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Differential response of AMPA and NMDA glutamate receptors of Purkinje cells to aging of the chicken cerebellum

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ABSTRACT

Aging can lead to cognitive, affective, learning, memory and motor deficits. Since the cerebellum and glutamatergic neurotransmission are involved in several of those functions, the present work aimed at studying the expression of AMPA and NMDA glutamate receptor subunits in the chick cerebellum during aging. Young (30 days old) and aged (ca. 4 years old) chickens (*Gallus gallus*) were used in order to evaluate the expression of GluR1, GluR2/3 and NR1 subunits. The cerebella of young and aged chickens were subjected to immunohistochemical and immunoblotting techniques. Numbers of GluR1, GluR2/3 and NR1-positive cells and optical density of the immunoblotting data were analyzed and submitted to statistical analysis using ANOVA and the Bonferroni post hoc test. Mean density of Purkinje cells stained for Giemsa, GluR1, GluR2/3 and NR1 in the cerebellum all showed a statistically significant decrease in aged animals when compared to the young animals (Giemsa, $P < 0.01$; GluRs and NR1, $P < 0.03$). However, the ratio of GluR1 and GluR2/3-positive Purkinje cells in relation the total number of Purkinje cells found in each time point decreased with aging (ca. 10%), whereas the ratio of NR1-positive cells increased (ca. 9%). The immunoblotting data showed a significant decrease of GluR1 (ca. 66%) and GluR2/3 (ca. 55%) protein expression with aging, but did not reveal changes for NR1. Our data suggest that aging can lead to differential changes in the pattern of expression of glutamate receptor subunits, which can underlie at least part of the cognitive and motor disorders found in aged animals.

1. Introduction

Aging could be defined as a dynamic and progressive physiological process that occurs asynchronically in several brain areas and include morphological, biochemical and physiological changes. Behaviourally, aged animals show impairment of cognitive (learning and memory), emotional (motivation) and motor functions [10]. Morphological changes in aged brains include shrinkage and loss of neurons in several areas, such as the neocortex, hippocampus, substantia nigra [5] and cerebellum [20,1]. In particular, numbers of Purkinje cells in older animals have been shown to be reduced. At least in mice, this decline of Purkinje cell numbers appears to be uniform throughout the cerebellar cortex [20], but in the human cerebellum it occurs especially in the anterior lobe [2]. These anatomical changes contribute to the well-known cognitive and motor decline associated with age, but mechanisms of pathophysiology and neurodegeneration during aging are unknown. One such mechanism may involve excitotoxicity produced by endogenous glutamate.

It has been suggested that glutamatergic transmission is involved in a number of cerebral functions, such as learning and memory, emotion and motivation [4], and motor functions [18]. In the cerebral cortex and striatum, several studies have shown unchanged glutamate release but a reduced glutamate uptake capacity in aged rats [17,19]. This could be interpreted as a loss of high-affinity glutamate transporters in glutamatergic terminals in these brain areas.

The expression and distribution of neurotransmitter receptors are also affected during aging. In particular, the number of neurons expressing certain ionotropic glutamate receptor subunits of the AMPA and NMDA subtypes is significantly reduced during aging [8,13]. Quantitative analysis of the distribution of GluR2 and NR1 in long and short corticocortical connections in monkeys, and several memory-associated structures in rats, revealed a down-regulation of the expression of both receptor types with aging [8]. Decreased NMDA receptor density has also been described in most cortical areas, striatum and hippocampus [13,21]. However, a study employing RT-PCR assays has not revealed differences in

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alternative splicing of GluR1–3 (flop/flip ratios) in the cortex, hippocampus, hypothalamus, and striatum, between young and aged CBA/J mouse [3]. In addition, autoradiographic [^{3}H]MK-801 and [^{3}H]CNQX binding in the Purkinje cell layer rose from the foetal period to reach a plateau by the age of 10 years and there was no apparent further change during aging in humans [6].

The studies mentioned above have investigated age-related changes of AMPA and NMDA receptor subunit expression in different brain regions, and the overall findings are quite controversial. Since the cerebellum and glutamatergic neurotransmission are involved in the processing of motor learning, motor coordination and cognitive functions, all of which become altered with age [16], the present work aimed at studying the expression of the GluR1–3 subunits of the AMPA receptors and NR1 subunits of the NMDA receptors in the Purkinje cells of the aging chick cerebellum.

2. Materials and methods

Ten young adult (3–4-week-old), and 10 aged (about 4 years old) free range chickens (*Gallus gallus*) were anesthetized with intramuscular injections of xylazine (1 mg/100 g of body weight) and ketamine (5 mg/100 g of body weight) for immunohistochemical and immunoblotting procedures. All animal procedures were performed following the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Society for Neuroscience Guidelines. The experimental protocol was also approved by the Ethics Committee of the City University of São Paulo, Brazil. For immunohistochemical experiments, five animals from each age (young and aged) were transcardially perfused with buffered saline followed by a solution of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4). The brains were postfixed in 4% PFA for 5 h, and placed in a 30% sucrose solution made in PB for cryoprotection. Brains were blocked in a tissue freezing medium (TBS, Durham, NC) and sectioned in the sagital plane at 30 μm on a cryostat. The sections were sequentially collected into six separate compartments, and stored at 4 °C until processed. Free floating sections were incubated overnight with commercial antibodies (Chemicon International, Temecula, CA) against GluR1, an antibody that recognizes a common epitope to GluR2 and GluR3 subunits (GluR2/3), and an antibody against NR1. Their concentrations varied from 1:250 to 1:1000, in a solution containing 5% normal serum in 0.3% Triton X-100 in 0.1 M PB, pH 7.4. The sections were then rinsed 3 times in PB (10 min each), and incubated with a biotinylated secondary antiserum (Jackson ImmunoResearch Labs, Westgrove, PA) diluted at 1:250 in PB with 0.3% Triton X-100 at 22 °C for 90 min. After three washes in PB, the sections were processed with the avidin–biotin–peroxidase complex (ABC Elite kit, Vector Labs., Burlingame, CA) for 1 h. Labeling was developed with 0.05% diaminobenzidine tetrahydrochloride and 0.03% (final concentration) hydrogen peroxide. The sections were rinsed in PB, mounted on subbed slides, and the labeling was intensified by immersion in a 0.05% solution of osmium tetroxide in PB. The sections were dehydrated, cleared in xylene, and coverslipped with Permount (Fisher Sci. Co., Pittsburg, PA). Controls for the specificity of immunostaining were performed by the omission of the primary antibodies and their substitution for normal rabbit or mouse sera. No staining was obtained in either control condition. The material was then analyzed on a light microscope and the identification of structures of the chick cerebellum was based on a stereotaxic atlas [12,22]. The density of Purkinje cells labeled for GluR1, GluR2/3, and NR1 was measured in five sections from young and aged animals in several lobules (II, IV, VI, and IX) to analyze possible differential patterns between lobules. These lobules represent areas of the paleocerebellum (II and IV), neocerebellum (VI) and archicerebellum (IX), as previously described [22]. Four areas of 10,000 μm^2 from each section were analyzed. Quantification of Purkinje cell abundance was made in sections of young and aged cerebella stained with Giemsa in order to determine the mean density of cells within the cerebellum.

Ten chickens (five from each age group) were deeply anesthetized and quickly decapitated. The cerebella were rapidly collected and homogenized at 4 °C in an extraction buffer (Tris, pH 7.4, 100 mM; EDTA 10 mM; PMSF 2 mM; aprotinin 0.01 mg/ml). The homogenates were subjected to centrifugation at 12,000 × *g* for 20 min, and the protein concentration of the supernatant was determined using a protein assay (Bio-Rad, Hercules, CA). Samples from the homogenate containing 100 μg protein were subjected to a 10% acrylamide gel containing sodium dodecyl sulfate and electrotransferred to nitrocellulose membranes using a Trans-Blot cell system (Bio-Rad). The nitrocellulose membranes were subsequently blocked in blot buffer (150 mM NaCl, 30 mM Tris–HCl buffer, pH 7.4, and 3% bovine serum albumin) for 2 h at 24 °C. Membranes were then incubated with the rabbit antisera against GluR1 (1:250) and GluR2/3 (1:500), and the mouse antiserum against NR1 subunit (1:1000) in blot buffer for 3 h at 24 °C. Loading control with β-actin was conducted in all experiments by using an anti-β-actin antibody (1:10,000) (Sigma, St. Louis, MO). The specifically bound antibody was visualized using a chemiluminescence kit (ECL Kit; Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom). Finally, the blots were densitometrically analyzed with Scion Image 4.0.2 (Scion Corporation, Frederick, MD) and statistically treated as described for immunohistochemistry. Because there were no β-actin changes under our conditions, the optical density of the GluR1, GluR2/3 and NR1 bands was first normalized in relation to the corresponding β-actin bands in each experiment. Subsequently, the normalized data were treated to evaluate protein changes in the young and aged cerebella. The immunohistochemical and immunoblotting data were statistically analyzed with ANOVA and the Bonferroni post hoc test when appropriate (SPSS, version 15.0). A 5% significance level was adopted.

3. Results

Purkinje cells of the chicken cerebellum expressed GluR1, GluR2/3, and NR1 subunits at both ages. Fig. 1 summarizes the results concerning the distribution of GluR1, GluR2/3, and NR1 immunoreactivities in the cerebellum of young and aged animals. No differences were noted in either stage between the different cerebellar lobules examined here.

The expression of GluR1, GluR2/3 and NR1 in Purkinje cells was observed as moderate to intense immunoreactivities in cell bodies and slight to intense staining in the dendritic arborization in the young animals. Despite the fact that overall staining was in some cases uneven, it appeared to substantially decrease in aged animals, going from slight to moderate in the perikarya of Purkinje cells and almost absent to slight in their dendritic arborizations (Fig. 1).

In Giemsa-stained material, we found a significant decrease (ca. 27%) in the number of Purkinje cells ($P < 0.001$) in the aged chicken cerebellum. Purkinje cell numbers were 9.9 ± 2.0 per mm^2 in young chickens and 7.2 ± 2.0 in aged chickens. Mean density of the Purkinje cells labeled for GluR1, GluR2/3, and NR1 in the cerebella presented a statistically significant decrease ($P < 0.03$) in aged animals (Fig. 2). Cerebella from young chickens contain 9.7 ± 1.6 GluR1-positive Purkinje cell per mm^2, 7.7 ± 1.5 GluR2/3-positive cells, and 7.7 ± 1.9 NR1-positive cells, whereas cerebella from aged chickens possess 6.4 ± 1.5 GluR1-positive Purkinje cells (a decrease of 34.1%), 5.7 ± 1.2 GluR2/3-positive cells (a decrease of 25.4%), and 6.2 ± 1.6 NR1-positive (a decrease of 19.8%) cells per mm^2. Therefore, the ratio of Purkinje cells that express GluR1 in relation to the total number of Purkinje cells detected in each time point showed