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AGING-DEPENDENT CHANGES IN THE CELLULAR COMPOSITION OF THE MOUSE BRAIN AND SPINAL CORD

Y. FU,[a,b] Y. YU,[a] G. PAXINOS,[a,b] C. WATSON[c] AND Z. RUSZNÁK[a]*

[a] Neuroscience Research Australia, Sydney, NSW 2031, Australia
[b] The University of New South Wales, Sydney, NSW 2052, Australia
[c] Faculty of Health Sciences, Curtin University, Perth, WA 6845, Australia

Abstract—Although the impact of aging on the function of the central nervous system is known, only a limited amount of information is available about accompanying changes affecting the cellular composition of the brain and spinal cord. In the present work we used the isotropic fractionator method to reveal aging-associated changes in the numbers of neuronal and non-neuronal cells harbored by the brain and spinal cord. The experiments were performed on 15-week, 7-month, 13-month, and 25-month-old female mice. The major parts of the brain were studied separately, including the isocortex, hippocampus, cerebellum, olfactory bulb, and the remaining part (i.e., 'rest of brain'). The proliferative capacity of each structure was assessed by counting the number of Ki-67-positive cells. We found no aging-dependent change when the cellular composition of the isocortex was studied. In contrast, the neuronal and non-neuronal cell numbers of the hippocampus decreased in the 7–25-month period. The neuronal cell number of the olfactory bulb showed positive age-dependence between 15 weeks and 13 months of age and presented a significant decrease thereafter. The cerebellum was characterized by an age-dependent decrease of its neuronal cell number and density. In the rest of brain, the non-neuronal cell number increased with age. The neuronal and non-neuronal cell numbers of the spinal cord increased, whereas its neuronal and non-neuronal densities decreased with age. The number of proliferating cells showed a marked age-dependent decrease in the hippocampus, olfactory bulb, and rest of the brain. In contrast, the number of Ki-67-positive cells increased with age in both the cerebellum and spinal cord. In conclusion, aging-dependent changes affecting the cellular composition of the mouse central nervous system are present but they are diverse and region-specific. **© 2015 Published by Elsevier Ltd. on behalf of IBRO.**

Key words: isotropic fractionator, NeuN, neuron number, hippocampus, olfactory bulb, Ki-67 protein.

INTRODUCTION

As evidenced by progressive deficits in memory, cognitive function, and behavior, aging has a significant impact on the function of the human brain. Finding morphological changes that may explain the functional decline has been in the focus of attention since the 1950s (e.g., Brody, 1955, 1970; Colon, 1972; Shefer, 1973; Cragg, 1975; Vijayashankar and Brody, 1979; Henderson et al., 1980; Mani et al., 1986; Hansen et al., 1987). Because of the importance of the cerebral cortex and hippocampus in both memory and cognitive processes, most research efforts aimed at establishing if aging is associated with significant changes of the cellular composition of these structures.

Before the advent of unbiased stereology, most studies concluded that there was a significant aging-related decrease of the number and/or density of cortical neurons (Brody, 1955, 1970; Colon, 1972; Shefer, 1973; Devaney and Johnson, 1980; Henderson et al., 1980). A concomitant increase of the number of neuroglial cells was also suggested (Hansen et al., 1987). In contrast, studies using more advanced cell-counting methods demonstrated no or only modest aging-associated changes (e.g., Peters et al., 1998; Long et al., 1999). For example, Pakkenberg and Gundersen (1997) estimated less than 10% reduction in the number of cortical neurons when comparing samples taken from subjects aged 20 and 90 years. Further, no aging-associated reduction of the neuronal number was found when female patients aged 65–105 years were studied (Fabricius et al., 2013). The most recent view, therefore, is that aging in humans is not associated with a massive reduction of the neuronal cell number in the cortex. Unbiased stereology experiments also indicated that the number of glia does not increase with age in the human cortex but, in fact, may be reduced (Pakkenberg et al., 2003). The decreased glial number is mostly attributable to the reduced number of oligodendrocytes (Pelvig et al., 2008). Because the primary function of the oligodendrocytes is the production of myelin, this observation is consistent with earlier findings demonstrating an aging-associated reduction of the volume of the white matter in the brain (e.g., Pakkenberg and Gundersen, 1997; Tang et al., 1997).

Many studies have attempted to identify aging-dependent changes that affect the human hippocampus.

*Corresponding author. Tel: +61-2-9399-1096.
E-mail addresses: y.fu@neura.edu.au (Y. Fu), yuyou@yeah.net (Y. Yu), g.paxinos@neura.edu.au (G. Paxinos), c.watson@curtin.edu.au (C. Watson), z.rusznak@neura.edu.au (Z. Rusznák).
† Permanent address: Department of Paediatric Surgery, The First Affiliated Hospital of Harbin Medical University, Harbin 150001, China.
Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; NeuN, neuronal nuclear protein; PBS, Phosphate- buffered saline; ROB, rest of the brain.

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Similar to the neocortex, the aging-dependent reduction of the neuronal cell number reported by early articles (e.g., Ball, 1977; Shefer, 1977; Anderson et al., 1983) was not unequivocally confirmed by more recent studies that employed unbiased stereology. West and Gundersen (1990) found a significant reduction of the neuronal cell number only in the CA1 region in a study on 47–85-year-old subjects, whereas West (1993) reported significant aging-related neuronal loss in the subiculum and hilus of the dentate gyrus. Later, West et al. (1994) demonstrated a decreased neuronal cell number in the hilus and subiculum along with a relatively stable neuronal number in the CA1 region, while Simic et al. (1997) found significantly reduced neuronal numbers in the CA1 region and subiculum, but no change in the hilus or elsewhere. A possible explanation of these non-uniform data may be the high inter-individual variability of the assessed parameters.

Although studies conducted on the rodent brain cannot substitute those using human tissue samples, the advantages of working on rodents are obvious: mice and rat colonies are easy to maintain, and the size of their brain and spinal cord makes them straightforward to study. Further, inter-individual variability is less pronounced because rodent brains are not affected by the same complex social activities as humans, and because inbred strains have a more homogeneous genetic background. Despite these, data obtained in rodent studies are not fully concordant: while several articles reported on the absence of prominent aging-related cell loss (e.g., Rapp and Gallagher, 1996; Rasmussen et al., 1996; Calhoun et al., 1998), significant and progressive neuron loss was reported in a more recent publication (Morterá and Herculano-Houzel, 2012). The authors of the latter article suggested that the neuronal cell number starts to decline in the rat hippocampus, cortex, cerebellum, and olfactory bulb as early as three months of age.

Using the isotropic fractionator method, we have previously demonstrated that the cellular composition of the brain and spinal cord of the C57BL/6J mouse shows age-dependent changes during adolescence (Fu et al., 2013a). In the present study we have extended our observations to aging-related changes using female mice aged 15 weeks, 7 months, 13 months, and 25 months. The previously implied aging-dependent reduction of the proliferating cell number in the central nervous system (e.g., Seki and Arai, 1995; Kuhn et al., 1996; Cameron and McKay, 1999; Lichtenwalner et al., 2001; Kempermann et al., 2002; Jin et al., 2003; Nacher et al., 2003) was addressed by determining the number of Ki-67 expressing cells. Our results are relevant to studies conducted on C57BL/6J mice aimed at discovering the effects of aging, adult neurogenesis, and/or neurodegeneration.

EXPERIMENTAL PROCEDURES

Animals

Female C57BL/6J mice were obtained from the Animal Resources Centre (Canning Vale, WA, Australia; $n = 18$) or received as a gift from Dr. Jana Vukovic (Queensland Brain Institute, The University of Queensland, Queensland, Australia; $n = 6$). All mice were maintained under virtually identical conditions, including the size of the cages and the type of chow. All mice belonged to one of the following four age groups: 15 weeks ($n = 7$), 7 months ($n = 6$), 13 months ($n = 5$), and 25 months ($n = 6$). The experiments were approved by the Animal Care and Ethics Committee of the University of New South Wales (11/75A) and were carried out in accordance with the relevant Australian and institutional guidelines on the care of research animals.

Perfusion, tissue dissection, and quantification of the total, neuronal, and non-neuronal cell numbers using the isotropic fractionator technique

Mice were euthanized by injection of a lethal dose of pentobarbitone sodium (0.24-mg/g body weight) and perfused through the left ventricle with saline, followed by cold (4 °C) 4% paraformaldehyde prepared in phosphate-buffered saline (PBS; pH 7.4). The meninges as well as the cranial and spinal nerve rootlets were removed, followed by separation of brains and spinal cords just caudal to the pyramidal decussation, and their removal from the skull and spine. After two weeks post-fixation, brains were dissected into the olfactory bulb, isocortex, hippocampus, cerebellum, and 'rest of the brain' (ROB—comprising the diencephalon, mesencephalon, and axial rhombencephalon) by the same member of the team.

The number of neuronal and non-neuronal cells was determined using the isotropic fractionator method (Herculano-Houzel and Lent, 2005). Cell numbers obtained with this method are compatible with those determined by unbiased stereology (Bahney and von Bartheld, 2014). Each tissue sample (i.e., the dissected isocortex, hippocampus, cerebellum, olfactory bulb, the remaining part of the brain, and the spinal cord) was mechanically homogenized in a dissociation solution (1% Triton X-100 in 40 mM trisodium citrate) until an isotropic nuclear suspension was obtained. Special care was taken to ensure that the cell nuclei were not broken up during homogenization. As the result, the number of fragmented nuclei was insignificant in all samples tested. Eighteen mice were used for the isotropic fractionator experiments: $n = 4$ for both the 15-week- and 7-month-old groups, and $n = 5$ for each of the remaining groups.

The total cell number was determined by adding 4′,6-diamidino-2-phenylindole (DAPI) dilactate (Invitrogen, Mulgrave, VIC, Australia) to the nuclear suspension. Four to twelve aliquots of this suspension, each with a volume of 10 μl, were then loaded onto a hemocytometer chamber, and the number of cell nuclei was determined using an Olympus BX51 fluorescence microscope (Olympus, Tokyo, Japan). The DAPI signal was observed using a U-MWU2 filter set (Olympus). The coefficient of variation of the counts was typically < 0.10, and always less than 0.15. The total number of cells of each investigated structure was determined on the basis of the total volume and average cell number of the homogenized tissue sample prepared from the relevant part of the central nervous system (i.e., the dissected isocortex, hippocampus, cerebellum, olfactory bulb, the remaining part of the brain, and the spinal cord).

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