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Region-specific glial hyperplasia and neuronal stability of rat lateral geniculate nucleus during aging

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ABSTRACT

The normal aging process is accompanied by functional declines in image-forming and non-image forming visual systems. Among the components of these systems, the thalamic lateral geniculate nucleus (LGN) offers a good model for aging studies since its three anatomical subdivisions, namely dorsal lateral geniculate nucleus (dLGN), intergeniculate leaflet (IGL) and ventral lateral geniculate nucleus (vLGN), receives light information from retina and projects to different brain areas involved in visual-related functions. Nevertheless, there is very little data available about quantitative morphological aspects in LGN across lifespan. In this study, we used design-based stereology to estimate the number of neurons, glial cells, the glia/neuron ratio and the volume of the LGN of Wistar rats from 3, 13 or 23 months of age. We examined each LGN subdivision processed by immunohistochemistry for NeuN and Nissl counterstain. We observed no significant age-related neuronal loss in any nuclei and a 21% and 33% significant increase in dLGN and IGL glial cells of 23 month-old rats. We also observed the glia/neuron relation increases in dLGN of 13 month-old rats and in dLGN, IGL and vLGN internal portion of 23 month-old ones. Moreover, we report an age-related increase in IGL volume. These results show region-specific glial hyperplasia during aging within LGN nuclei, perhaps due to compensatory responses to inflammation. In addition, we observed the glia/neuron ratio as a more sensitive parameter to quantify age-related alterations. Hence, we provide an updated and expanded quantitative characterization of these visual-related thalamic nuclei and its variability across lifespan.

1. Introduction

Classical morphometric approaches aim to quantify histochemical features in 2-dimensional images, despite real anatomical structures follows Euclidean geometry rules within a 3-dimensional space. In neuroscience, the bias of these methods led to misinterpretations of important quantitative aspects of nervous system structure, notably regarding the brain aging process (Long et al., 1999; von Bartheld et al., 2016). While early studies of brain aging reported a major loss of neuronal number in several species (Brody, 1955; Brody, 1970; Ordy et al., 1978; Brizzee et al., 1980; Heumann and Leuba, 1983), further investigations using unbiased stereological methods for cell counting stated there is no profound loss of neural cells in most brain nuclei (Madeira et al., 1995; Rapp and Gallagher, 1996; Giannaris and Rosene, 2012; Roberts et al., 2012). Even so, recent works point to age-related global reductions in neuronal number of entire regions such as hippocampus of rats and mice (Morterá and Herculano-Houzel, 2012; Fu et al., 2015). Probably, these global findings reflect the sum of several non-significant alterations with significant changes restricted to certain sub-regions. Thus, studying this process in a region-specific manner is crucial to describe in detail the anatomical correlates of aging impacts on nervous system.

Abbreviations: ANOVA, analysis of variance; *asf*, area sampling fraction; CE, coefficient of error; DAB, diaminobenzidine; dLGN, dorsal lateral geniculate nucleus; GFAP, glial fibrillary acidic protein; GNR, glia/neuron ratio; IGL, intergeniculate leaflet; LGN, lateral geniculate nucleus; m.o, month-old; SCN, suprachiasmatic nucleus; *ssf*, section sampling fraction; str, superior thalamic radiation; SURS, systematic uniform random sampling; PB, phosphate buffer; vLGN, ventral lateral geniculate nucleus; vLGNe, ventral lateral geniculate nucleus external layer; vLGNi, ventral lateral geniculate nucleus internal layer; ZIc, zona incerta caudal portion

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It is noteworthy that several of brain aging quantitative morphological studies are focused on the visual system components, such like retina (Esquiva et al., 2017), optic nerve (El-Sayyad et al., 2014), visual cortex (Yates et al., 2008; Giannaris and Rosene, 2012) and thalamus (Satorre et al., 1985; Ahmad and Spear, 1993; Diaz et al., 1999). The latter, in rodents, encloses the lateral geniculate nucleus (LGN) which is subdivided in three distinct nuclei, namely dorsal lateral geniculate nucleus (dLGN), the intergeniculate leaflet (IGL) and the ventral lateral geniculate nucleus (vLGN). The vLGN is even further subdivided in an external (vLGNe) lateral layer and an internal layer (vLGNi) located in a medial portion of vLGN (Nimi et al., 1963; Mitrofanis, 1992; Harrington, 1997). Although these three nuclei receive light information from retinal efferent projections, only the dLGN has a well-established part in visual sensory information relay to the cerebral cortex. Alternatively, IGL and vLGN are associated with the non-image forming visual system, in which IGL projects to hypothalamic suprachiasmatic nucleus (SCN) to adjust circadian rhythmicity and vLGN is involved in visuomotor functions such as pupillary reflexes (Harrington, 1997; Horowitz et al., 2004; Morin, 2013). Thus, from an anatomical perspective, the LGN offers a good model to understand the aging impact in visual-related functions since in the same brain region are nuclei involved with distinct aspects of visual and circadian systems.

Despite the morphological and neurochemical properties of LGN nuclei are fairly known, few studies addressed how this morphology is affected by the aging process in quantitative parameters, especially in IGL and vLGN (Fiuza et al., 2016). Thus, we undertook a design-based stereological study to determine the volume, total neuron number, total glial number and the numeric relationship between glia and neurons of LGN subfields in 3, 13 and 23 months-old rats.

2. Material and methods

2.1. Experimental subjects

A total of 15 adult male Wistar rats were housed in cages at 22 °C, 50% humidity in a 12:12 h light/dark cycle with food and water freely available. These animals were studied at the ages of 3, 13 or 23 months (n = 5 per age). All procedures were in accordance with Brazilian law number 11.794/2008 for animal experimental use. All experiments were approved by local ethic committee for animal use (CEUA-UFRN number 054/2015).

2.2. Tissue fixation

All animals were firstly anesthetized with sodium thiopental (40 mg/kg). Then, they were transcardiac perfused with a 300 ml NaCl solution (0.9%) followed by 300 ml formalin (10%) in a 0.1 M phosphate buffer (PB, pH 7.4). Following perfusion, the brains were removed, post-fixed with the same fixative overnight and cryoprotected in a solution containing 30% sucrose with 0.1 M PB (pH 7. 4) for 3 days. Then, brains were cut into coronal sections (50 μm) in a cryostat and the sections were sequentially collected in 96 well plates filled with antifreezing solution to be stored at − 20 °C until use.

2.3. Immunohistochemistry

Representative sections of LGN (see stereology topic for sampling details) were submitted to free-floating immunohistochemistry. Firstly, the sections were previously blocked for endogenous peroxidase activity in 0.3% hydrogen peroxide. Then, sections were incubated overnight with monoclonal mouse anti-NeuN primary antibody (MAB 377, Millipore) in a dilution containing 2% bovine serum albumin with 0.1 M PB in solution with 0.4% Triton X-100 (PBTX 0.4%). After rinsing, sections were incubated with biotinylated goat anti-mouse secondary antibody (115-065-003, Jackson ImmunoResearch) diluted with PBTX 0.4%. Then, sections were incubated in a 0.5% avidin-biotin solution (Vectastain standard ABC kit, PK-4000, Vector Laboratories), with 2.3% NaCl addiction, for 120 min. Then, brain sections were placed with a 2.5% solution of diaminobenzidine (DAB) diluted with 0.1 M PB. The final reaction was performed adding a 0.01% H2O2 solution, to reveal marked areas in brown colors resulting from DAB oxidation. The brain sections were mounted in gelatinized slides, dried, counterstained for Nissl substance with a 0.1% cresyl violet solution, dehydrated in graded ethanol solutions, cleared in xylene and coverslipped with DPX embedding matrix.

2.4. Antibody characteristics

The antibody used in this study (Clone A60, MAB 377, Millipore) was generated in mouse brain cell nuclei and recognizes both 46 and 48-kDa isoforms of Neuronal nuclei protein (NeuN). This antibody is sensitive and specific for neurons and is widely used to quantify neurons in animals, from similar or distinct age groups, since it provides highly correlated neuronal numbers with those obtained by Nissl technique (Wolf et al., 1996; Gittins and Harrison, 2004; Giannaris and Rosene, 2012). Prior to experiments, we performed pilot studies to establish optimal antibody concentration and incubation time. Also, we addressed negative controls by incubating sections with no addition of primary antibody. In these cases, we observed no immunoreactivity.

2.5. Stereology

Unbiased stereological analyses were performed in an optical microscope (AxioImager Z2, Carl Zeiss) fitted with an X-Y motorized staged, Z encoder and camera (CX 9000, MBF Bioscience). Prior to estimation of volume and cell number, a pilot study was performed in order to establish optimal parameters of section fraction, counting frame area, grid spacing size and disector height. Accordingly, we used a 1/4 section sampling fraction (*ssf*), corresponding to approximately 5 IGL and 6 dLGN and vLGN sections from an average of 24 LGN sections identified by an experienced researcher following the 6th edition of the rat brain atlas from Paxinos and Watson (2007) and the anatomical descriptions of Nimi et al. (1963). Thus, our systematic uniform random sampling (SURS) was established by randomly choosing the first section and analyzing every other LGN section in a 200 μm interval. All sections were blind-coded, so the experimenter had no information regarding the animal age in the stereological procedures. The estimation of cell numbers and volume was performed bilaterally in each LGN sub-nuclei.

2.5.1. Regions of interest

The combination of NeuN immunohistochemistry and cresyl violet counterstain for Nissl substance allowed us to distinguish the borders of each LGN subdivision. Considering there are no other nucleus lateral to LGN, the lateral boundary was evident and used to define the external contour of nuclei. In addition, the medial boundary was also clear to define since it is delimited by the superior thalamic radiation (str). We established the border between dLGN and IGL by the change in density and orientation of cells. Furthermore, strong NeuN immunoreactivity was observed at low magnification in vLGNe, which was used to distinguish from both IGL and vLGNi (Fig. 1a). These features were consistent delimitation points within the rostrocaudal extension. At rostral most levels, dLGN and vLGN appears as small clusters of cells positioned dorsal to reticular thalamic nucleus. At intermediate levels both dLGN and vLGN enlarges and are divided by IGL. At caudal levels the medial portion of IGL projects ventrally and fuses with Zona Incerta caudal portion (ZIc).

2.5.2. Estimation of neuron and glial numbers

Numbers of glial cells and neurons were estimated by the optical fractionator method (West et al., 1991). For these estimations, we firstly outlined the LGN subdivisions with a low magnification objective (2.5 ×) and counted cells at high magnification (40 ×). We identified