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A novel approach for integrative studies on neurodegenerative diseases in human brains

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HIGHLIGHTS

- Network-based approach for studying differential vulnerability in neurodegeneration.
- Allow to combine stereology, immunohistochemistry and 3D reconstruction.
- Suitable for humans and animal models.
- The approach is economical and requires shorter processing and analysis time.
- This method can make studies with human brain tissue more attractive.

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ABSTRACT

Despite a massive research effort to elucidate Alzheimer's disease (AD) in recent decades, effective treatment remains elusive. This failure may relate to an oversimplification of the pathogenic processes underlying AD and also lack of understanding of AD progression during its long latent stages. Although evidence shows that the two specific neuropathological hallmarks in AD (neuronal loss and protein accumulation), which are opposite in nature, do not progress in parallel, the great majority of studies have focused on only one of these aspects. Furthermore, research focusing on single structures is likely to render an incomplete picture of AD pathogenesis because as AD involves complete brain networks, potential compensatory mechanisms within the network may ameliorate impairment of the system to a certain extent. Here, we describe an approach for enabling integrative analysis of the dual-nature lesions, simultaneously, in all components of one of the brain networks most vulnerable to AD. This approach is based on significant development of methods previously described mainly by our group that were optimized and complemented for this study. It combines unbiased stereology with immunohistochemistry and immunofluorescence, making use of advanced graphics computing for three-dimensional (3D) volume reconstructions. Although this study was performed in human brainstem and focused in AD, it may be applied to the study of any neurological disease characterized by dual-nature lesions, in humans and animal models. This approach does not require a high level of investment in new equipment and a significant number of specimens can be processed and analyzed within a funding cycle.

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1. Introduction

The dramatic increase in life expectancy worldwide is being followed by an increase in the prevalence of Alzheimer's disease (AD), the leading cause of dementia (Hebert et al., 2003).

AD is characterized by two specific neuropathological hallmarks which are opposite in nature: (a) positive lesions caused by extracellular accumulation of β-amyloid peptides in neuritic plaques (NPs) and intracellular accumulation of phosphorylated tau protein in neurofibrillary tangles (NFTs) and (b) negative lesions (neuronal and synaptic loss) (Duyckaerts et al., 2009). Positive and negative AD lesions do not develop in parallel (Andrade-Moraes et al., 2013). Despite this complex pathogenesis, research effort has been directed to the elucidation of a limited number of candidate pathways involved in the positive lesions, such as the amyloid cascade (Korczyn, 2012). It may partly explain why a number of promising therapies for AD that appeared to have great efficacy in animal models fell short when tested in human AD subjects (Huang and Mucke, 2012). AD is associated with a highly characteristic spread pattern across specific neural networks (Seeley et al., 2009), accompanied by the activation of and/or damage to an increased number of cellular and molecular pathways. Studies show that potential compensation mechanisms among the components the vulnerable network ameliorate impairment of the system to a certain extent (Boxer et al., 2011), suggesting that research focusing on single structures is likely to render an incomplete picture of AD pathogenesis.

In 2009, using a postmortem brain collection including over 1500 controls (Grinberg et al., 2007), we demonstrated for the first time that a midbrain structure, the dorsal raphe nucleus, showed NFT pathology even before the entorhinal cortex, the structure previously considered to be the first affected by NFT. The dorsal raphe nucleus is part of the isodendritic core (IC) network, which also includes the cholinergic nucleus basalis of Meynert (NbM), the noradrenergic locus coeruleus and the dopaminergic substantia nigra (Ramon-Moliner and Nauta, 1966). These inter-connected nuclei share a similar neuron appearance when Golgi-stained, and modulate basic physiological processes and behavior. All IC components show AD changes during AD latent stage, years before the symptoms onset (Braak et al., 2011; Geula et al., 2008; Ohm et al., 1989; Parvizi et al., 2001; Sassin et al., 2000). Since AD treatment is likely to be more effective if started during its long latent stage (Jack et al., 2010), before a large percent of brain tissue has already been irrevocably damaged, studies on the IC during early AD is a top priority (Grinberg et al., 2009b).

With this vision, we aimed to take advantage of our large and ongoing postmortem brain collection to investigate IC involvement in early AD. However, aware of the complexities of AD pathogenesis, we aimed to develop an approach for enabling integrative analysis of the dual-nature AD lesions in all IC components simultaneously. This process was based on methods mainly described previously by our group (Ewers et al., 2011; Grinberg et al., 2008a, 2009a, c; Grinberg and Heinsen, 2007; Heinsen et al., 2000, 2004) that were optimized and complemented for this study. It combines unbiased stereology with immunohistochemistry and immunofluorescence, making use of advanced graphics computing for three-dimensional (3D) volume reconstructions.

2. Materials and methods

2.1. Subjects

Postmortem human brains from four subjects aged ≥50 years, with no cognitive decline (clinical dementia rating, CDR = 0) (Morris, 1993) or parkinsonism (Tanner et al., 1990) were supplied by the Brain Bank of the Brazilian Brain Aging Study Group (BBBABSG) (Grinberg et al., 2007; Table 1). All subjects showed latent AD (pathology was detected in the brains, but no clinical decline was present). After informed consent, clinical and functional information were obtained from knowledgeable informants who were interviewed by nurses specifically trained for the questionnaire application (Ferretti et al., 2010).

Table 1
Characteristics of cases.

Cases	Age (years)	Gender	Post-mortem interval (h)	Cause of death
1	69	Male	17	Hemopericardium
2	62	Male	17.5	Cachexia
3	59	Male	20	Myocardial infarction
4	56	Male	19.5	Broncopneumonia

2.2. Tissue processing

2.2.1. Initial brain processing

Brains were fixed by immersion in 8% paraformaldehyde for at least 4 weeks. The brainstem and thalamus were then severed from the brain as a unit and the cerebellum was removed to produce the brainstem block. A second block (basal forebrain block) was constituted of a 2 cm thick coronal brain slab encompassing the NbM. Both blocks were processed as described below.

For neuropathological assessment, specific brain areas were sampled according to the BBBABSG protocol (Grinberg et al., 2007).

2.2.2. Celloidin embedding

The brainstem and forebrain blocks were initially embedded in celloidin using the modified method developed by Heinsen et al. (2000). This method reduced tissue distortion and enabled production of thick histological sections, a requirement for the subsequent stereological studies. Prior to embedding, the meningeal and pial vessels were removed to reduce artifactual damage and the blocks were dehydrated in a graded series of ethanol solutions (70, 80, and 96% in dH_2O). The dehydrated blocks were then individually placed in glass boxes filled with 8% celloidin solution in 100% ethanol (Hacocell H 9 IPA 35%, Hagedorn, Osnabrück, Germany). We modified our original protocol by mixing 0.1% Sudan black B (Sigma–Aldrich, St. Louis, MO) in a proportion of 50:1 with celloidin aiming to improve the contrast between the tissue and the embedding media. To ensure proper embedding, the volume of celloidin was at least double the block volume and the block did not come into contact with the walls of the box. The glass boxes were individually placed in a desiccator connected to a vacuum pump with pressure set at −600 mb (−60 kPa) or −400 mb (−40 kPa) until the celloidin contracted to half its original volume, becoming clear and semi-solid (Fig. 1). Blocks were then placed in a covered aquarium which volume was 30 times greater than the block, together with a 1000 ml beaker filled with 200 ml chloroform. The chloroform vapor hardened the celloidin to an India rubber consistency by promoting alcohol removal from the block (Fig. 2).

2.2.3. Sectioning and imaging of the celloidin-embedded brain tissue blocks

Excess celloidin was trimmed off the blocks with a knife before mounting on a polyamide base that was later attached to a microtome (Fig. 2). The same 8% celloidin solution was used to glue the blocks into the base. Blocks remained in the aquarium overnight, followed by exposure to 70% ethanol for a minimum of 2 h to ensure that the celloidin blocks were firmly attached to the base. The blocks were sectioned at 300 μm thickness using either a