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Effects of Ageing on Dendritic Arborizations, Dendritic Spines and Somatic Configurations of Cerebellar Purkinje Cells of Old Cat

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Abstract.- We investigated the morphological alterations in the Purkinje cells (PCs) of the cerebellar cortex of young adult (2–3 years old) and old cats (12–13 years old). Rapid Golgi staining was used to visualize dendritic arborizations and spines, and routine Nissl staining was applied to visualize the somatic configuration of the PCs. The height, width and area of the dendritic network were measured, the spine density was calculated, and the average volumes of the soma and nucleus of PCs were roughly estimated. Golgi staining demonstrated that old PCs exhibited significant retraction of dendritic arborizations and considerable desquamation of dendritic spines, indicating a remarkable decrease of information input to the ageing PCs. In addition, Nissl staining showed a distinct reduction in size of the soma and nucleus of old PCs. We suggest that retraction of dendritic arborizations and degeneration of the somatic configuration might lead to deficits in information processing and substance synthesis in senile cerebellar PCs.

Key words: Cerebellum, Purkinje cell, dendritic spines, somatic configuration, ageing.

INTRODUCTION

It is well known that cerebellar functions are impaired during normal ageing process (Hilber and Caston, 2001; Taniwaki *et al*., 2007; Paul *et al*., 2009). However, the mechanisms responsible for such impairments remain largely unclear. It was once widely hypothesized that age-related degeneration in cerebellar functioning could result from a considerable loss of neurons (Henrique *et al*., 2001; Zhang *et al*., 2006), especially the loss of Purkinje cells (PCs) (Pires *et al*., 2010; Woodruff-Pak *et al*., 2010). However, Andersen *et al*. (2003) reported only limited neuronal loss in the ageing cerebellar cortex, which is restricted to the anterior lobe. As an alternative, many neurons in cerebellum undergo significant morphological alterations during ageing (Quackenbush *et al*., 1990; Hadj-Sahraoui *et al*., 2001; Andersen *et al*., 2003), which may, to some extent, be responsible for age-related cerebellar dysfunction.

The PC, a typically large multipolar neuron, plays important roles in the cerebellar circuitry, and has been extensively studied in the ageing cerebellum (Huang *et al*., 2006a; Zhang *et al*., 2010). Its neuronal morphology and function have been found to alter with ageing. Atrophy in the dendritic arborizations (Quackenbush *et al*., 1990; Chen and Hillman, 1999; Hadj-Sahraoui *et al*., 2001), loss of the dendritic spines (Pentney, 1986), decrease in the cellular volume (Andersen *et al*., 2003), changes of receptor responses (Yew *et al*., 2009; Pires *et al*., 2010; Woodruff-Pak *et al*., 2010) and alterations in the electrophysiological properties (Rogers *et al*., 1980; Woodruff-Pak *et al*., 2010) have all been observed in the ageing cerebellum. These changes have been noted in mice, rats and humans. The cat is a higher-order mammal with a well-developed cerebellum. Our previous studies have shown a decrease in PC number in the anterior cerebellum along with obvious atrophy in neurofilament-immunoreactive (NF-IR) arborizations in the ageing cat (Zhang *et al*., 2006). This paper reports on age-related changes of the dendrites and somata in cat cerebellar PCs, which will further increase our understanding of the possible mechanisms underlying the cerebellar functional deficits (such as dysfunctions in balance control, motor coordination and motor learning) in the senescent individuals (Hilber and Caston, 2001; Shiozaki *et al*., 2008; Taniwaki *et al*., 2007).

MATERIALS AND METHODS

Animals and tissue treatments

Young adult cats (2–3 years old, n=4) and old

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cats (12–13 years old, n = 4) were used in this study. All the subjects were healthy domestic cats (*Felis domesticus*) with complete age and health-care records kept by our laboratory assistants. The experimental treatments met the standard of the National Institute of Health's Guide for the Care and Use of Laboratory Animals. After anesthesia and perfusion (Zhang *et al*., 2006; Zhang *et al*., 2011), the cat brain was exposed and the cerebellar hemispheres were dissected out, and immediately immersed in fixative solution.

Rapid Golgi staining

Blocks of tissue were trimmed to 2 cm × 1 cm × 1 cm size for rapid Golgi staining. The blocks were impregnated in a solution containing 2% potassium dichromate and 0.16% osmium tetroxide, and agitated in the dark for 2 weeks. After being washed in increasing concentrations (0.25%, 0.50%, 0.75%, and 1%) of silver nitrate for 5 min each, the blocks were agitated in 1% silver nitrate in the dark for another week, and subsequently washed in ethanol/acetone (1:1) for 2 h, in pure ethanol for 5 min, and in ethyl ether/ethanol (1:1) for 5 min. The blocks were then rapidly embedded in paraffin, and cut into 90 μm thick sections. Finally, the sections were washed in butanol for 10 min, in cedar wood oil twice for 10 min each, and in toluene twice for 5 min each, before being mounted onto slides.

Nissl staining

Another set of tissue blocks, selected from the remaining anterior zone were trimmed to a length of 1 cm from a lobule and fixed overnight in the above fixative solution at 4°C. The tissue blocks were then dehydrated in graded ethanol, transparentized in xylene and embedded in paraffin. Finally, 10 μm thick consecutive coronal sections were cut for toluidine blue staining.

Quantitative analysis

In each Golgi stained slide, PCs meeting the following criteria were selected for measurement: (1) the entire arborizations were completely and distinctly stained in the sections; (2) the cell was distinct from neighbouring cells; (3) the cell was located at the lobular flanks where accurate measurements could be made; and (4) the spines on the dendrites were discernible. For each animal, the first 10 PCs satisfying all these requirements were imaged by a Motic confocal microscope with Tsview software (Motic China Group Co., Ltd) at 400×. The height, width and area of the imaged PC dendrite as well as the local thickness of the molecular layer were measured. The height of the dendritic tree was measured from the hillock of the soma (by visual examination) to the top of the maximal canopy of the tree. The thickness of the molecular layer was measured by prolonging the line of the dendritic height to intersect with the pia. The width was measured as the distance between two lines tangential to either side of the dendritic field. The area of the dendrites was measured by circumscribing a polygon around the dendritic arborizations and then computing the area of the polygon (methods were similar to those previously described, see Chang *et al*., 1981). These measurements were made with the Tsview software. The number of the spines per 10 μm long dendrite from randomly chosen terminal branchlets was counted with an ocular micrometer at 1000×. Minor adjustments in the fine focus were made when necessary in order to clearly visualize the spines on a given segment.

In the Nissl stained slides, the diameters of PC soma and nuclei were directly measured from cells with a clear border, distinct nucleus and distinguishable nucleolus at 1000×. A total of 20 PCs was measured for each cerebellum. The longitudinal (AA' for the soma and aa' for the nucleus, Figures 3 and 4) and latitudinal (BB' for the soma and bb' for the nucleus, Figures 3 and 4) axes of each soma and nucleus were measured and the mean diameter was calculated (Muche *et al*., 2006). The diameter of a PC soma was estimated as: $D = \sqrt{AA' \cdot BB'}$, and the diameter of the nucleus was: $d = \sqrt{aa' \cdot bb'}$. Each measurement was made 3 times and the mean values were taken as the diameters of a single PC, and then the volumes of PC soma and nucleus were roughly estimated as: $V_D = 4/3 \cdot \pi \cdot (D/2)^3$ and $V_d = 4/3 \cdot \pi \cdot (d/2)^3$, respectively. All the measurements were conducted by a researcher blinded to animal age.

Statistical analysis

The data were expressed as mean ± standard

error of the mean (SEM). Statistical comparisons were made by two-way analysis of variance (ANOVA). A *P* value of <0.01 was considered significant.

RESULTS

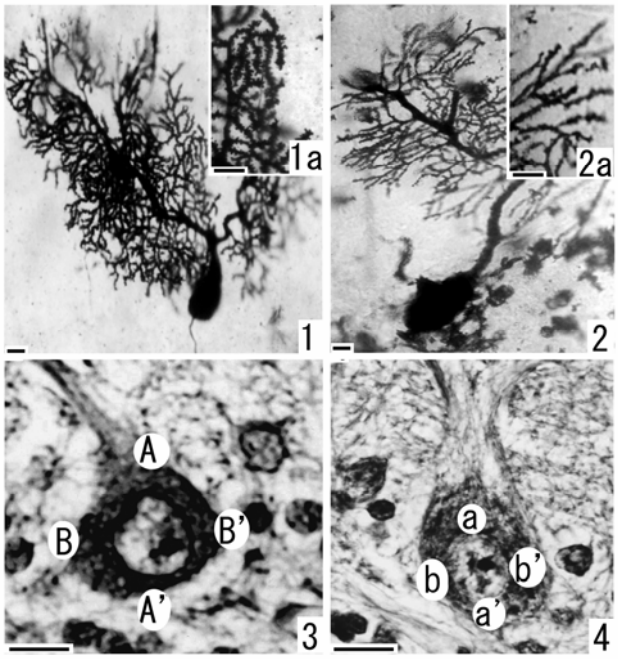
Dendritic arborizations and spines visualized by rapid Golgi staining

The appearance of the dendritic arborizations of PCs between cats belonging to the two age groups showed obvious differences. Young PCs had profuse arborizations vertically approaching the pial surface, while old PC arborizations appeared significantly atrophied in the molecular layer (Figs. 1, 2). The branches from young PCs were nearly homogeneous in each segment, while old dendrites possessed distinct swellings and nodulations in many segments (see Figures 1a and 2a). Quantitative evaluation of age-related changes in the mean height, width, dendritic area and thickness of the molecular layer are shown in Table I. In the young cerebellum, the height of PC dendrites occupied 94.1% of the thickness of the molecular layer, while old PC dendrites occupied only 73.7% of the thickness of the molecular layer, despite a significant decline of 12.2% in molecular layer thickness during cerebellar ageing (Table I). In addition, the arborizations in old PC dendrites were significantly atrophied. When compared with young PC dendrites, the mean height, width and area of old PC dendrites were significantly less (31.2%, 34.2% and 45.6% respectively, see Table I, $P<0.01$).

Under high magnification, Golgi staining revealed small spines on the dendrites of PCs in both the age groups (Figs. 1a and 2a). Age-related changes in spine density are shown in Table 1. The mean number of spines per 10 μm long segment was 40.7% less in aged arborizations compared with those in the young adult group.

Somatic configuration from Nissl staining

The somatic appearance and morphology of PCs differed between the young adult and old groups. Old PCs showed a remarkable loss of Nissl granules in the perikaryon. In addition, Nissl granules in the old PCs were weakly stained compared with those of young PCs (Figs. 3 and 4).



Figs. 1-4. Morphological changes in dendritic arborizations and somatic configurations of cerebellar Purkinje cells (PCs) in young and old cats. **1 and 2** cerebellar Purkinje cells (PCs) of a 2-year-old cat (young) and a 12-year-old cat (old) with rapid Golgi staining. Old PCs showed significant retraction in dendritic arborizations. **1a, and 2a** show branchlets of the PC dendrites of young and old cats. The PC branchlets of old cats showed fewer spines, and nodulations or swellings in some segments. **3 and 4** show the PC bodies in the two age groups stained with toluidine blue. Old PCs showed a decrease in size of the soma and nucleus, as well as a loss of Nissl granules in the perikaryon. The longitudinal (AA' for the soma and aa' for the nucleus) and latitudinal (BB' for the soma and bb' for the nucleus) axes of each soma and nucleus was linearly measured. The diameter of the PC soma was calculated as: $D = \sqrt{AA' \cdot BB'}$, and the diameter of the karyon was: $d = \sqrt{aa' \cdot bb'}$, and then the volumes of PC soma and nucleus were roughly estimated as: $V_D = 4/3 \cdot \pi \cdot (D/2)^3$ and $V_d = 4/3 \cdot \pi \cdot (d/2)^3$, respectively. **(1, 1a and 3)** young cat; **(2, 2a and 4)** old cat. Scale bar: 10 μm.

In comparison with young PCs, the average volumes of the soma and nucleus of old PCs were decreased by 29.3% and 27.4%, respectively (see Table I, $P<0.01$). It should be noted that the PC soma and

nucleus are presumed as spheres for convenience of calculation, which might be somewhat biased to the actual situations, and therefore, the volumes of PC soma and nucleus calculated in this study are rough estimation. However, the results should not be affected since the same criteria are used for both the groups.

Table I.- Morphological parameters of Purkinje cells (PCs) in the cerebellar cortex of the young and old cats[*]

	Young cerebellum	Old cerebellum
Thickness of the molecular layer (μm)[$]	316.7±36.3	278.2±38.5[*]
Height of PC dendritic arborizations (μm)	298.0±40.4	204.9±46.6[*]
Width of PC dendritic arborizations (μm)	330.0±69.9	217.1±81.6[*]
Area of PC dendritic arborizations (μm^2)	29908.1±6649.1	16256.2±3762.5[*]
Spine number/10 μm long terminal branchlets of PC	8.6±2.6	5.1±1.7[*]
Volume of PC soma (μm^3)	9380.2±3771.9	6634.0±2974.3[*]
Volume of PC nucleus (μm^3)	1731.7±763.7	1257.2±492.3[*]

[*] $P<0.01$ vs old cat

[$] This measurement was made at the positions where the chosen PCs locate, while a similar measurement in our previous Nissl-stained sections was done randomly at the lobular flanks (Zhang *et al*., 2006). This measurement is necessary for an accurate comparison on age-related changes of the ratio of PC dendritic height to molecular layer thickness in Golgi-stained sections.

DISCUSSION

The present paper reports age-related cytoarchitectonic differences of Purkinje cells in a more advanced mammalian model. Information about ageing in the cat may serve as a bridge to a better understanding of human ageing.

Age-related changes in dendritic arborizations

Significant declines in the height, width and area of dendritic arborization in the PCs of senescent cat cerebellar cortex support previously reported results from mice and rats (Quackenbush *et al*., 1990; Hadj-Sahraoui *et al*., 2001). The cause of age-related loss of PC dendrites is a topic of much current research. The most interesting part is the effect of an age-related decrease in input from parallel fibres of the granule cells to the PCs (Dlugos and Pentney, 1994; Huang *et al*., 1999; 2006b). The parallel fibres that bifurcate from the axons of granule cells play a key role in regulating PC response properties (Huang *et al*., 2006a; Zhang *et al*., 2010) and interact with PCs reciprocally in the cerebellar circuitry (Kim *et al*., 2002; Heck and Sultan, 2002). It is, therefore, reasonable to speculate that age-related loss of parallel fibres (Huang *et al*., 1999) or granule cells (Zhang *et al*., 2006) may trigger the dendritic loss in ageing PCs (Zhang *et al*., 2010). Furthermore, Grill and Riddle (2002) proposed that an age-related decline in trophic support from the brain vasculature contributes to neuronal dendritic regression. As an alternate hypothesis, the noticeable swellings and nodulations in the ageing dendritic segments found in this and the other studies (Chen and Hillman, 1999) may hinder substance transport to the distal terminals. A loss of trophic factors may account for the preferential loss of distal branchlets during dendritic ageing (Quackenbush *et al*., 1990).

Age-related changes on the dendritic spines

Dendritic spines play a vital role in receiving neuronal input. Thus, any alterations in the dendritic spines would greatly influence the signaling process (Duan *et al*., 2003; Penzes *et al*., 2009). Ageing results in significant morphological alterations to the dendritic spines, such as a decline in the number of spines (Duan *et al*., 2003), aberrance in the geometry of the spines (Andrews *et al*., 1996), and degeneration of the inner organelles (Itzev *et al*., 2003). Our results show a 40.7% decline in spine density on the branchlets of old cerebellar PCs, indicating a significant loss of synapses during ageing. This loss would attenuate transmission of neuronal information and may induce age-related dysfunctions in the senescent cerebellum. The cause for spine defoliation on existing arborizations is still under investigation. One possible explanation may be the loss of presynaptic terminals during ageing (Wong *et al*., 2000). Recent reports suggest that an age-related decline in the expression of microtubule-associated protein 2 (MAP2), a marker of dendrites, correlates with a significant contraction of ageing

spines and associated spine number decrease (Himeda *et al*., 2005; Shimada *et al*., 2006; Shiozaki *et al*., 2008). As the dendritic spines of Purkinje cells associates with motor learning (Lee *et al*., 2007), age-related alteration in the dendrites might also impair cerebellar motor learning abilities in the aged individuals.

Age-related changes to the somatic appearance of PCs

The ageing process results in significant alterations in the neuronal body. Previous reports have demonstrated a significant decrease of 33% in the PC somatic volume in aged human beings (Andersen *et al*., 2003), and significant age-related alterations in PC ultrastructure in mice and rats (Monteiro, 1991; Shiozaki *et al*., 2008). We also found significant alterations in the somatic structure of cat PCs during normal ageing. The reduction of Nissl granules, which stain the rough endoplasmic reticulum and free ribosomes, suggests a significant decline in substance synthesis, possibly resulting in trophic deficiencies in the aged PCs. Significant shrinkage of both the soma and nucleus from this study may be caused by loss of the cytoplasmic matrix and collapse of cellular organelles (Monteiro, 1991; Monteiro *et al*., 1992), which would lead to neuronal dysfunction during ageing.

In summary, our study shows that cat cerebellar PCs undergo significant morphological alterations during the normal ageing process. Diminution of dendritic arborizations and a decrease in the number of dendritic spines may attenuate neuronal information input, while degeneration of the somatic configuration may reduce substance synthesis and energy provision. These alterations may lead to cerebellar dysfunctions such as balance control, motor coordination and motor learning in senescent organisms.

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