Age-related changes of structures in cerebellar cortex of cat

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We studied the structures of the cerebellar cortex of young adult and old cats for age-related changes, which were statistically analysed. Nissl staining was used to visualize the cortical neurons. The immunohistochemical method was used to display glial fibrillary acidic protein (GFAP)-immunoreactive (IR) astrocytes and neurofilament-immunoreactive (NF-IR) neurons. Under the microscope, the thickness of the cerebellar cortex was measured; and the density of neurons in all the layers as well as that of GFAP-IR cells in the granular layer was analysed. Compared with young adult cats, the thickness of the molecular layer and total cerebellar cortex was significantly decreased in old cats, and that of the granular layer increased. The density of neurons in each layer was significantly lower in old cats than in young adult ones. Astrocytes in old cats were significantly denser than in young adult ones, and accompanied by evident hypertrophy of the cell bodies and enhanced immunoreaction of GFAP substance. Purkinje cells (PCs) in old cats showed much fewer NF-IR dendrites than those in young adults. The above findings indicate a loss of neurons and decrease in the number of dendrites of the PCs in the aged cerebellar cortex, which might underlie the functional decline of afferent efficacy and information integration in the senescent cerebellum. An age-dependent enhancement of activity of the astrocytes may exert a protective effect on neurons in the aged cerebellum.

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1. Introduction

The cerebellum is a region of the brain that is important for a number of motor and cognitive functions, including motor learning, time perception and precise movement (Thach 1998; Salman 2002; Matsumura *et al* 2004). The cortex has become the focus of particularly intense research because it is presumed to be responsible for planning movement and adapting to special conditions, and is also involved in storing memories over various time-periods (Gray *et al* 1993; Attwell *et al* 2002). In fact, aspects of structural configuration, neuronal elements, fibre communications and neurotransmitters in the cerebellar cortex have been extensively investigated. The cerebellar cortex undergoes age-related morphological changes, which have been studied mainly in rats, human beings, *Macaca nemestrinas* and gerbils (Nandy 1981; Amenta *et al* 1991; Hara *et al* 1992; Sabbatini *et al* 1999; Jernigan *et al* 2001). The results of these studies show that in senile individuals there is a decline in the thickness of the cerebellar cortex as well as loss of neurons, and hypertrophy and hyperplasia of the astrocytes. The cat, as a kind of higher order mammal, is widely raised as a pet so that it is easily available and frequently used in various biology studies. Previous researches indicate that the cat's brain does undergo considerably functional and/or morphological changes during ageing (Hua *et al* 2006; Iontov and Shefer 1981; Zhang *et al* 2000). In view of this, we compared the thickness of the cortical layers, density of the neurons, number and size of the astrocytes in the granular layer, as well as the dendritic arborizations of Purkinje cells (PCs) in

Keywords. Cat; cerebellar cortex; age-related changes; glial fibrillary acidic protein; neurofilament; astrocyte.

Abbreviations used: ANOVA, analysis of variance; CNS, central nervous system; DAB, diaminobenzidine; GFAP, glial fibrillary acidic protein; GFAP-IR, GFAP-immunoreactive; NF-IR, neurofilament-immunoreactive; PBS, phosphate buffer saline; PC, Purkinje cell

the cerebellum of young adult and old cats to look for any age-related changes in the cerebellar cortex of the cat and determine how this change occurs. We also collected data for exploring the mechanisms of functional retrogression (such as diminution in motor control and motor learning) underlying these changes in the senescent cerebellum (Caston *et al* 2003; Hilber and Caston 2001; Hue *et al* 2004).

2. Materials and methods

2.1 *Animals and tissue treatment*

Young adult cats (2–3 years old, $n = 4$) and old cats (12–13 years old, $n = 4$) were used in this study. All the subjects were healthy domestic cats with a history of age and healthcare filed by veterinarians in the Animal Hospital. The animals were deeply anaesthetized with ketamine hydrochloride (40 mg/kg i.p.) and 0.9% NaCl was perfused through the ascending aorta till the liver became pale, followed immediately by 500 ml of a fixative solution containing 4% paraformaldehyde in 0.1 M PBS (pH 7.4). After perfusion, the skull was opened and the cerebellar hemispheres were dissected out. For convenience of comparison among individuals, an equivalent area in the anterior lobe of each cat was chosen and cut into about 1 cm in length. After further fixation for 20–24 h in the perfusing fixative, the samples were washed in PBS, dehydrated in ethanol, transparentized with xylene and embedded in paraffin. Consecutive coronal sections 8 μm in thickness were cut and mounted for histological staining and immunostaining on microscope slides that had been treated with 3-aminopropyl triethoxysilane (APES; Sigma, USA; dilution: 1:50 in acetone).

2.2 *Histological staining and immunohistochemical staining*

In each series, 30 sections were taken at intervals of about 200 μm for immunohistochemical labelling of neurofilaments (NF) and glial fibrillary acidic protein (GFAP). Adjacent sections were used for Nissl staining (0.5% thionine 37°C, 40 min) as well as for immunohistochemical control samples. Sections were deparaffinized in xylene, hydrated through a graded series of ethanol and washed for 5 min in distilled water. After treatment at room temperature for 10 min with 3% hydrogen peroxide to inhibit endogenous peroxidase activity, sections were treated with the blocking kit of Biotin/Avidin System reagents (Vector Laboratories, USA). The avidin solution was mixed with 10% goat serum to suppress background staining. Sections were then incubated for 1 h at 37°C with anti-GFAP (dilution 1:400) or NF (dilution 1:200) monoclonal antibody (Sigma) in PBS. Thereafter, sections were washed 3 times in PBS for 10 min each (the same washing procedure was performed between each of the following steps) and incubated for 30 min at 37°C with biotinylated anti-mouse IgG (Sigma) antibody diluted 1:800 in PBS. Then, sections were treated with a preformed avidin–biotin–peroxidase complex for 30 min at 37°C. After washing in tap water, sections were incubated for 10 min in 0.05% 3,3′-diaminobenzidine (DAB)/0.01% hydrogen peroxidase in PBS solution (pH 7.4). For the negative controls, the anti-GFAP/NF antibody was replaced by mouse IgG.

2.3 *Quantitative analysis*

Under an Olympus BX51 microscope, quantitative measurement was done by technicians who were not provided with information regarding the age of the samples. In each Nissl stained slide, at a magnification of 100×, the thickness of the molecular and granular layers as well as that of the total cortex was measured randomly for 8 visual fields at the lobular flanks where the cortical layers were almost parallel to the panel of white matter. The number of PCs was counted using an eyepiece micrometer and their linear density calculated (cell number/mm). At 1000×, the number of neurons in the molecular layer and granular cells were counted in a calibrator (50 μm×50 μm) and the density (cells/mm^2) was calculated. The criterion for acceptance as a neuron was a clear differentiation from background staining of a soma and the presence of a nucleus. The astrocytes in the granular layer labelled by GFAP immunoreaction were also examined and the density calculated as above. Age-related changes in the dendritic arborizations of PCs were observed through NF immunoreaction. The number of branches of apical dendritic arborizations was counted and the average of 8 randomly selected PCs in each slide was considered. All the data were expressed as mean ± SEM. The significance of the differences between the means was evaluated by a two-way analysis of variance (ANOVA), and a P value of < 0.05 was considered significant.

3. Results

3.1 *Nissl staining*

From the outside to the inside, the cerebellar cortex is distinctly divided into 3 layers: molecular layer, PC layer and granular layer. There was a significant difference between young adult and old cats when the average thickness of the molecular and granular layers as well as that of the total cortex was compared ($P < 0.01$) (figures 1, 4). Compared with young adults, the total cortical thickness in old cats decreased by 7.05% and that of the molecular layer by 10.93%. In the granular layer, the thickness increased by 7.82% (table 1). The density of neurons in the various

layers of old cats also showed statistical significance ($P < 0.01$); in the molecular layer the density decreased by 26.38%, in the granular layer by 22.57% and the linear density of PCs by 25.63% (table 1).

It should be noted that our measurements of layer thickness might be biased to an unknown degree by a factor ignored in the tissue preparation, the shrinkage, which is higher in young tissues than in old ones. However, this factor may not have affected the results significantly, because we roughly estimated the shrinkage parameter of each cerebellum by measuring the thickness of a lobular flank in fresh tissue and in Nissl stained sections (following the same procedure described in §2). There was no significant difference in shrinkage of tissues between young and old cats. This has also been proved in the human cerebellum by different methods (Braendgaard *et al* 1990; Andersen *et al* 2003).

3.2 *GFAP immunostaining*

The sections of the cerebellar cortex processed for GFAP immunoreactivity showed dark-brown astrocytes of different sizes in the granular layer. Compared with those of young adults, astrocytes in old cats not only displayed significant hypertrophy and hyperplasia but the intensity of GFAP immunoreaction was also markedly increased (figures 2, 5 and table 1). The processes of adjacent astrocytes were interwoven with each other, especially in old cats. Near the bodies of the PCs, a different kind of astrocyte was seen, called Bergmann glial cells. These send vertically oriented processes that reach the outer portions of the molecular layer. However, no evident differences in the general morphological characteristics were detected between young adult and old cats (figures not shown).

3.3 *NF immunostaining*

The sections processed for NF immunostaining showed a positive immunoreaction in the cerebellar layers of cats of both age groups, but the most obvious immunoreaction was in the PCs, where the peroxidase reaction product was deposited heavily in the dendrites and perikaryon. Compared with young adult cats, a dramatic reduction in apical dendritic arborizations was seen in old cats (figures 3, 6), with a significant loss of 58.26% of their number (table 1).

4. Discussion

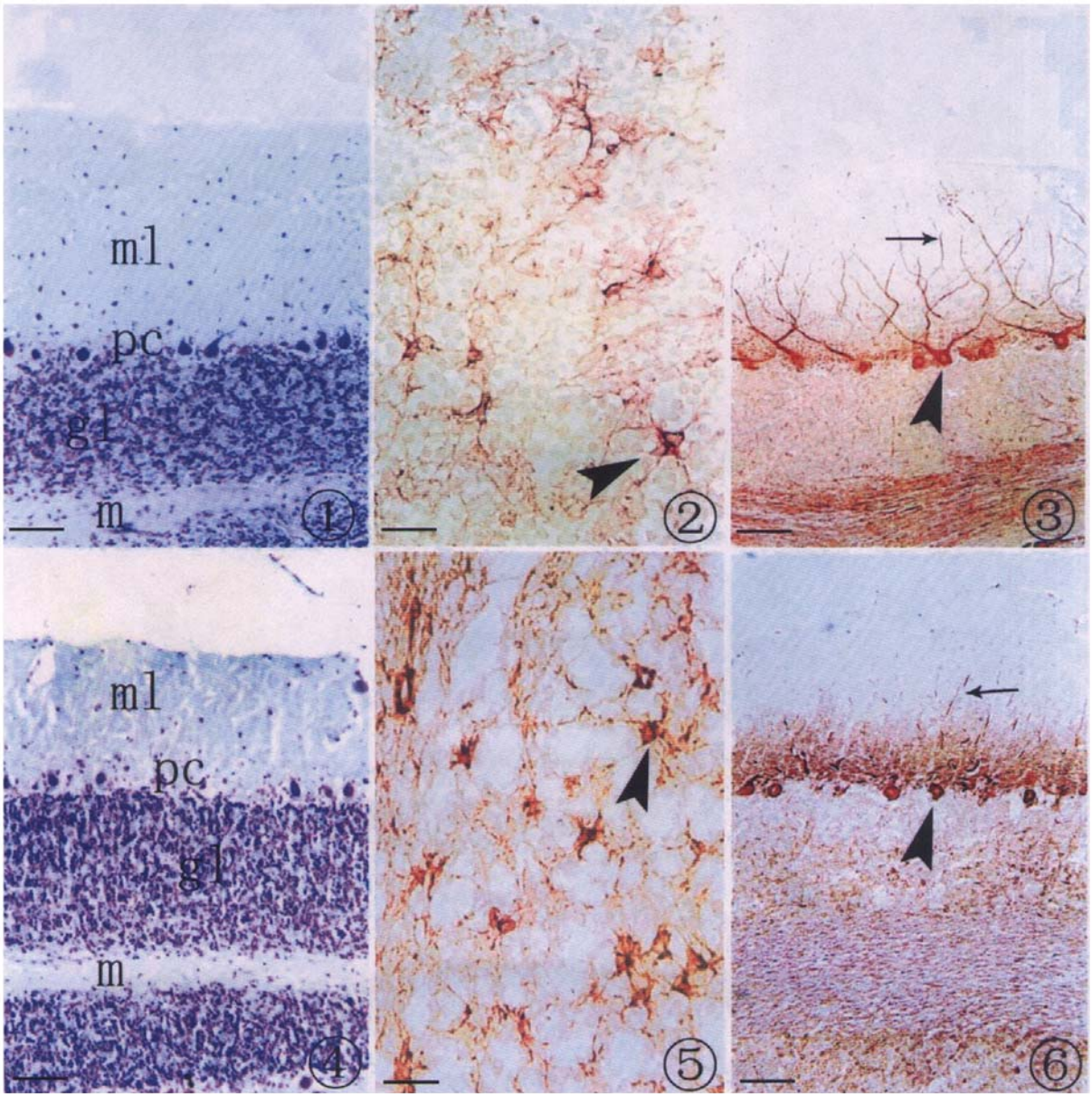
4.1 *Age-related changes in cortical thickness and density of neurons*

Numerous studies have documented the relationship between morphological change and functional decline in the central nervous system (CNS). The loss of cortical thickness and neurons is viewed as the main cause of decline in brain function during ageing. In our study, we observed a significant decrease in thickness of the molecular layer, which ultimately led to a decrease in total cortical thickness. The loss of thickness in the molecular layer was caused largely by the progressive defoliation of dendrites in the ageing PCs (Hadj-Sahraoui *et al* 2001), whereas a slight increase in the thickness of the granular layer was mainly due to proliferation of the astrocytes in it. The exact functional relevance of the decrease in cortical thickness is not clear, but a reduction in the number of neurons leading to global motor behaviour impairment has been largely proved (Hilber and Caston 2001; Caston *et al* 2003). The present study showed a large amount of neuronal loss in the molecular layer of the aged cerebellum. These neurons (basket cells and stellate cells) make inhibitory synapses with the PCs. Such a loss may weaken the inhibitory stimuli to the PCs. The presence of age-related changes in the granular cells is disputed; some researches reported no age-related changes in aged *Macaca nemestrina* (Nandy 1981) and rat (Bakalian *et al* 1991), while other authors found significant changes in aged rats (Amenta *et al* 1991) and humans (Renovell *et al* 1996).

Table 1. Morphological parameters of the cerebellar cortex in the young adult and old cats[a].

	Old cat (n = 4)	Young adult cat (n = 4)
The thickness of cerebellar cortex (μm)	611.22±60.50	657.58±49.31[b]
The thickness of molecular layer (μm)	274.10±46.45	307.73±43.81[b]
The thickness of granular layer (μm)	316.13±55.84	293.19±49.69[b]
Number of neurons in molecular layer/mm^2	734.71±153.02	998.03±175.82[b]
Number of granular cells/mm^2	10890.32±1399.84	14064.52±1873.60[b]
Number of PCs/mm	10.01±2.64	13.46±2.72[b]
Branch of the apical dendrites/PC	4.81±1.33	11.52±2.67[b]
Number of astrocytes in granular layer/mm^2	697.11±79.15	427.15±76.24[b]

[a]Data are the means ± SEM and were obtained as indicated in § 2.3.
[b]$P < 0.01$ vs. old cat.



Figures 1–6. (**1**, **4**) Sections of the cerebellar cortex of a 2-year-old cat (young adult) and a 12-year-old cat (old) stained with thionine. The old cat showed a decrease in total cortical thickness and the molecular layer, but an increase in thickness of the granular layer. The density of neurons in the various layers is also decreased in the old cat. ml, molecular layer; pc, Purkinje cell; gl, granular layer; m, medulla. **(2, 5)** Sections processed for GFAP immunohistochemistry. Cell bodies of GFAP-IR astrocytes (arrowhead) were found in the granular layer. In the old cat not only did the GFAP-IR astrocytes increase in size and number, but also in the intensity of GFAP immunoreaction. **(3, 6)** Sections processed for NF immunohistochemistry. PCs (big arrowhead) displayed stronge immunoreaction in the two age groups. The dendritic arborizations (small arrowhead) of the PCs showed an evident atrophy in the old cat. [Bar scale: **(1, 3, 4, 6)** 100 μm; **(2, 5)** 25 μm]. **(1–3)** Young cat. **(4–6)** Old cat

However, Andersen *et al* (2003) reported that both these phenomena were observed in the ageing human cerebellum within different zones. Our results showed a significant decrease of 22.57% in the density of granular cells in the ageing cat (anterior lobe). Considering that we found a large loss of neuron density (22.57%) and only a slight increase in thickness (7.82%), we postulate that the granular cells undergo age-related neuronal loss. The axons of the granular cells bifurcate into parallel fibres, which send major excitatory inputs to the PCs. The loss of granular cells may

directly lessen the excitatory inputs to the PCs in aged animals, and because the granular cells (along with their parallel fibres) are related to the storage of memories over different time-periods, regulate the dynamics of movement and allow bidirectional changes in movement amplitude (Attwell *et al* 2002; Boyden *et al* 2004), their loss may greatly affect motor learning in ageing individuals. PCs are the sole efferent neurons in the cerebellar cortex that are prone to ageing. It appears from this and other studies that the number of PCs is significantly reduced in ageing animals (Nandy 1981; Hara *et al* 1992).

Loss of neurons is not the only mechanism by which age may affect the cerebellum. A reduction in the dendritic branches of PCs may also interfere with information exchange in spite of the presence of surviving cell bodies. Our results are in agreement with Hadj-Sahraoui *et al* (2001) that the dendritic arborizations of PCs are obviously atrophied in senile animals. PCs receive synaptic inputs mainly from parallel fibres through their dendritic network and send projections to the deep cerebellar and vestibular nuclei via their long axons. The loss of arborization in the PCs could directly cut down the amount of information input, reduce afferent efficacy, affect information integration and signal transmission in the aged cerebellum. These changes would lead to a reduction in motor control and accurate motor coordination (Hilber and Caston 2001; Hue *et al* 2004; Porras-Garcia *et al* 2005).

4.2 *Age-related changes in GFAP immunoreaction*

GFAP is a special marker for astrocytes. It has been reported that the number and size of GFAP-IR astrocytes in the CNS increases following certain types of brain injury and in various pathological conditions (Davis *et al* 2002; German *et al* 2002; Sulkowski *et al* 2002). GFAP-IR astrocytes are also sensitive to ageing and display age-related proliferation, which has been investigated in many mammalian species (Jalenques *et al* 1997; Sabbatini *et al* 1999; Wu *et al* 2005). The cerebellar cortex has a particular glial architecture, with large astrocytes located in the granular layer. Age-related changes in the astrocytes of the cerebellar cortex have been reported only in the rat (Sabbatini *et al* 1999). The findings of our study provide evidence that ageing is also accompanied by changes in the astrocytes of the cat cerebellar cortex, including an increase in their size and number, and intensity of immunoreactivity. Our finding that there is an obvious loss of neurons in the ageing cerebellar cortex suggests that proliferation of the astrocytes might be triggered by the neurodegenerative changes during ageing. The significance of age-related changes in the expression of GFAP in the astrocytes is not clear. Gliosis may be a reaction to intrinsic modifications in the CNS such as degeneration of neighbouring dendrites or entire neurons (Geinisman *et al* 1978; Niquet *et al* 1996), and may be an attempt to promote long-term neuronal survival during ageing through the production of neurotrophic factors (Schmalenbach and Muller 1993). Since trophic interactions exist between astrocytes and neurons (Araque *et al* 2001; Kirchhoff *et al* 2001), we suggest that the hypertrophy and hyperplasia of the astrocytes may slow down the dying process of ageing neurons and the degeneration in the cerebellar cortex. We also suggest that this may be a response to offset the decrease in cortical thickness and fill up the gap caused by the loss of neurons.

In summary, the cerebellar cortex undergoes age-related structural changes. Our findings in the cat confirm the results in other species, indicating a common phenomenon for age-related structural changes in the mammalian cerebellar cortex. The loss of cortical neurons and the reduction in dendritic arborizations of the PCs might be the main reason for the functional decline in motor control and motor learning in senescent animals. The proliferation of astrocytes may exert a protective effect on neurons and offset the decrease in cortical thickness of the aged cerebellum.

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References

Amenta F, Del Valle M, Vega J A and Zaccheo D 1991 Age-related structural changes in the rat cerebellar cortex: Effect of choline alfoscerate treatment; *Mech. Ageing Dev.* **61** 173–186

Andersen B B, Gundersen H J and Pakkenberg B 2003 Aging of the human cerebellum: A stereological study; *J. Comp. Neurol.* **466** 356–365

Araque A, Carmignoto G and Haydon P G 2001 Dynamic signaling between astrocyte and neurons; *Annu. Rev. Physiol.* **63** 795–813

Attwell P J, Cooke S F and Yeo C H 2002 Cerebellar function in consolidation of a motor memory; *Neuron* **34** 1011–1020

Bakalian A, Corman B, Delhaye-Bouchaud N and Mariani J 1991 Quantitative analysis of the Purkinje cell population during extreme ageing in the cerebellum of the Wistar/Louvain rat; *Neurobiol. Aging* **12** 425–430

Boyden E S, Katoh A and Raymond J L 2004 Cerebellum-dependent learning: The role of multiple plasticity mechanisms; *Annu. Rev. Neurosci.* **27** 581–609

Braendgaard H, Evans S M, Howard C V and Gundersen H J 1990 The total number of neurons in the human neocortex unbiasedly estimated using optical dissectors; *J. Microsc.* **157** 285–304

Caston J, Hilber P, Chianale C and Mariani J 2003 Effect of training on motor abilities of heterozygous staggerer mutant (Rora(+)/Rora(sg)) mice during aging; *Behav. Brain Res.* **141** 35–42

Davis S, Thomas A, Perry R, Oakley A, Kalaria R N and O'Brien J T 2002 Glial fibrillary acidic protein in late life major depressive disorder: An immunocytochemical study; *Exp. Toxicol. Pathol.* **54** 31–38

Geinisman Y, Bondareff W and Dodge J T 1978 Hypertrophy of astroglial processes in the dentate gyrus of the senescent rat; *Am. J. Anat.* **153** 537–543

German D C, Liang C L, Song T, Yazdani U, Xie C and Dietschy J M 2002 Neurodegeneration in the Niemann–Pick C mouse: Glial involvement; *Neuroscience* **109** 437–450

Gray C, Perciavalle V and Poppele R 1993 Sensory responses to passive hindlimb joint rotation in the cerebellar cortex of the cat; *Brain Res.* **622** 280–284

Hadj-Sahraoui N, Frederic F, Zanjani H, Delhaye-Bouchaud N, Herrup K and Mariani J 2001 Progressive atrophy of cerebellar PC dendrites during aging of the heterozygous staggerer mouse (Rora+/sg); *Brain Res. Dev. Brain Res.* **126** 201–209

Hara H, Onodera H, Kato H and Kogure K 1992 Effects of aging on signal transmission and transduction systems in the gerbil brain: Morphological and autoradiographic study; *Neuroscience* **46** 475–488

Hilber P and Caston J 2001 Motor skills and motor learning in Lurcher mutant mice during aging; *Neuroscience* **102** 615–623

Hua T M, Li X R, Zhou Y F, Wang Y C and Leventhal A G 2006 Functional degradation of visual cortical cells in old cats; *Neurobiol. Aging* **27** 155-162

Hue O A, Seynnes O, Ledrole D, Colson S S and Bernard P L 2004 Effects of a physical activity program on postural stability in older people; *Aging Clin. Exp. Res.* **16** 356–362

Iontov A S and Shefer V F 1981 Age-related changes in the dendrites in the cat cerebral cortex (an electron microscopic study); *Arkh. Anat. Gistol. Embriol. (Russian)* **81** 11–16

Jalenques I, Burette A, Albuisson E and Romand R 1997 Age-related changes in GFAP- innunoreactive astrocytes in the rat ventral cochlear nucleus; *Hearing Res.* **107** 113–124

Jernigan T L, Archibald S L, Fennema-Notestine C, Gamst A C, Stout J C, Bonner J and Hesselink J R 2001 Effects of age on tissues and regions of the cerebrum and cerebellum; *Neurobiol. Aging* **22** 581–594

Kirchhoff F, Dringen R and Giaume C 2001 Pathways of neuron–astrocyte interactions and their possible role in neuroprotection; *Eur. Arch. Psychiatry Clin. Neurosci.* **251** 159–169

Matsumura M, Sadato N, Kochiyama T, Nakamura S, Naito E, Matsunami K, Kawashima R, Fukuda H and Yonekura Y 2004 Role of the cerebellum in implicit motor skill learning: A PET study; *Brain Res. Bull.* **63** 471–483

Nandy K 1981 Morphological changes in the cerebellar cortex of aging *Macaca nemestrina*; *Neurobiol. Aging* **2** 61–64

Niquet J, Gillian A, Ben-Ari Y and Represa A 1996 Reactive glial cells express a vitronectin-like protein in the hippocampus of epileptic rats; *Glia* **16** 359–367

Porras-Garcia E, Cendelin J, Dominguez-del-Toro E, Vozeh F and Delgado-Garcia J M 2005 Purkinje cell loss affects differentially the execution, acquisition and prepulse inhibition of skeletal and facial motor responses in Lurcher mice; *Eur J Neurosci.* **21** 979–988

Renovell A, Giner J and Portoles M 1996 Loss of granule neurons in the aging human cerebellar cortex; *Int. J. Dev. Biol.(Suppl.)* **1** 193–194

Sabbatini M, Barili P, Bronzetti E, Zaccheo D and Amenta F 1999 Age-related changes of glial fibrillary acidic protein immunoreactive astrocytes in the rat cerebellar cortex; *Mech. Ageing Dev.* **108** 165–172

Salman M S 2002 The cerebellum: It's about time! But timing is not everything – new insights into the role of the cerebellum in timing motor and cognitive tasks; *J. Child Neurol.* **17** 1–9

Schmalenbach C and Muller H W 1993 Astroglia–neuron interactions that promote long-term neuronal survival; *J. Chem. Neuroanat.* **6** 229–237

Sulkowski G, Bubko I, Struzynska L, Januszewski S, Walski M and Rafalowska U 2002 Astrocytic response in the rodent model of global cerebral ischemia and during reperfusion; *Exp. Toxicol. Pathol.* **54** 31–38

Thach W T 1998 A role for the cerebellum in learning movement coordination; *Neurobiol. Learn. Mem.* **70** 177–188

Wu Y, Zhang A Q and Yew D T 2005 Age related changes of various markers of astrocytes in senescence-accelerated mice hippocampus; *Neurochem. Int.* **46** 565–574

Zhang J H, Sampogna S, Morales F R and Chase M H 2000 Age-dependent changes in the midsized neurofilament subunit in sensory–motor systems of the cat brainstem: An immunocytochemical study; *J. Gerontol. A. Biol. Sci. Med. Sci.* **55** 233–241

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