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(Article begins on next page)

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Context-Dependent Toxicity of Amyloid-β Peptides on Mouse Cerebellar Cells

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Abstract. Alzheimer's disease (AD) is the major cause of dementia in old people. AD pathology is characterized by amyloid-β (Aβ) deposits in several regions of the brain, and links have been hypothesized between Aβ toxicity and apoptosis. Cerebellar granule cells (CGCs) have been widely used as *in vitro* tools for molecular studies correlating apoptosis with AD, although the cerebellum is a relatively spared area of the brain *in vivo*. We have used mixed neuronal-glial cerebellar cultures (NGCCs) and organotypic cerebellar cultures (OCCs) obtained from postnatal mice to assess the toxic effect of the Aβ oligomer 1-40 ($A\beta_{1-40}$) after propidium iodide uptake *in vitro*. Our results demonstrate that NGCCs, which are primarily composed of CGCs, are resistant to $A\beta_{1-40}$ challenge (5–10 f.LM) when cultured in physiological (5 mM) extracellular KCl ($[K^+]_e$) concentrations, i.e., in a condition in which CGCs undergo full maturation. Conversely, when 10 f.LM $A\beta_{1-40}$ is given to NGCCs cultured in elevated (25 mM) $[K^+]_e$ (and thus maintained in an immature state), there is a statistically significant increase in cell death. Cell death is by apoptosis, as demonstrated by ultrastructural examination. OCCs are resistant to Aβ challenge in any of the conditions tested (variation of $[K^+]_e$, presence or absence of serum, or addition of the neprilysin blocker phosphoramidon). Altogether these observations lead us to conclude that cerebellar cells in an organotypic context may be less susceptible to damage by Aβ, raising the question whether isolated CGCs are a reliable assay in drug discovery studies of AD.

Keywords: Alzheimer's disease, amyloid, apoptosis, cerebellum, *in vitro* studies, organotypic cultures

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INTRODUCTION

Alzheimer's disease (AD) is a progressive neurode-generative disease and the major cause of dementia in old people. AD pathology is characterized by amyloid-β (Aβ) deposits in certain

regions of the brain in five subsequent phases, and involves the neocortex (phase I); the allocortical brain regions (phase II); the diencephalic nuclei, the striatum, and cholinergic nuclei of the basal forebrain (phase III); a number of brainstem nuclei (phase IV); and the cerebellum (phase V) [1]. The cerebellum is apparently spared from significant Aβ toxicity, although plaques are also found in this area of the brain [2] in the final stage of the disease and many patients die before reaching this condition. Aβ deposits in the cerebellar cortex are primarily found in the molecular layer, much less frequently in the granule cell layer [1], and take the form of diffuse plaques composed of non-fibrillar Aβ [3]. In addition, cerebellar neurons have been reported to be more resistant to soluble oligomeric Aβ, which also displays potent neurotoxic effects [4] as compared to other neurons in brain [5].

Cultured cerebellar granule cells (CGCs) have been widely used as *in vitro* models for molecular studies that correlate apoptosis with AD [6]. Numerous lines of evidence have, in fact, linked AD neurode-generation with apoptosis [7]. In fact, it has been suggested that caspases represent a bridge between amyloid and tau Alzheimer pathologies, i.e., Aβ pep- tides activate caspases which, in turn, cleave tau, and, via phosphorylation of tau, initiate tangle pathology in both AD and other tauopathies [8]. In addition, data are accumulating in support for a role of intracellular calcium dysregulation [9] and disturbances in beclin 1-mediated autophagy [10] in AD.

We have previously studied apoptosis in the normal postnatal cerebellum. Studies *in vitro* on organotypic cerebellar cultures (OCCs) have helped us to shed some light on the mechanisms of programmed cell death affecting CGCs and other types of neurons during the maturation of the cerebellar cortex. In addition, we have recently demonstrated that a calcium-related post-translational regulation of BCL2, one of the most intensively investigated anti-apoptotic factors, acts as a mechanism of neuronal survival in CGCs [11], probably through a beclin 1-mediated autophagic process in normal [12] and diseased [13] conditions. Therefore, OCCs represent a useful model for analysis of the cellular and molecular mechanisms of Aβ toxicity under controlled experimental conditions.

Since the cerebellum is only affected in the last stage of AD in humans, and since rodents are incomplete models [14] in that they only exhibit functional anomalies similar to those observed in the preclinical stages of AD [15], it is important to assess the suitability of current *in vitro/ex vivo* rodent preparations for further studies on Aβ toxicity in cerebellum. There- fore, we have investigated here the response of cultured mouse cerebellar neurons to toxic Aβ peptide fragments. Our results demonstrate a context-dependent toxicity of Aβ not only in relation to species, but also to the type of cultures (dissociated cells versus organotypic), medium composition and the concentration of extracellular potassium.

MATERIALS AND METHODS

All experiments were performed in agreement with the Italian and European Union regulations on animal welfare and have been authorized by the Italian Ministry of Health (ref. 600.8/82.20/AG1826) and the Bioethics Committee of the University of Turin.

Animal and tissue preparation

CD1 mice at postnatal day 6–7 (P6-P7) were euthanized with an overdose of sodium pentobarbital (60 mg/100 g body weight). The brain was quickly removed and placed in ice-cooled Gey's solution supplemented with glucose and antioxidants (for 500 ml: 50% glucose 4.8 mL, ascorbic acid 0.05 g, sodium pyruvate 0.1 g).

Chemicals and general reagents

Eagle Basal Medium, Hanks balanced salt solution, Gey's solution, CMF-Tyrode's solution, ascorbic acid, sodium pyruvate, horse and fetal calf serum, bovine deoxyribonuclease I (DNase I), trypsin 0.05% with EDTA 0.02%, antibiotic antimycotic solution, propid- ium iodide (PI), glucose, phosphoramidon, cytosine β-D-arabinofuranoside (ARA-C), and all other chemicals were from Sigma-Aldrich (St. Louis, MO, USA). Neurobasal Medium A and B27 supplement were from Gibco® (Invitrogen, Carlsbad, CA, USA). β-amyloid protein 1-40 and 40-1 were from Bachem (Bubendorf, Switzerland). Millicell-CM inserts were from Millipore (Billerica, MA, USA).

Mixed Neuronal-Glial Cerebellar Cultures (NGCCs)

After isolation from the brain, the cerebellum was minced into small pieces while still submerged in ice-cooled CMF-Tyrode's solution, digested in a solution containing 0.05% trypsin (with 0.02% EDTA) and DNAse I for 14 min at room temperature (RT), triturated in CMF-Tyrode's solution in the presence of 10% fetal calf serum. Cells were finally suspended in: i) 50% Eagle basal medium, 25% horse serum, 25% Hanks balanced salt solution, 0.5% glucose, 0.5% 200 mM L-glutamine, 1% antibi- otic/antimycotic solution (*medium 1*) or ii) 90% Eagle basal medium, 10% fetal calf serum, 0.1% glucose, 0.5% 200 mM L-glutamine, 1% antibiotic/antimycotic solution (*medium 2*). Cells were then plated at a density of 2×10^5 per cm^2 onto poly-lysine (0.05 mg/mL)-

coated multi-well plates. Four days after plating, 10 f.LM ARA-C was added to culture medium, to limit glial proliferation. This type of preparation mainly con- tains CGCs with less than 10% glia [16].

Organotypic Cerebellar Cultures (OCCs)

While the brain was kept submerged in ice-cooled Gey's solution, the cerebellum was isolated and immediately cut in 400 μm-thick parasagittal slices with a McIlwain tissue chopper (Metrohm USA, Riverview, FL, USA). Slices were plated onto Millicell-CM inserts (three slices per insert). Each insert was subsequently placed inside a 35-mm Petri dish containing 1 mL of culture medium, and incubated at 34°C in 5% CO_2 for up to 8 days *in vitro* (DIV). Culture *medium* 3 was: 50% Eagle basal medium, 25% horse serum, 25% Hanks balanced salt solution, 0.5% glucose, 0.5% 200 mM L-glutamine, 1% antibiotic/antimycotic solution. Culture *medium* 4 was: 95% Neurobasal Medium A, 2% B27 supplement 50X, 2% 100 mM L-glutamine, 1% antibiotic/antimycotic solution.
In both NGCCs and OCCs, KCl concentration in media varied from 5 to 25 mM according to different sets of experiments (Table 1). Media were changed twice a week, and sections were allowed to equilibrate to the *in vitro* conditions for at least 6 days before A β treatment.

Incubation with Aβ peptides

Synthetic peptides $A\beta_{1-40}$ (neurotoxic active form) and $A\beta_{40-1}$ (inactive form) were dissolved in sterile distilled water at a concentration of 6 mg/mL and then diluted to 1 mg/mL (231.5 μM) with phosphate- buffered saline (PBS). Prior to use, they were incubated for 5 days at 37°C to induce fibril formation. In OCCs, a 100 μL aliquot of medium supplemented with $A\beta_{1-40}$ or $A\beta_{40-1}$ was applied directly to the top of slices at the beginning of experiment to ensure sufficient exposure to the aggregated peptide. Slices remained submerged with fluid only for a few minutes, as the medium rapidly diffuses to the bottom of dish. In all preparations, $A\beta_{40-1}$ served as an internal control.

Assessment of cell death Propidium iodide (PI) uptake

Cell toxicity was determined in function of the extent of neuronal uptake of the fluorescent dye PI. NGCCs and OCCs were stained with PI (7.5 μM in culture medium) for 20 min, prior to imaging and analysis. Cells exhibiting red fluorescent nuclei were considered as apoptotic.
NGCCs analysis was carried out by directly counting the number of PI-labeled cells in 5 fields (10X magnification, 1.2 mm^2) for each culture dish. The average density of dead cells per mm^2

and the ratio between the number of PI-labeled cells and the total cell number were then calculated for each sample. Values were finally averaged among samples. For OCCs, four inserts (i.e., a total of 12 slices) were subjected to the same treatment in each experiment.

PI uptake was observed with a fluorescence microscope (CTR 6000, Leica Microsystems, Wetlzar, Germany), using a Texas red filter set. Photographs at 10X were obtained with a Leica DFC 310 digital camera. Cell counting and slice areas were calculated with the ImageJ software (NIH, Bethesda, MA, USA).

Electron Microscopy

After treatment with Aβ peptides, 8 DIV NGCCs were fixed with 2% glutaraldehyde and 1% paraformaldehyde in phosphate buffer (PB – 0.1 M, pH 7.4) for 30 min at RT. Cells were then washed in PB, removed from cover slides with a cell scraper and separated by centrifugation for 40 min at 130 g. The pellet was post-fixed in osmium ferrocyanide (1 volume of 2% aqueous osmium tetroxide : 1 volume of 3% potassium ferrocyanide) for 1 h at 4°C, washed in maleate buffer (pH 6) for 20 min at 4°C, dehydrated in increasing concentrations of ethanol, washed in propylene oxide and embedded in Araldite. Ultrathin sections were collected on grids and observed with a CM 10 electron microscope (Philips, Amsterdam, NL).

Statistical analysis

Statistical analysis was performed on raw data only, with the SPSS software (IBM, Armonk, NY, USA). Student's *t*-test for independent groups was used when two conditions at a time were matched. ANOVA was applied to compare data divided in more than two groups. Data were reported as means ± SEM, with *n* indicating the number of samples processed. P values <0.05 were considered statistically significant.

RESULTS

The rationale and results of experiments are summarized in Table 1.

NGCCs

Under conventional culture conditions [17] (Table 1 Exp. 1), incubation with 5 or 10 μM Aβ 1-40

resulted in a clearly evident cell injury at the light (Fig. 1A–D) and electron microscopic level (Fig. 1E–H). After quantitative analysis (Fig. 2A left), the number of dead cells in cultures treated with the active Aβ fragment (5 μM) was significantly higher than in control condition [33.90 ± 2.05 cell/mm^2 (Aβ $_{1-40}$) versus 20.97 ± 2.05 cell/mm^2 (Aβ $_{40-1}$); $n = 5$, $p < 0.01$]. The application of 10 μM β $_{1-40}$ (Fig. 2A right) produced a similar effect [76.51 ± 4.45 cell/mm^2 (Aβ $_{1-40}$) versus 46.19 ± 5.01 cell/mm^2 (Aβ $_{40-1}$); $n = 13$, $p < 0.001$]. Both conditions induced a 60% increase in cell death, thereby underlining a non- significant dose-dependent response to Aβ $_{1-40}$ at 5 and 10 μM.

Table 1

Ab challenge to murine NGCCs and OCCs *in vitro*

	Aβ		**NGCCs**		**OCCs**
Exp #	**challenge**	Culture conditions	Experiment rationale/ RESULTS	Culture conditions	Experiment rationale/ RESULTS
1	Aβ40-1 and Aβ1-40 5 and 10 μM	Medium 1 (horse serum 25mM KCl)-6DIV *followed by* Medium1 Aβ-2 DIV	Testing response to Ab under conventional culture conditions(depolarizing)/ **INCREASE IN PI UPTAKE (Fig. 2A)**	Medium 3(horse serum 5mM KCl) 6 DIV *followed by* Medium 3 (horse serum **5mM KCL) Aβ-2DIV** Aβ 2DIV	Testing response to Ab under conventional culture conditions (depolarizing)/ **NEGATIVE (Fig. 2B)**
2	NO	Medium 1 (horse serum or 25mM KCl) 2, 4 or 8 DIV	Testing survival in depolarizing (25mM)and non-depolarizing (5mM) conditionsin relation to DIV/ **INCREASE IN PI UPTAKE (25mM versus 5mM) at 8 DIV (Fig. 2C)**	Medium 1 (horse serum 5 *or* 25mM KCl) - 8 DIV	Testing survival in depolarizing (25mM) and non-depolarizing (5mM) conditions/ **INCREASE IN PI UPTAKE (25 mM versus 5 mM) at 8 DIV (Fig. 3D)**
3	Aβ40-1 and Aβ1-40 10 μM	Medium 1 (horse serum 5mM KCl)-6DIV *followed by* Medium1 (horse serum 5 mM KCl) Aβ 2 DIV	Testing response to Aβ under better survival (non-depolarizing) conditions/ **NEGATIVE (Fig. 2E)**	Medium 3(horse serum 25mM KCl) DIV *followed by* Medium 3 (horse serum 25mM KCL) Ab-2DIV	Testing response to Ab under depolarizing conditions/ **NEGATIVE (Fig. 2F)**
4	NO	Medium 2 (calf serum 5 *or* 25 mM KCl)- 6 DIV	Testing survival in depolarizing (25mM)and non-depolarizing (5mM) conditionsin relation to serum/ **DECREASE IN CELL NUMBER AND INCREASE IN PI UPTAKE(25 mM versus 5 mM) (Fig. 2G-H)**	N/A	N/A
5	Aβ40-1 and Aβ1-40 5 and 10 μM	Medium 2 (calf serum 5 mM KCl)- 6 DIV *followed by* Medium 2 (calf serum 5mM KCl) Aβ-2 DIV	Testing response to Aβ in best survival conditions of Exp. 4/**NEGATIVE (Fig. 2I)**	Medium 3(horse serum 5mM KCl)6 DIV *followed by* Medium 4 (serum free 5mM KCl)	Testing response to Ab under serum free conditions/ **NEGATIVE**

6	Aβ40-1 and Aβ1-40 10 μM	N/A	N/A	Medium 3(horse serum 5mM KCl)6 DIV *followed by* Medium 3 (horse serum 5mM KCL) Aβ/ Phosphoramidon-2 DIV	Testing response to Ab under tissue metalloprotease block/ **NEGATIVE (Fig. 2J)**

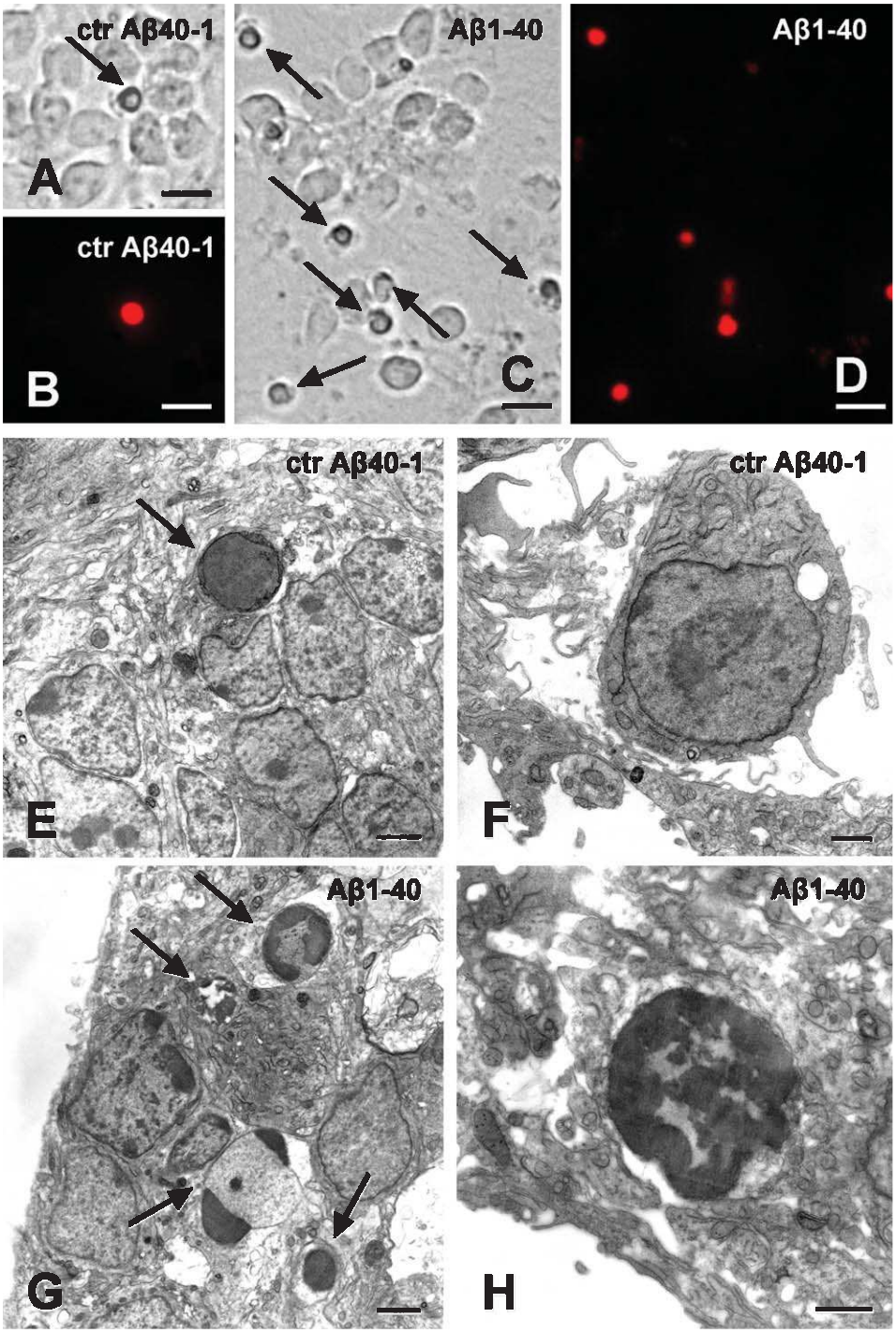

Fig. 1. A β-induced cell death in mouse NGCCs. A, C) Dying cells observed by differential interference contrast microscopy (arrows) are darker and display a certain degree of shrinkage. B, D) The same cells examined under fluorescence microscopy, display an intense PI nuclear staining. Note the higher number of dead cells after challenge with A β 1-40 . E–H) TEM demonstrates that NGCCs for the most contain CGCs easily recognizable by typical ultrastructural features: very small size, nucleus with large clumps of heterochromatin at periphery, an evident nucleolus,

and a scant cytoplasmic rim. Apoptotic CGCs (arrows) display chromatin condensation and margination, cytoplasm and nuclear shrinkage. An apoptotic CGC with two characteristic semilunar clumps abutted to the nuclear envelope is shown in G. A healthy (F) and a dying (H) CGC are shown at higher magnification. A β = amyloid- β; CGC = cerebellar granule cell; NGCCs = neuronal-glial cerebellar cultures; PI = propidium iodide. TEM = transmission electron microscopy. Scale bars: A–D = 15 μm; E, G = 1μm; F, H = 2 μm.

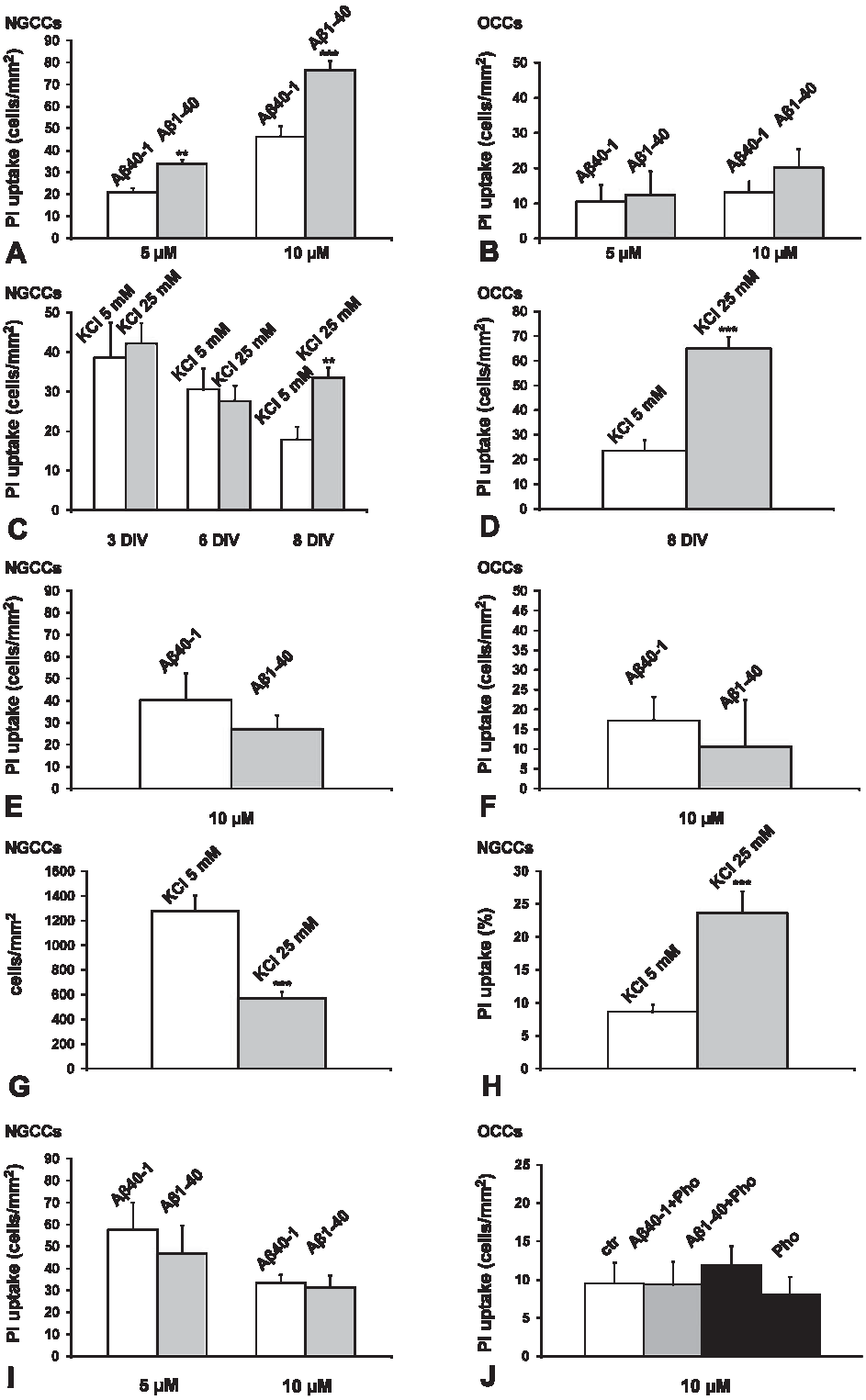

Figure 2. Quantitative analysis of PI uptake and cell density in mouse NGCCs and OCCs after A β -induced cell death. NGCCs and OCCs have been cultivated in medium 1 and medium 3 respectively unless otherwise stated and challenged with the inactive (control: A β 40-1) or the active peptide fragment (A β 1-40). For NGCCs, *n* = number of samples (for each sample the average cell density was calculated in 5 different microscopic fields from the same culture). For OCCs, *n* = number of inserts (each insert containing 3 slices). A) Left: PI uptake (cells/mm^2) in NGCCs after 5 f.LM A β 40-1 (white bar) versus 10 f.LM A β 1-40 (grey bar). Student's *t*-test, *n* = 5, $^{**}p < 0.01$.

Right: PI uptake (cells/mm^2) in NGCCs after 10 f.LM Aβ_{40-1} (white bar) versus 10 μM Aβ_{1-40} (grey bar). Student's *t*-test, $n = 13$, ***$p < 0.001$. B) Left: PI uptake (cells/mm^2) in OCCs after 5 μM Aβ_{40-1} (white bar) versus 10 μM Aβ_{1-40} (grey bar). Student's *t*-test, $n = 7$, $p > 0.05$. Right: PI uptake (cells/mm^2) in OCCs after 10 μM Aβ_{40-1} (white bar) versus 10 μM Aβ_{1-40} (grey bar). Student's *t*-test, $n = 7$, $p > 0.05$. C) PI uptake (cells/mm^2) in NGCCs grown in 5 mM KCl (white bars) and 25 mM KCl (grey bars) at 3, 6, and 8 DIV. Student's *t*-test, $n = 10$, **$p < 0.01$. D) PI uptake (cells/mm^2) in OCCs grown in 5 mM KCl (white bar) and 25 mM KCl (grey bar) at 8 DIV. Student's *t*-test, $n = 9$, ***$p < 0.001$. E) PI uptake (cells/mm^2) in NGCCs grown in 5 mM KCl after 10 μM Aβ_{40-1} (white bar) versus 10 μM Aβ_{1-40} (grey bar). Student's *t*-test, $n = 9$, $p > 0.05$. F) PI uptake (cells/mm^2) in OCCs grown in 25 mM KCl after 10 μM Aβ_{40-1} (white bar) versus 10 μM Aβ_{1-40} (grey bar). Student's *t*-test, $n = 9$, $p > 0.05$. G) Cell survival (cells/mm^2) in 8 DIV NGCCs grown in medium 2 at 5 mM KCl (white bar) and 25 mM KCl (grey bar). Student's *t*-test, $n = 15$, ***$p < 0.001$. H) Cell survival (percentage of PI uptake) in 8 DIV NGCCs grown in medium 2 at 5 mM KCl (white bar) and 25 mM KCl (gray bar). Student's *t*-test, $n = 10$ ***$p < 0.001$. I) Left: PI uptake (cells/mm^2) in NGCCs after 5 f.LM Aβ_{40-1} (white bar) versus 10 μM Aβ_{1-40} (grey bar). Student's *t*-test, $n = 5$, $p > 0.05$. Right: PI uptake (cells/mm^2) in NGCCs after 10 μM Aβ_{40-1} (white bar) versus 10 f.LM Aβ_{1-40} (grey bar). Student's *t*-test, $n = 5$, $p > 0.05$. J) PI uptake (cells/mm^2) in OCCs under control condition (ctr, white bar), 10 μM Aβ_{40-1} + 10 μM phospho-ramidon (grey bar); 10 μM Aβ_{1-40} + 10 μM phosphoramidon (black bar) and 10 μM phosphoramidon (striped bar). ANOVA, $n = 6$, $p > 0.05$. Aβ = amyloid-β; ctr = control; NGCCs = neuronal-glial cerebellar cultures; OCCs = organotypic cerebellar cultures; Pho = phosphoramidon; PI= propidium iodide.

In a second set of experiments (Table 1 Exp. 2 and Fig. 2C), the number of dead cells in 5 and 25 mM KCl was not significantly different at 3 and 6 DIV [3 DIV: 41.80 ± 8.96 cell/mm^2 (5 mM) versus 43.15 ± 5.28 cell/mm^2 (25 mM); 6 DIV: 30.55 ± 5.28 cell/mm^2 (5 mM) versus 27.62 ± 3.92 cell/mm^2 (25 mM); $n = 10$, $p > 0.05$], whereas at 8 DIV, a significant increase in cell death was observed in 25 mM KCl [17.89 ± 3.21 cell/mm^2 (5 mM) versus 35.74 ± 2.58 cell/mm^2 (25 mM); $n = 10$, $p < 0.01$]. Therefore the effect of Aβ_{1-40} (10 μM) was tested under these conditions of better cell survival (Table 1 Exp. 3 and Fig. 2E) and Aβ_{1-40} was found to be ineffective as the number of dead cells was not different when compared to control conditions [27.05 ± 6.27 cell/mm^2 (Aβ_{1-40}) versus 40.17 ± 12.21 cell/mm^2 (Aβ_{40-1}); $n = 9$, $p > 0.05$]. NGCCs were then grown with a different serum. The degree of cell death was first evaluated in the absence of Aβ (Table 1 Exp. 4) and in relation to cell density (Fig. 2G) and percentage of PI uptake (Fig. 2H). In this set of experiments, a rise to 25 mM KCl reduced cell density [571.02 ± 52.05 cell/mm^2 (25 mM KCl) versus 1275.88 ± 127.24 cell/mm^2 (5 mM KCl); $n = 15$, $p < 0.001$], and augmented the percentage of dead cells [8.16 ± 0.90% (25 mM) versus 22.46 ± 2.69%; $n = 10$, $p < 0.001$] in a dose-dependent fashion (Fig. 3). When Aβ_{1-40} (Table 1 Exp. 5 and Fig. 2I) was applied, it was still ineffective in inducing cell death [5 μM: 46.81 ± 12.83 cell/mm^2 (Aβ_{1-40}) versus 57.65 ± 12.61 cell/mm^2 (Aβ_{40-1}); $n = 5$, $p > 0.05$, and 10 μM: 31.28 ± 5.71 cell/mm^2 (Aβ_{1-40}) versus 33.48 ± 3.71 cell/mm^2 (Aβ_{40-1}); $n = 5$, $p > 0.05$].

OCCs

When OCCs were conventionally cultured [18] (Table 1 Exp. 1), the number of dead cells after challenge with $A\beta_{1-40}$ (Fig. 2B) was not significantly higher than in control conditions [5 μM: 12.45 ± 6.67cell/mm^2 ($A\beta_{1-40}$) versus 10.48 ± 4.79cell/mm^2 ($A\beta_{40-1}$); $n = 7$, $p > 0.05$, and 10 μM: 20.09 ± 5.42 cell/mm^2 ($A\beta_{1-40}$) versus 13.22 ± 3.11 cell/mm^2 ($A\beta_{40-1}$); $n = 13$, $p > 0.05$]. It is worth noting that these culture conditions were more favorable to cell survival than maintenance in 25 mM KCl (Table 1 Exp. 2 and Fig. 2D) [PI uptake: 23.58 ± 4.34 cell/mm^2 (5 mM) versus 64.95 ± 4.81 cell/mm^2 (25 mM); $n = 9$, $p < 0.001$]. However, even in 25 mM KCl (Table 1 Exp. 3 and Fig. 2F), OCCs were resistant 10 μM $A\beta_{1-40}$ [10.71 ± 2.94 cell/mm^2 ($A\beta_{1-40}$) versus 17.25 ± 5.97 cell/mm^2 ($A\beta_{40-1}$); $n = 9$, $p > 0.05$], as they were when maintained in serum-free medium (Table 1 Exp. 5) [15.27 ± 3.75 cell/mm^2 ($A\beta_{1-40}$) versus 13.98 ± 4.12 cell/mm^2 ($A\beta_{40-1}$); $n = 6$, $p > 0.05$]. Notably, the density of dead cells was not affected by the medium.

In a last set of experiments (Table 1 Exp. 6 and Fig. 2J), incubation with the metalloprotease inhibitor phosphoramidon (10 μM) alone or in combination with $A\beta_{1-40}$ (10 μM) had no effect on the density of dead cells [9.51 ± 2.70 cell/mm^2 (ctr); 11.85 ± 2.52 cell/mm^2 ($A\beta_{1-40}$ + phosphoramidon); 9.36 ± 3.02 cell/mm^2 ($A\beta_{40-1}$ + phosphoramidon); 8.026 ± 2.37 cell/mm^2 (phosphoramidon); $n = 6$, $p > 0.05$].

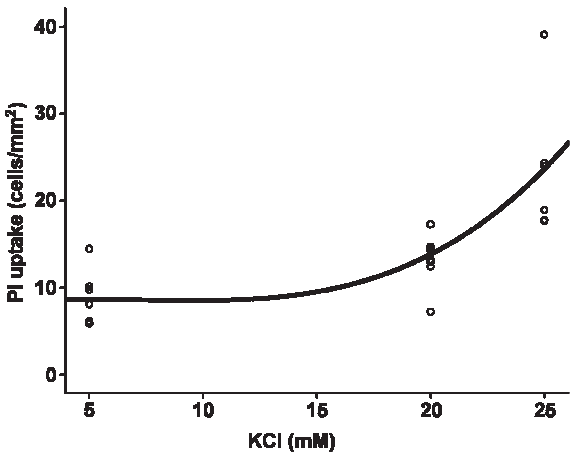

Fig. 3. Dose-dependent toxicity of KCl on mouse NGCCs. Regression analysis of the ratio between PI^+-nuclei and the total cell number in 8 DIV NGCCs grown in medium 2 at 5, 20, and 25 mM KCl demonstrates a negative effect of KCl on cell survival ($R^2 = 0.617$). NGCCs = neuronal-glial cerebellar cultures; PI = propidium iodide.

DISCUSSION

This study demonstrates that *in vitro* Aβ toxicity on cerebellar cells is strictly dependent upon the type of preparation (isolated versus organotypic), medium composition, and concentration of extracellular potassium. This highlights the caution that should be exercised when using CGCs as tools for analysis of AD neurodegeneration.

Response of NGCCs to Aβ 1-40

Aβ protein toxicity has extensively been studied in primary cultures of several types of CNS cells including cortical [19, 20], hippocampal pyramidal [21–23], striatal [24], basal forebrain [25, 26], and cerebellar [27–31] neurons. Previous work on isolated CGCs or NGCCs has highlighted a strict correlation between Aβ and apoptosis, since it was initially demonstrated that Aβ induces apoptosis [27], and also that an increased amyloidogenic secretion occurs in apoptotic CGCs [32]. In addition, multiple caspases, the effector proteases in apoptosis, are activated in cultured CGCs undergoing cell death [33].

Rat CGCs die when maintained *in vitro* in physiological KCl (3.5–5 mM) under non-depolarizing conditions, and, to avoid this, they are usually cultivated in high KCl (25 mM) under sustained depolarization for use in apoptosis studies [27–31]. We show here that mouse NGCCs are susceptible to 5–10 μM Aβ 1-40 only when maintained in 25 mM KCl. Interestingly, it was previously demonstrated that, differently from rat, isolated mouse CGCs can also survive *in vitro* in the presence of 5 mM KCl [34]. However, in both species, these neurons develop and become mature in a temporally regulated manner under physiological KCl, whereas elevated KCl blocks their differentiation during immaturity [35–39]. Growth of CGCs in elevated KCl profoundly alters the expression/repression pattern of a wide number of mRNAs and maintains high levels of expression of mRNAs typical of immature neurons [39].

Thus our data demonstrate that mouse CGCs are sensitive to Aβ challenge only when kept in an immature state. Because work on rat CGCs has fundamentally been carried out in comparable conditions of immatu- rity, it might be of interest to consider the possibility that mature rat CGCs are also resistant to Aβ challenge.

*Response of OCCs to Aβ*1-40

Much of the work on Aβ peptide toxicity in organotypic cultures has been carried out on hippocampal neurons [40–44]. These studies have focused on some cellular mechanisms involved in the neurodegeneration induced by Aβ, which involves activation of caspases 3 [45] and 12

[46]. Bruce and colleagues have highlighted the fact that only cultures equivalent to postnatal day 35 (defined as mature) were damaged by exposure to various concentration (from 10 to 50 μM) of $A\beta_{1-42}$ or $A\beta_{1-40}$, whereas those equivalent to postnatal day 14 (defined as immature) were resistant to amyloid challenge in serum-free medium [40]. However, other serum-free studies have shown that, even after a relatively short incubation *in vitro* (7 DIV), cultures were sensitive to the same Aβ peptides [41, 44, 45, 47]. When a different Aβ peptide (25–35) was used, still after a short maintenance *in vitro*, results were also quite heterogeneous with very limited effects (in some cases only after a long incubation time) according to some authors [43, 46], but much stronger according to others [48, 49]. In another study [50], toxic effects were similar for $A\beta_{1-42}$ and $A\beta_{25-35}$. There are no data available about the responses of OCCs to Aβ. In this paper, mouse OCCs were resistant to 5–10 μM $A\beta_{1-40}$ challenge, independent of culture conditions. In keeping with our experiments, it is worth noting that no toxicity was found on hippocampal organotypic cultures treated with 1–10 μM Aβ in fibrillary and/or oligomeric forms after an estimation of cell death with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide reduction assay [51]. These authors then concluded that failure of Aβ penetration into tissue was not responsible for their negative result. Another possible explanation for the resistance of OCCs to Aβ could be related to the presence of tissue proteases in cultured brain slices. The zinc-dependent metalloprotease neprilysin plays a role in regulating Aβ protein clearance in brain, and it was demonstrated that the production of neprylisin is increased in the aged cerebellum [52], as compared to other brain regions that are earlier targets of AD pathology [1]. However, we have demonstrated here that neprylisin is not responsible for the lack of toxicity in OCCs, since inhibition by phosphoramidon does not affect resistance to $A\beta_{1-40}$. Altogether, these observations lead us to speculate that when cerebellar neurons are cultured in a complex environment, they are less susceptible to damage by Aβ, again raising some cautions against the consideration of isolated CGCs as a reliable assay in drug discovery studies in AD.

Probing the biology of AD in transgenic mice has provided valuable insights into specific aspects of AD pathogenesis [14, 53, 54]. Specifically, these studies have pointed to the roles of Aβ and tau, and support a model in which Aβ is not directly responsible for neuronal death which is instead triggered by formation of abnormal tau species. With respect to the cerebellum, Aβ deposition in AD patients is a very late event, and it will be necessary to devote further experiments to better clarify the susceptibility of aged CGCs to Aβ.

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