controlling an animal reaction to threatening and stressful stimuli. It is also one of the main components of the descending pain inhibitory system. Due to its connections to the hypothalamus, it is also involved in sexual behaviour and affects cardiovascular activity. There is little information about OLs in the PAG. The available literature does not provide data on the number, morphology, or quantification of the expression of the OL protein MBP in this region of the CNS. The aim of this study was to perform a comparative analysis: the location and morphology of OLs, the cellular and regional distribution of iron, and the number of OLs in PAG and CC of adult male and female sheep.

2. Material and methods

2.1. Animals

The Animals were 6-month-old had constant access to food, adequate temperature and humidity and water ad libitum. All implementation procedures have been approved by “Guide for the care and use of laboratory animals” approved by the National Research Council and our local law regulations. All efforts were made to minimize the number of animals used and their suffering. The approval was assigned by the 2nd Local Ethical Committee in Lublin No 78/2014.

The study was carried out on sixteen male and sixteen female adult animals.

The animals were kept on an experimental farm in Uhrusk, belonging to the University of Life Sciences in Lublin. Staff were property educated and trained before performing animal killings. Animals were killed intraperitoneally with Euthanandal vet 400 mg/ml, at a dose of 0.35 ml/kg b.w (FATRO S.p.A. - Industria Farmaceutica Veterinaria, Kobierzyce, Poland). These animals were killed in accordance with the requirements laid down in Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. No formal randomisation procedures was used. After dissection of the skulls, the brains were removed and weighed using a digital scale.

2.2. Sectioning and histology

After the animals were sacrificed by cervical decapitation to avoid stress conditions, the brains were removed from the skulls, weighed, and placed in 4 % buffered formaldehyde pH 7.4 (Biosolve Chimie, Dieuze, France). After fixation in, the brain was dehydrated in ethyl alcohol
(Sigma Aldrich, St. Louis, MO, USA) and embedded in paraffin blocks, then exhaustively cut in a randomly chosen sagittal plane on the microtome into a series of 20 μm thick sections. The sections were stained with 0.5 % cresyl violet Nissl staining solution (Merck, Darmstadt, Germany) in distilled water (ratio 1:4). The mounted sections were placed in the staining solution until an adequate intensity of staining was achieved. The sections were differentiated in 96 % alcohol (Sigma Aldrich, St. Louis, MO, USA) until a relatively light background was obtained. Then, they were dehydrated and mounted in DPX (Merck, Darmstadt, Germany).

2.3. Morphological criteria for differentiation between glial cells and neurons

To determine the differences in OLGs and astrocytes, we used the Nissl’s method. This method uses basic dyes to colour the rough endoplasmic reticulum in neurons. The aggregates of the reticulum are called Nissl’s corpuscles.

It can be observed that OLGs have small round or ovoid nuclei with dense, compact chromatin and dark cytoplasm. Typically, OLGs have round and pale stained nuclei with heterochromatin localized mainly in the nuclear cytoplasm and relatively clear. The example of morphological characteristics of OLGs and astrocytes was example on the Fig. 1.

2.4. Stereological analyses

All analyses were performed blind for diagnosis using a stereological workstation, consisting of a modified light microscope Leica DM6000B, motorized sample stage for automatic sampling, electronic microcator, high resolution camera Leica DFC 450C, PC with frame grabber board, morphometric software module LAS 4.7 Montage (Leica Application Suite, Germany) and 20’ monitor. For each case, the sections obtained were analyzed apart to span the length of the PAG and each CC region. Cell counts were performed separately in the PAG and in the CC defined according to the atlas of Franklin and Paxinos (Franklin and Paxinos, 1997). The sections containing areas under study were selected randomly from each group of animals (males and females). For every section, 10 photos of the PAG and the CC were taken. A grid of squares of 2.5 × 10–3 mm2 was imposed on randomly chosen microphotographs. The size of the squares was selected in such a way that the test area was the same for all layers studied in both age groups. To count the cells, squares which entirely covered a particular layer were chosen, but not more than 3 squares per photo in one layer. The OLGs were counted until the results from 100 squares were obtained from every layer and every area of the brain investigated (Kennedy et al., 2020; Schmitz and Hof, 2005). A count of OLGs that came into focus within the unbiased virtual counting spaces (González-Rodríguez et al., 2021) distributed in a systematic random fashion throughout different regions of interest. The estimated cell numbers were calculated from the number of cells counted and the sampling probability. The predicted coefficient of error (CE) of the number of OLGs was evaluated using the prediction methods described by Schmitz and Hof (Schmitz and Hof, 2005). The ratter was blinded for the diagnosis and the PAG and CC measurements were repeated. The density of OLG was estimated as that of OLGs per 1 mm3.

2.5. Impregnation of nervous tissue with silver salts

The paraffin blocks were cut to 20 μm thickness using a Leica RM2255 microtome (Leica Germany). The slides were deparaffinized in three changes of fresh Xylene (Sigma Aldrich, St. Louis, MO, USA), rehydrated in a series of decreasing concentrations of alcohol (Sigma Aldrich, St. Louis, MO, USA) and water (Sigma Aldrich, St. Louis, MO, USA). After hydration, the sections were dried at room temperature. The next sections were placed on slides and transferred to a 5 % water solution of potassium-silver cyanide (Sigma Aldrich, St. Louis, MO, USA) for 24 h at room temperature. The sections were rinsed with distilled water and ammoniacal pyridine solution prepared according to Ogawa et al. (Ogawa et al., 1975). After the precipitate had been filtered, 3 drops of pyridine anhydrous (Sigma Aldrich, St. Louis, MO, USA) were added to the obtained solution. The sections were then reduced to 10 % formalin, rinsed with distilled water, and fixed in 5 % sodium thiosulfate (Sigma Aldrich, St. Louis, MO, USA). In the next stage, the sections were dehydrated by passing them through a range of alcohols and mounted with DPX (Merck, Darmstadt, Germany).

2.6. Detecting the presence of iron ions by means of a histochemical method

Histochemical staining of iron detection was performed by the Perls method with diaminobenzidine intensification (Wawrzyniak-Gacek, 2002). The 20 μm thick sections were deparaffinized in xylene, rehydrated in a series of alcohols, transferred to distilled water, and dried at room temperature. The sections were then transferred to the freshly prepared mixture containing 0.25 N HCl (Sigma Aldrich, St. Louis, MO, USA), 2 % potassium ferricyanide (K3Fe(CN)6) (Sigma Aldrich, St. Louis, MO, USA) and 8 % Triton X-100 (Sigma Aldrich, St. Louis, MO, USA) for 30 min. The sections were then rinsed in 60 mg of 3.3’-diaminobenzidine (DAB; Sigma Aldrich, St. Louis, MO, USA) in 300 ml of 0.01 M Tris-Cl at pH 7.6 (Sigma Aldrich, St. Louis, MO, USA) with the addition of 600 μl of 30 % H2O2 (Sigma Aldrich, St. Louis, MO, USA). The incubation was carried out for three hours in complete darkness at room temperature. After incubation, the sections were rinsed in PBS (Sigma Aldrich, St. Louis, MO, USA) solution for 30 min. The tissue was then dehydrated and mounted on DPX (Merck, Darmstadt, Germany). For the negative control, the endogenous peroxidase activity was determined by processing the representative sections in DAB without treatment with the Perl reaction. All staining procedures were carried out within 24 h after sectioning. Then they were observed and photographed under the Leica DM6000B light microscope equipped with the Leica DFC 450C camera (Leica Germany).

2.7. Single immunofluorescence staining for the myelin basic protein

The fixed tissue was embedded in paraffin after dehydration and 5 μm sections were cut on a microtome (Leica, Germany) and then...
mounted on slides. To investigate the OLGs, immunofluorescence was performed. After rehydration of the sections, antigen retrieval was achieved by microwave using sodium citrate solution at pH 6.0. After incubation with a blocking reagent (1% bovine serum diluted in PBS containing 0.1% Triton X-100; Sigma Aldrich, St. Louis, MO, USA) for 1 h at room temperature, all sections were incubated with mouse anti-\( \beta \)-MBP (Millipore MAB 382, 1:50; (Merck, Darmstadt, Germany) overnight at 4 °C. The PBS rinses served as negative controls. The sections were then incubated with the secondary antibody: Rhodamine Affini-Pure goat anti-rabbit IgG (Jackson Immunoresearch 115-025-003, 1:200; West Grove, PA 19390, USA) for 1 h at 37 °C. Rinsing steps in PBS (Sigma Aldrich, St. Louis, MO, USA) containing 0.1% Triton X-100 (Sigma Aldrich, St. Louis, MO, USA) were applied between each of these steps. The sections were counterstained with DAPI (Sigma Aldrich, St. Louis, MO, USA). Sections were embedded in an in-glycerol gel. Fluorescence signals were visualized using the Leica DM6000B light microscope equipped with the Leica DFC 450C camera (Leica Germany).

2.8. Statistical analysis

The two-tailed Student's \( t \) test was used unpaired. A significant difference was considered when \( p < 0.05 \). Results are expressed as mean ± SEM. The mean coefficient of error (CE) for the estimation of total number of oligodendrocytes in the CC and PAG of male and female sheep was calculated according to Gundersen et al. (1999). Differences between means were analysed using the Student's \( t \) test. A \( p < 0.05 \) was taken as the significance criterion. All statistical analyses were performed using STATISTICA 13 software (StatSoft, Inc., Cracow, Poland).

2.9. Photomicrographic production

The high magnification photomicrographs of the OLGs were taken with a Leica DFC 450C digital camera controlled by the LAS program v4.7 (Leica Germany) on a Leica DM6000B microscope. As some important details were poorly seen on the row images to improve the quality of photomicrographs, the global functions (AutoLevels, AutoContrast, and AutoColor) of Adobe Photoshop CS4, v. 11.0 (Adobe, San Jose, CA, USA), which changes the contrast and gray levels on the whole image. These functions substantially improved the readability of the photomicrographs without changing details.

3. Results

3.1. Brain weight

After dissection of the skulls, the brains were removed and weighed using a digital scale. The males brain weight was statistically significant higher than female (165.33 g ± 12.64 g vs 138.00 g ± 4.19 g; \( p = 0.0023 \); \( t \)-Student's \( t \) test).

3.2. Nissl staining

In single or arranged PAGs, OLGs accompanying neurons, astrocytes, or blood vessels were seen in female and male sheep. In CC, the OLGs formed characteristic rows and accompanied numerous nerve fibers. No morphological differences were observed between males and females (Figs. 2-3 A-B, A-J).

3.3. Impregnation with silver salts

The application of impregnation with silver salt enabled the observation of the distribution and localization of dark-brown stained OLGs. In the PAG area dark brown cellular bodies OLGs with few short processes could be seen considering different kinds of neurons, blood vessels, or astrocytes. Most frequently they were arranged in pairs, a few next to each other, but rarely single. In CC, the OLGs are arranged in series, parallel to each other. Bunches of nerve fibres could be seen between them. In some cases, OLGs accompanied the blood vessels. The slides did not show differences in the morphology or localization of the OLGs in women and men (Figs. 2-3, CD, KL).

3.4. Histochemical detection of iron presence in OLGs

The histochemical reaction, which allows iron-containing compounds to be localized histochemically, is a useful stain for OLGs. In the material we examined, the stained OLGs occur principally within the white matter; few of the oligodendrocytes in the grey matter were stained. OLGs have round to oval cell bodies and processes with smooth and regular outlines. Because of the DAB reaction, the cytoplasm of the stained OLGs is dark brown in color, but within the cell body the nucleus is unstained, and it may be located either centrally or to one side of the perikaryon. In material the cell bodies of the OLGs are round or oval in shape, and a number of thin processes can be seen to radiate from the perikarya. In addition to the staining of the OLGs, there is also some light staining of myelin sheaths in these slides.

In PAG, the iron-positive distribution of OLGs, in females and males, corresponded to their arrangement from the slides impregnated with silver salts. A lot of the stained OLGs lie adjacent to blood vessels, and the remainder either lie next to the cell bodies of neurons or lie free in the neuropil. Morphologically, in PAG the OLGs were similar to those observed in CC. In the white matter there is an obvious concentration of OLGs associated with the thick horizontal band of myelinated fibers. OLGs arranged in series or rows, as well as nerve fibers, revealed the reaction product in CC. No differences between men and women were observed in the location of these cells and the intensity of the histochemical reaction in the OLG and myelin sheaths (Figs. 2-3, EF, MN).

3.5. Immunofluorescence staining

Fluorescent assigned OLGs were characterized by a round green cellular body; short and subtle processes were sometimes visible. Blue cell nuclei were identified as a result of the tinting of the DAPI slides. The immunoreactivity of the brains studied in males and females depended on the number of OLG positive for MBP. In PAG, OLGs exhibited a similar localization pattern, and their intensity and distribution overlapped the localization pattern observed in silver, histochemical, and Nissl staining. In CC in men and women, the reaction product was also visible in nerve tissue that had a myelin sheath (Figs. 2-3, GH, OP).

3.6. Determination of the number of OLGs in the selected areas of the CNS

Table 1 presents the data from the OLG density measurements in the selected brain areas in sheep. The result was given as the number of OLGs per mm³. In the study, the number of OLGs in male and female PAG was similar (183587.02 ± 21781.22 vs 185705.17 ± 23119.64; \( p > 0.05 \)). However, there were statistically significant differences in the number of OLG cells between different areas of the brain (101.146 ± 22450.43 vs 412.5849 ± 89584.66; \( p < 0.05 \); Table 1).

4. Discussion

The essential of OLGs is share in myelination, therefore very few studies of people, monkeys, rodents, as well as dogs included older individuals (Peters and Connor, 2014; Zhou et al., 2020; Slater et al., 2019; Won et al., 2021). PAG is the area of the CNS mainly built up by neurons and blood vessels, nerve fibres, as well as glia cells. Nerve fibres, glia cells, and blood vessels are predominant in CC, whereas neurons are a rare population. Of all types of glial, OLGs are the most numerous cell population in the CNS in both white and gray grain substances (Bauermann and Pham-Dinh, 2001). The OLGs present in the gray matter of the
Fig. 2. OLGs of the doestic sheep PAG. A-B. Nissl staining (scale Bar 20 μm). C-D. Glial tissue impregnation with silver salts. Characteristic arrangement of OLGs next to each other (scale Bar 20 μm). E-F. Histochemical method for detection of iron in OLGs (scale Bar 20 μm). G-H. Immunofluorescence staining for MBP (scale Bar 50 μm). Blue nuclei stained with DAPI. Notations: OLGs ↓ - black; processes of OLGs ↓ - red; (C-D); N - neuron; A - astrocyte, blood vessel ↓ - blue (D-F); description in the text. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Fig. 3. OLGs of the domestic sheep CC.
I-J. Nissl staining (scale Bar 20 μm). K-L. Glial tissue impregnation with silver salts. The traditional arrangement of OLGs in rows (scale Bar 20 μm). M–N. Histochemical method for detection of iron in OLGs (scale Bar 20 μm). O–P. Immunofluorescence staining for MBP (scale Bar 50 μm). Blue nuclei stained with DAPI. Notations: OLGs ↓; processes of OLGs ↑; N - red); blood vessel ↓- blue (N); the nerve fibers ↓- light green (K-M); description in the text. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
The highest iron content. The role of iron in myelin genesis and regeneration of studied animal species is consistent with observations in man, rat, and similar studies in other species as well as data from humans, rats, and mice. Some authors (Jhaveri et al., 1992) and Sturrock (Sturrock, 1991) indicate that a significant difference between the iron content in male and female rats is consistent with observations in man, rat, and other species. Cellular separation of iron-related proteins from the brains of rats and mice was arranged parallel to nerve fiber bunches. OLGs were sometimes observed in the close vicinity of blood vessels or near other glial cells. A similar distribution was shown in the studies on rats by Berger et al. (Berger et al., 1991) and in hamsters by Jhaveri et al. (Jhaveri et al., 1992). Some authors (Jhaveri et al., 1992; Sturrock, 1987) suggest that part of OLGs whose body and processes dye higher on the slides belong to their younger form, which did not start myelination. It is commonly known that the main CC cells are mature forms of OLGs darker dyed on slides, which are responsible for myelin sheath formation (Yshii et al., 2022; Castelli et al., 2019). With aging, the susceptibility to remyelination slows down or is completely lost even though the brain is capable of self-reparation (Nave, 2010). The results of the own investigation and those of the other authors in CC of adult individuals suggest that even after myelination OLGs can react with myelin sheaths (Nave, 2010).

Several observations suggest that iron is an essential factor in myelination and OLG biology. The specific role of iron in these processes is still being elucidated. Its role could be an essential cofactor in the metabolic process or a regular transcriptional or translational (Møller et al., 2019). Cellular separation of iron-related proteins from the brains of studied animal species is consistent with observations in man, rat, and mouse (LeVine and Macklin, 1990; Connor, 2004). The investigations confirmed that OLGs are included in the preservation of the myelin sheath of the synthesis. They are the main cells in the CNS that contain iron (Thomason et al., 2020). In addition to OLGs, iron is also present in nerve fibres due to the fact that in rats, areas such as circumventricular organs, Calleja islands, globus pallidus, substantia nigra, interpeduncular nucleus, as well as dentate nucleus were characterized by the highest iron content. The role of iron in myelin genesis and regeneration of the myelin sheath indicates that iron deficiency in mammals is related to hypomyelination (Leitner and Connor, 2012). Some authors have shown that iron accumulation by OLGs contributes largely to myelination (Piñero and Connor, 2000). According to other authors, iron is present in the body and neuron processes and its distribution in the brain is related to the metabolism of neurotransmitters and coincides with the standard of γ-aminobutyric acid, encephalin, and luteinizing hormones (Hill and Switzer, 1984). In this paper an attempt was made to compare with the regional distribution of iron in the brain of adult female and male sheep. The results obtained confirmed that the bodies and processes of OLGs contained the product of a histochromatic reaction to the presence of iron. The observation of iron was like those made by Fukunaga et al. (Fukunaga et al., 2010) in humans and by Le Vine and Torres (LeVine and Macklin, 1990) in mice. In PAG, iron containing OLGs were visible mainly in neurons and close to blood vessels suggesting that they can participate in iron transport to and from nerve cells and contribute to control of iron passage from plasma to the brain. The iron distribution is different and the observation of PAG and CC is different from that in the human brain (Simpson et al., 2015). Similar studies of the presence of iron were carried out in humans in the areas: cerebral cortex, cerebellum, olfactory bulb, striatum, hippocampus, and amygdala. Iron was proved to be located mainly in the cytoplasm of OLGs (Piñero and Connor, 2000). In the strongly myelinated area, that is, the CC product of the histochromatic reaction was visible in nerve fibres and in both female and male sheep. Similar results were described in other species by Francois et al. (Francois et al., 1981); Gilissen et al. (Gilissen et al., 1998), Oz Schulz et al. (Schulz et al., 2011). In humans, iron-positive cells, particularly in areas rich in myelinated fibres, form characteristic lobes (Piñero and Connor, 2000). The observations made by Hill and Switzer (Hill and Switzer, 1984) prove that OLG rich areas are the cells rich in iron and are accompanied by nerve fibres. The iron-rich areas are involved in motor controls, as confirmed by their involvement in motor impairment, which is the reason for iron deficiency during growth. This, in turn, was proved by studies in humans suffering from Parkinson’s disease (PD) (Visanji et al., 2013). OLG containing ions colour intensively on slides compared to other cells of the normal adult brain (Connor, 2004; Piñero and Connor, 2000). The most common neurological symptoms of iron deficiency are poor learning results, diminished cognitive ability, and educational problems (Grantham-McGregor and Ani, 2001). Most of the above symptoms in children with iron deficiency can be due to hypomyelination, and neurological consequences are observed even after iron supplementation (Lynch et al., 2018;148(suppl_1):1001S–1067S.). Understanding the role of iron in myelination processes and iron-OLG interactions is essential to get to know, reason and mechanisms of numerous neurodegenerative disease action (Connor, 2004; Piñero and Connor, 2000). In early embryonic development, iron is present in OLGs located just next to blood vessels. Then, with CNS development, OLGs are located a bit further from blood vessels, still in close contact with them (Ma et al., 2021; Rahimian et al., 2021). It was suggested that there should be close relations between OLGs containing iron and nerve fibres, as well as accumulation of iron and myelin production. The acquisition of iron by OLGs is probably associated with their energetic metabolism. Iron accumulation can be due to oxidative stress that affects neurodegeneration and myelin decomposition (Piñero and Connor, 2000). This can be found in diseases such as Alzheimer’s disease (AD), Huntington’s disease (HD), or Parkinson’s disease (PD) (Piñero and Connor, 2000; Quintana Rodríguez et al., 2008; Bartzokis et al., 2007). It is not yet known whether iron deficiency or the amount of OLGs affects iron transport to the brain barrier (BBB) by TF (Fisher et al., 2007). TF is present in the brain mainly in OLG and plays a key role in the transport of iron to different cells. Iron transport to the brain is believed to proceed through the blood–brain barrier (BBB) by TF (Fisher et al., 2007).
role during their creation (Connor, 2004). It was shown that the expression of OLGs of receptor TF decreases with aging (Hulet et al., 2002). Furthermore, TF injected into rats was found to increase myelin deposition in the optic nerve and CC (Marta et al., 2003). Disturbances in myelin density may be due to the irregular composition of myelin and, first, to the structure of its basic proteins, i.e.: MBP and the proteolipid protein PLP (Marta et al., 2003). TF is a protein that accumulates iron and is treated as a cytoplasmic iron buffer due to the ability to mask excess iron and prevent free radical toxicity. It is present in OLGS and can bind around a third of the total iron amount in the brain (Visanji et al., 2013). TF consists of two chains: heavy (H) and light (L) (Ringer et al., 2022). Neurons have mainly H-Ft, microglia L-Ft, and OLGS, both L and H-Ft, and the decomposition in these cells is consistent with their function and iron distribution pattern (Visanji et al., 2013). The presence of the receptor Ft was found in bonds of various types of cells, e.g., in human, rat, and pig hepatocytes as well as in guinea pig reticulocytes (Adams et al., 1988; Adams et al., 1988). Hulet et al. (Hulet et al., 2002) studies in humans and rodents showed that the presence of the H-Ft receptor H-Ft in white matter was unique for OLGS. H-Ft can play an essential role as a protein that provides iron to the brain, which is specific for OLG. BBbis one of the potential sources of direct transport (Zhang et al., 2014), and the other can be microglia as it begins to accumulate iron in the white matter even before myelination starts (Zhang et al., 2014). Some researchers suggest that microglia contribute to iron inflow during myelination, supporting OLGS (Todorich et al., 2009). In the own studies, no morphological differences in PAG and CC were observed in male and female sheep.

In this study, the number of OLGS in PAG and CC was estimated using morphometric methods. Cells were counted when the first visible picture of the nucleus was sharp in the optical disector (Raji and Potter, 2021). In this study, the number of OLGS in PAG in females and males was similar. However, in CC, females possessed a larger number of OLGS compared to males. Statistical analysis did not show significant differences between the CNS area studied and between men and women assuming significance p < 0.05. The myelin sheath of the CNS is formed by membranes that extend from the OLGS and wrap concentrically around the nerve fibers, thus insulating them and facilitating the rapid transmission of nerve impulses (Baumann and Pham-Dinh, 2001; Arroyo and Scherer, 2000). As follows from recent investigations, in addition to myelination, OLGS play a key role in maintaining nerve fiber integrity (Saab et al., 2013). In mice with PLP or 2‘3‘-cyclic nucleotide-3‘-phosphohydrolase (CNP) deficiency, no irregularities were observed in the structure of the same myelin. However, disturbances in axonal transport were found, and the destruction of OLGS caused an anomaly in the morphology of nerve fibers (Edgar et al., 2010). Studying the density of axons, some authors found that the presence of myelin or OLGN scan contributes to changes and regulation of nerve fiber energy (Traka et al., 20102010). In addition to the metabolic support of nerve fibers, OLGS can regulate the survival time of neurons through the production of neurotrophic factors. Investigations showed that OLGS possess brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and neurotrophin-3 (NT-3) (Zhang et al., 2014). It was also shown that BDNF and NT-3 in vitro in the forebrain evolved by OLGN enhance the basic functions of neurons by increasing the expression of choline acetyltransferase (ChAT) (Dai et al., 2003). OLGS can also contribute to the formation of glial cell-derived neurotrophic factor (GDNF) and insulin-like growth factor (IIF1), which increase the survival rate of collagen cells (Zhang et al., 2014). In his studies, Sturrock (Sturrock, 1987) found that the number of OLGS in the CC area in mice increases between 22 and 25 months after birth, whereas the number of increases in the number of OLGS in the optic nerve of old monkeys Rhesus compared to young individuals (De Luca et al., 2020). The drop in the number of OLGS with age was observed in strongly myelinated areas in rats (Chen et al., 2011). Studying CC in mice, Sturrock (Sturrock, 1987) stated that the number of OLGS and myelinated fibers decreases between 9 and 12 months at birth, then increases between 22 and 25 months after birth. Qualitative and qualitative research in old rats revealed a differentiated domination of OLGS depending on the CNS area and the age of the rats. OLG dominance was observed from the first month of life in CC, from the third month in the cerebral cortex, and between the second and fifteenth month in the cerebellum. The electron microscope, studies of three types of OLG in the CC area in old rats showed that dark cytoplasm were the dominant forms, but bright cytoplasm were the smallest. Increased number of glial cells may be associated with the possibility of metabolic support for neurons and nerve fibers (Sherwood et al., 2006). Thus, the animals of large volume of neurons also require a larger number of neuroglia cells. An increased number of glia cells may reflect their role in the process of neuronal development, and therefore the function performed by neurons (Mortensen et al., 2014). The number of glia cells may depend on the living conditions of a given animal. The participation of OLGS in the myelination process and the maintenance of myelin sheaths is very complicated. OLGS are formed throughout individual life and contribute mainly to the formation of myelin reconstruction. Myelination is a complex structure and can play a significant role in the learning process and memorization. Disturbances in myelin sheath formation are closely correlated with serious diseases of the nervous system. However, the role of OLGS in different CNS diseases has not been fully studied. Current knowledge about the function of OLGS focuses mainly on myelination processes in young people, but little is known about the role they play in the complicated network of combinations between neurons and when myelination is over. Morphological and morphometric studies of OLGS in different areas of the brain in the CNS are essential in demyelination diseases. Morphological changes in OLGS and myelin can play a significant role in cognitive function disorders that can be associated with the degeneration of specific OLG proteins and myelin. Better knowledge and understanding of the biology of glia cells can be a key element in developing new therapeutic strategies for numerous neurodegenerative diseases.

5. Conclusions

In conclusion, using a powerful morphometric approach, it was possible for the first time to estimate the number of OLG in PAG and CC in male and female sheep. Research results indicate that the localization of OLGS in nerve fibers, blood vessels, and neurons indicates that they are involved in metabolic changes of accompanying neurons to the same extent, regardless of the gender and age of the individuals. These results can be used as a morphological basis for understanding OLGS biology.

6. Ethics approval and consent to participate

The approval was assigned by the 2nd Local Ethical Committee in Lublin No 78/2014.

These animals were killed in accordance with the requirements laid down in Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

7. Consent for publication

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CRediT authorship contribution statement

Agata Wawrzyńiak: Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing. Krzysztof Bala: Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing. Roman Lalak: Writing – review &
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