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Cytoarchitectural Characterization of the Medial Geniculated Body of Young Wistar Rats (Rattus Norvegicus) Through Immunohistochemistry for S100B

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Medial Geniculate Body;
Protein S100B.

Abstract

Several molecular, cytoarchitectural and neurochemical works study the Medial Geniculate Body (MGB), a thalamus structure related to the auditory pathway. This region is composed of several nuclei of neurons, mainly of retransmission to the auditory cortex, in addition to astrocytes and axon fibers. In order to make MGB's histophysiological knowledge solid, several astrocyte markers are commonly used, such as GFAP, S100B and metabolic enzyme aldehyde dehydrogenase 1 (Al-

dh1L1). We studied the pattern of distribution and immunoreactivity for S100B in the MGB of young Wistar rats and we were able not only to delimit and characterize the topography as a whole of this area, but also to obtain the subdivision of some sub-regions. In this sense, we found that immunohistochemistry against S100, classically, can be used to characterize the MGB, as our results indicated that it is a reliable marker for cytoarchitectonic studies in this area in young rats.

Introduction

The thalamus makes up the diencephalon and is macroscopically constituted as two ovoid masses, interconnected by intertalamic adhesion, located in the most central portion of the skull, between the brain and the brainstem (Duré et al. 2009). Morphologically, the thalamus has an anterior and posterior extremity, called the anterior and pulvinar tubercles, respectively. It has four regions: medial, lateral, upper and lower. The thalamus nuclei are divided into anterior, medial, median and lateral (Machado, 2013). The thalamus also has several nonspecific relay nuclei correlating, for example, with the Ascending Reticular Activation System (SARA) and specific relay nuclei of sensorimotor information to the cortex (Moreira, 2017). Thus, the thalamus is a vital, highly complex region involved in numerous functions and, when affected, causes a range of diseases, such as thalamic infarctions (Duré et al. 2009).

The microscopic anatomy of the Medial Geniculate Body (MGB), which is a postero-inferior structure of the thalamus, has four subdivisions: dorsal zone (MGD), medial zone (MGM), ventral zone (MGV) and marginal zone (MGMZ) (Vertes et al. 2006). The CGM is constituted as a center with several nuclei of neurons, whose basic function is the transmission of auditory impulses. It receives afferent and ascending fibers from the lateral lemniscus and the inferior collicullus (IC) and descending projections from the Primary Auditory Cortex and Reticular Thalamic Nucleus (RTN) (Caspary and Llano, 2017) **(figure. 1)**.

S100 proteins are a group of small acidic calcium-binding proteins. It consists of two subunits

A and B (Al-Ayadhi and Mostafa, 2012). The protein S100 beta (S100B) is one of the most abundant members of this family of proteins, found in the cytoplasm of astrocytes and that act in several biochemical processes, whether intracellular, or extracellular (SHAPIRO et al, 2010 and CHEONG et al, 2014).

Astrocytes, as well as oligodendrocytes and microglia, are part of the family of glial cells in the CNS, and play essential roles for the functioning of the whole brain (Boron and Boulpaep, 2015).

Astrocytes are extremely complex cells that act by giving mechanical support to neurons (Purves et al., 2010), in the buffering of calcium (Nortley and Attwell, 2017) and potassium ions (Bellot-Saez et al., 2017), processing and storage of energetic substrate for neurons, as well as the maintenance of adequate PH (Deitmer et al., 2019), release of antioxidant molecules (Fernandez--Fernandez et al. 2012), modulation of synaptic plasticity (De Pittà et al. 2016) and neurogenesis (Duan et al. 2015).

The concentration of the S100B protein rises in the serum under conditions of tissue hypoxia resulting from traumatic brain injury, ischemia or vascular injury to the CNS (Gattaz, 2000). In addition, their serum levels are often elevated in the elderly and newborns (Costa et al. 2013). High levels of the S100B protein have already been identified in serum or CSF in numerous perinatal inflammatory conditions, in Multiple Sclerosis (MS), in Subarachnoid Hemorrhage (SH), in Rheumatoid Arthritis (RA) as well as in thalamus pathologies (Stochero et al. 2010; Katsumasa, 2012; Barateiro , 2016, Baptista et al., 2017 and Santos G, 2018). At the cellular level, it is neurotrophic, gliotrophic and mitogenic, thus promoting the maintenance of CNS homeostasis (Gattaz, 2000).

The biological importance of this protein correlates with its concentration, given that low or physiological levels are characterized by neuroprotection (Chong, 2016). Then, in the presence of pathological conditions, such as those mentioned above, senescence or cell stress, for example, in physical activity, the release of this protein in serum or CSF can occur in significant amounts to be identified by immunohistochemical methods (Stochero et al, 2010).

Thus, it is a potential multifactorial marker used with a diverse range of functions and tissues (Sedhagat, 2008).

This study aimed to characterize the cytoarchitecture of the Medial Geniculate Body by means of immunohistochemistry against S100B. The specific objective was to delimit the location of the MGB, describe the subcomponents of the area of interest and compare them with the rat brain atlas (Watson and Paxinos, 2007).

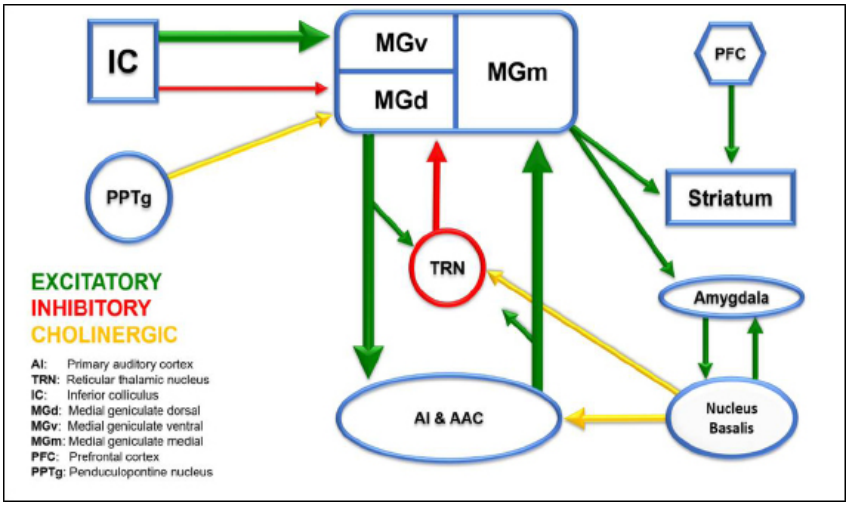

Figure 1. Schematic drawing of the MGB and some sub-regions showing the main connections with the inferior colliculus (IC), primary auditory cortex and reticular thalamic nucleus (RTN), Tonsillar nucleus and striatum (caudate nucleus and putamen) inspired in Caspary and Llano (2017).

METHODS

In this study, immunohistochemistry for S100B was used to characterize the cytoarchitecture of MGB in young Wistar rats. After obtaining. From the images, the photomicrographs were identified and analyzed in the sequence of the rostral, medium and caudal levels in the 4X, 10X and 40X objectives. In parallel, these regions have been described and compared

with the rat brain atlas (Paxinos and Watson, 2007). The nomenclature used was based on the work on mice performed by Full and collaborators in 2012 and on the atlas described above. At the end, the results of each level were described and characterized, focusing on the areas and subareas of the Medial Geniculate Body, detailing the histological and cytoarchitectonic peculiarities found.

Animals and perfusion

This research was carried out by the Experimental Neurology Laboratory of the Faculty of Medicine of the State University of Rio Grande do Norte (FACS / LabNeuro / UERN). In this research a total of 10 young male Wistar rats (Rattus norvegicus) were used, weighing about 250g, with 3 months of age. The animals came from the Central Vivarium of the Department of Biomedical Sciences of the State University of Rio Grande do Norte (DCB / UERN), after approval by the Ethics Committee on Animal Experimentation - CEEA, through opinion No. 07/2016. Every effort has been made to reduce the suffering of the animals. They were housed in airy shelves, at a temperature between 23 to 26º C, kept in a standard polypropylene cage of dimensions 49x34x16 cm, lined with wood shavings. The luminosity control was maintained, with light / dark cycles of 12 hours each. The animals were anesthetized with Ketamine (10mg / kg) in combination with Xylazine (5mg / kg)). After reaching the anesthetic plane, a thoracotomy was performed on the animals, and then cardiopuncture. A cannula was used in the ascending aorta connected to a peristaltic pump (Cole Permer). A puncture was made in the left ventricle followed by an incision in the right atrium, 300 mL of 0.9% saline solution was infused with heparin (Parinex, Hipolabor 2mL / 1000mL) for approximately six minutes. Then, 300mL of 4% paraformaldehyde was administered at pH 7.4, in a 0.1M phosphate buffer.

Tissue processing

The brains were removed by the appropriate dissection technique, then stored for a period of 24 to 48 h in a 30% sucrose solution in 0.1 M phosphate buffer, pH 7.4 at 4º C. After that, the brains were frozen and sectioned in a microtome, ob-

taining coronal sections of standard thickness of 50µm. The samples were collected and stored in a liquid environment with buffer previously mentioned. The slices were distributed in six compartments, sequentially, in order to maintain the distance between one cut and another of 300 µm. These compartments were finally transferred to an antifreeze solution and kept at a temperature of - 20º C.

Immunohistochemistry and osmium intensification

At first, all antibodies were tested for false positive control and pretreatment was performed in order to avoid tissue artifacts. To perform immunohistochemistry using 0.1M phosphate buffer, approximately 100mL added in a container containing glass tubes with a filter. In this place the samples were stored delicately, taking care to place them in the tubes previously identified. On an orbital table, five washes were performed for five minutes on an orbital shaker at 70 revolutions per minute (RPM), replacing the 0.1M phosphate buffer with each wash. At the end of the washing process, 100µL of 30% hydrogen peroxide (H2O2) was added to a container containing 0.1M phosphate buffer and pH 7.4. With this solution, another wash was performed for 20 minutes. After this procedure, five more washes were performed for five minutes each.

The second step is the incubation of the cuts in primary antibody. For this, the duly identified test tubes were separated, then a solution containing 450 µL Bovine Serum Albumin (5% BSA) (Prothermo®) was prepared with 8550 µL of 0.4% Triton X-100, totaling 9000 µL , aims to make the membrane more porous. A pipette was used, in which 9 µL of the anti-S100B antibody obtained in mice (Sigma®) was aspirated at a dilution of 1: 10,000, being 0.5. µL of the antibody. Then, the primary antibody was homogenized for 12h (overnight). At the end of the overnight, cuts were transferred to test tubes containing the solution containing the primary antibody followed by washes for 5 minutes in 0.1M phosphate buffer, pH 7.4.

The third moment of immunohistochemistry consisted in the incubation of the secondary antibody, for this purpose the anti-conjugated antibody with peroxidase produced

in goat (GOAT α Ms) AbCAM® in slow rotation for 90 minutes, followed by 5 successive washes of 5 minutes each in phosphate buffer 0.1M, pH 7.4. Then, a 2% incubation in 80 µL of the Avidin Biotin Complex (ABC) and Triton X-100 NaCl was performed in a slow rotation for 90 minutes. Subsequently, 5 more washes were performed per 5 minutes each. Finally, immunohistochemistry was revealed by tetrahydrochloride-diaminobenzidine (DAB-3,3 ', 4,4'). After that, the cuts were mounted on silanized slides, immersed in distilled water for 30 seconds, then in a 0.05% osmium trioxide solution for 20 seconds. Subsequently, the slides were dehydrated and diaphanized in xylol for five minutes each slide. It was proceeded by assembling the coverslips, which were delicately assembled using the Erv-Mount mounting medium, finally dried in the open air and cleaned.

Photomicrography

The sections of the brain submitted to immunohistochemistry for S100B were examined under an optical microscope (Olypus BX41) in a bright field. Digital images were obtained from representative sections using a digital video camera (Nikon, DXM1200) 4x objective was used in the images that revealed the entire area. And, 10x, 20x, possibly 40x objectives to detail the sub-regions found at the rostral, medium and caudal levels of young rats. Then, the images were analyzed, corrected for brightness and contrast, as well as, schematic drawings were assembled using Adobe Photoshop CS5® and Adobe Illustrator CS5® software.

Results

Rostral level:

At this level **(figure 2),** it is possible to observe that the areas of the Medial Geniculate Body exhibit cells stained diffusely with average intensity of staining. Bundles of fibers were seen that cross the medial part to the regions of the Medial Geniculate Body, ventral zone (MGV); Medial Geniculate Body, medial zone (MGM); Medial Geniculate Body, dorsal zone (MGD) and Medial Geniculate Body, marginal zone (MZMG). These fibers are charac-

terized in the form of longitudinal clusters, conspicuously separating the marked astrocytes between the study region and the most medial periphery (figures 2A and 2D). Also, there is a change in color from medium intensity to strong intensity between the areas that separate the CGM from the more peripheral neighboring region, in this case, the lateral geniculate body (LGB) (figures 2A and 2B) The posterior nucleus of the thalamus (PoT) it was observed and exhibits similarities in immunohistochemistry in the same section plane, however, with a subtly smaller mark compared to CGM (figure 2D).

In the 10X objective, a dense population of S100B immunoreactive astrocytes, in greater detail, is visualized in greater detail, both in the MGB and in the most peripheral region, which is the lateral geniculate body (LGB) (figure 2B). However, there is a border area between the MGB and the LGB, notably recognized as the Intergeniculate Booklet (IGL) (figure 2D). IGL are multipolar interneurons that make up the lateral geniculate body (LGB) in which, in view of the immunohistochemistry for S100B, they present themselves as a weakly colored area, in order to be characterized as an obvious split between these two regions. (figure 2B). A panoramic view of this section shows an oval area with well-defined edges and not continuous pale in the center (figure 2A).

At the same cut-off level, but increasing to the 40x objective (figure 2C), it is possible to identify the immunoreactive astrocytes from MGB. we can see that both the body and its extensions are intensely colored, revealing a starry shape with typical spiky projections. Thus, it appears that the S100B protein is a characterizer of cell morphology, as it was possible to individualize it, allowing not only to describe it, but also to describe the region that permeates it.

In contrast, in a slightly more rostral section (figure 2A), there is little fiber interference. When analyzing this image and overlaying it with the Atlas Paxinos of the rat, the histological aspect was corroborated with the histochemistry. In other words, that is why the region in question proves to be homogeneously marked.

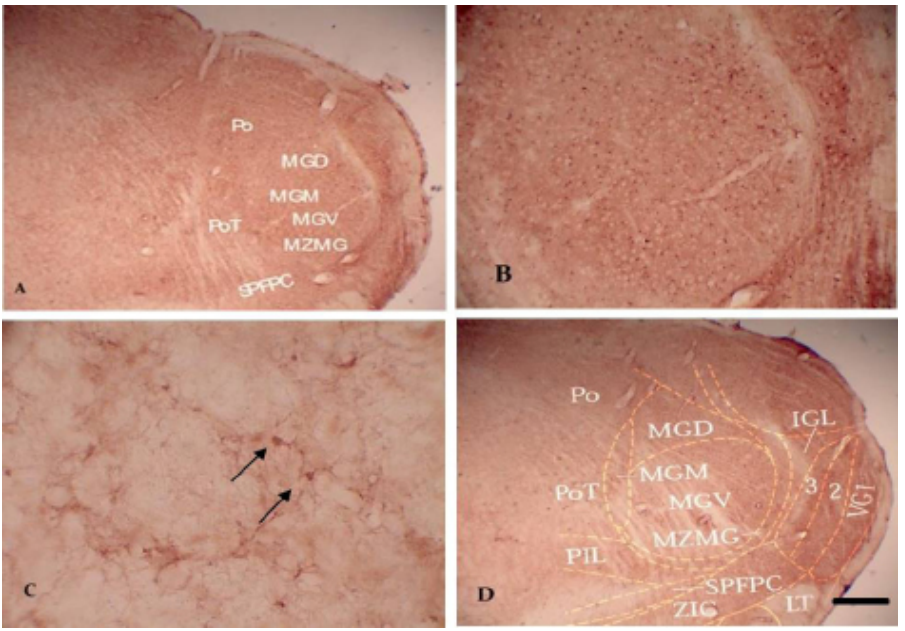

Figure 2: Light field photomicrograph of MGB, (A) rostral level, 4x objective, (B) same cut in the 10x objective (C) the same cut in the 20 objective showing the astrocyte morphology, highlighting the body and projections (arrow) (D) rostral level immediately above, revealing the streaky fibers in the CGM subregions. Scale: 300 um (2A and 2D), 260 um (2B); 130 um (2C).

Middle level

At this medium level, a similar pattern of marking is maintained, with areas diffusely marked, throughout the study's topography. There is a slight difference in staining intensity between MGM and PoT, IGL and posterior thalamic nuclear group (Po). However, it was not possible to visualize the sub-regions of the MGB due to poor marking. The immunohistochemistry characteristic is very similar to the rostral level, as it is present with homogeneous coloring, sometimes with the insertion of longitudinal fibers entering, without a pattern, the areas and subareas of the MGB.

Caudal level

It is observed at this level of cut that the marginal region was more clearly differentiated from the central part. That is, MZMG is of these unique ones, the only two subdivisions of MGB at this level, the presence of immunoreactive and heterogeneously distributed astocytes is visible.

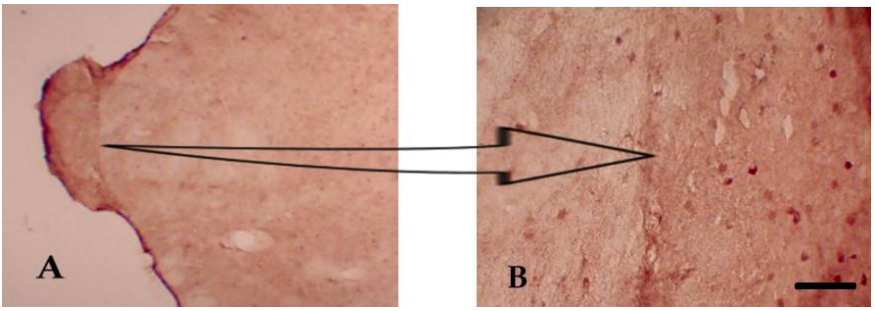

Figure 3: Photomicrograph of the Geniculate Body at caudal level, with an increase in the 4x and 20x objective showing the transition between MGB and LGB and immunoreactive astrocytes in both areas. Scale: 300 um (3A), 260 um (3B).

In the 10x objective lens, there is a clear limit at the border between the marginal zone of the CGM and the Lower Coliculum Arm (biC). The schematic drawing of Atlas Paxinus better elucidates the boundaries between the analyzed and adjacent areas. What is observed is that both regions, the astrocytes of both are immunoreactive, however, the staining is notoriously different.Therefore, at the caudal level, in addition to the clear division of MGV and MGZM

Discussion

The S100B protein belongs to a group of proteins partially soluble in 100% saturated ammonium sulfate solution at neutral pH. They are known as Ca2 + binding proteins, but their importance is recognized with other divalent ions such as Cu2 +, and Zn2 +, (Wheller et al., 2016). It performs important functions both in the intracellular and in the extracellular environment. In the astrocyte cytosol, it regulates cell prolifera-

tion, acts in protein phosphorylation and transcription, in the dynamics of microtubules, in enzymatic reactions, in Ca2 + homeostasis and in a series of activities involving target proteins within the cytoplasm. In the extracellular environment, it acts as a receptor for advanced glycation products (RAGE), stimulates differentiation, proliferation, as well as apoptosis and degradation of neurons, but its effects vary according to local concentration (Al-Ayadhi, 2012; Grupta et al. 2013).

The elevated serum or CSF level of the S100B protein is important as a biomarker, as it is often associated with pathological conditions such as Parkinson's, Multiple Sclerosis, Thalamus diseases and / or physiological conditions in which oxidative stress is involved (Shappiro et al. 2010; Stochero et al 2010). It is also known that this protein is located in different types of cells, such as, for example, in cardiomyocytes, exercising tissue functions that vary, in a complex way, according to the medium and the genetic expression of S100. Cu2 + and Zn2 + acting in cell maintenance (Donato et al. 2009).

Regarding immunohistochemistry, in general, it is known that S100B is an efficient astrocyte marker, but that it varies the pattern of immunoreactivity due to the functionality of the CNS tissue, because different neuronal cells have different functions, therefore, the localized astrocytes close to these neurons promote the maintenance of cellular demands according to the need of these neurons. The degree of specialization of these cells allows, therefore, to respond to all forms of aggression to the CNS through a process called reactive astrogliosis. (Sofroniew et al. 2010; Muneoka et al. 2012; Gross et al. 2014). Thus, both fibrillar glial acid protein (GFAP) and S100B protein are the most frequently used astrocyte markers (Wei et al. 2018). It should be remembered that astrocytes are the most abundant cells in the CNS (Azevedo et al. 2009), and can be subdivided into: protoplasmic astrocytes, constituents of the gray matter; fibrous astrocytes, belonging to the white substance, veiled, in the cerebellum and projection (Gomes et al. 2013).

Thus, GFAP is very specific for fibrous astrocytes, whose normal

function is to provide the stability of the white matter and the fullness of the blood-brain barrier. In addition, GFAP, in most protoplasmic astrocytes, is not expressed enough to be detected by immunohistochemistry, and in general, astrocytes in the gray matter are not immunoreactive. (Li et al. 2012).

In studies by Kawano (2015) on the chemoarchitecture of nerve cells in the optical tract of rats, using GFAP and the enzime Glutamine Synthetase (GS). They corroborated that GFAP is an important astrocyte immunostaining marker, in several territories of the CNS of man and laboratory animals. Immunoreactivity for GFAP was strong, especially in some regions not myelinated, but the immunostaining varied according to the subareas analyzed. Regarding GS, the pattern of immunoreactivity occurred, predominantly, in glial cells of the oligodendrocyte type in the myelinated region.

On the other hand, S100B is very specific for astrocytes that are correlated with the intra or extracellular maintenance of CNS neurons (Li et al, 2012). To illustrate, the S100B protein proved to be a great tool to characterize Substantia Nigra, Pars Compacta (SNPc), Substantia Nigra Reticulate (SNPr), Cerebral Peduncle (CP), Medial Lemnisco (ML), in addition to the Medial Geniculate Body (MGB) (Arrais, 2018). But it is almost a consensus, among many studies, that the combined use of two or more markers can be useful in the cytoarchitectonic characterization of nervous territories with astrocytes.

The function of the MGB is discrimination and auditory attention. (Amaral, 2014). CGM connections in mammals are extremely complex, and, depending on the sub-regions, different neurons and astrocytes are recognized (Antunes and Malmierca, 2014). The medial and dorsal zones receive fibers from the external dorsal portions of the inferior Colliculus (IC), while the ventral zone receives fibers from the central nucleus of the IC. The neurons of the MGV are bipolar, are related to frequency and project to the Primary Auditory Cortex (Barry, et al. 2017).

Otherwise, MGD neurons are varied, do not relate to a typical frequency, and send projections to the secondary auditory cortex (Araújo, 2008; Ak et al. 2017). Paralleling this study, it was observed that MGV, the

main nucleus of the CGM, presented itself at a more intensely marked rostral section and at a weakly marked caudal level compared to MGMZ.

Finally, although it was not the objective of this work to correlate the function of certain sub-areas of the CGM with patterns of greater or lesser immunoreactivity. It was also possible to establish an association between histology and immunohistochemistry, through the overlapping of Atlas Paxinos (2007).

Conclusion

From the results obtained in this research, it was possible to conclude that the Medial Geniculate Body has immunohistochemical marking for S100B. This marking varies, according to the sub-regions and the cut level analyzed, in addition, it was possible to visualize both the body and the astrocyte projections, glimpsing its morphology.

For all these reasons, the S100B proved to be an efficient marker, in addition to being an important CGM structure splitter, being able to contribute as a potential tool for future cytoarchitectural studies both in this area and in other regions of the CNS in Young Rats.

Ethical statement

Handling of animals was carried out in accordance with the guidelines of the ethic commission of animals experimentation of the State university of Rio Grande Do Norte (CEEA/UERN) for Use and Care of Animals.

Conflict of interest´s declaration

This paper has no conflict of interests for the authors.

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