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Excitotoxin-induced changes in transglutaminase during differentiation of cerebellar granule cells

D. Caccamo [1], **M. Currò** [1], **G. Cusumano** [2], **G. Crisafulli** [1], and **R. Ientile** [1]

[1] Department of Biochemical, Physiological and Nutritional Sciences, University of Messina, Messina, Italy
[2] Department of Emerging Pathologies, University of Palermo, Palermo, Italy

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Summary. Excitotoxicity induced by NMDA receptor stimulation is able to increase the activity of many enzymes involved in neuronal cell death. Primary cultures of rat cerebellar granule cells were used to elucidate the role of transglutaminase reaction in the excitotoxic cell response, and to evaluate the role of glutamate receptors in cell survival and degeneration. Granule neurons, maintained *in vitro* for two weeks, were exposed to NMDA at different stages of differentiation. Following NMDA receptor activation, increases in transglutaminase activity were observed in cell cultures. The levels of enzyme activity were higher in cells at 5 days *in vitro* than in those at 8–9 or 13–14 days *in vitro*. Moreover, NMDA exposure up-regulated tTG expression in neurons as young as 5 days *in vitro*. These cultures also exhibited morphological changes with clear apoptotic features. Results obtained demonstrate that susceptibility of granule cells to excitotoxicity depends on the developmental stage of neurons.

Keywords: Transglutaminase – Excitotoxicity – Neurodegenerative diseases – Apoptosis – Glutamate – Cerebellar granule neurons

Abbreviations: FC, monofluorescein cadaverine; NMDA, N-methyl-D-aspartic acid; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*, *d*]-cyclohepten-5,10-imine hydrogen maleate; GYKI 52466, 1-(4′-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine; PBS, phosphate buffered saline; tTG, tissue transglutaminase.

Introduction

Evidence has accumulated that over-stimulation of glutamate receptors is responsible, at least in part, for the neuronal death occurring after ischemic brain injury as well as in a variety of neurodegenerative disorders (Loo et al., 1993; Portera-Cailliau et al., 1995; Lipton, 1997; Doble, 1999). Neuronal cell damage may apparently be mediated by a sustained increase in neuron cytosolic free Ca^{2+}, caused by exposure to excitatory amino acids. Two mechanisms are mainly involved in excitotoxicity leading to cell death. The first is an acute neuronal swelling caused by depolarization-mediated influx of Na^{+}, Cl^{-} and water. The second is a slowly occurring neuronal degeneration that appears to depend on changes in free cytosolic Ca^{2+} induced by glutamate receptor activation. A dramatic consequence of Ca^{2+} influx into the cells is the modification of several enzyme activities (lipases, proteases, etc.), finally triggering excitotoxic damage. There is strong evidence that such alterations in intracellular Ca^{2+} homeostasis mediate the toxicity of glutamate and NMDA for cerebellar granule cells. In particular, it has been reported that a short exposure of cultured neurons to relatively mild insults, such as low concentrations of NMDA, causes delayed apoptotic cell death (Bonfoco et al., 1995; Tenneti and Lipton, 2000; Ientile et al., 2001). Although excitotoxicity initiated by over-stimulation of NMDA receptors has been associated with excessive Ca^{2+} influx and overload, the subsequent biochemical events leading to cell death are poorly understood. Recently, we have demonstrated that NMDA-induced excitotoxicity increases the expression of tissue transglutaminase (tTG) at high levels (Ientile et al., 2002). tTG is a cytosolic form of transglutaminase (TGase), which likely plays a role in numerous cellular processes. Protein modifications following TGase-mediated cross-linking reaction have significant effects on neuronal functions. tTG is a highly regulated and inducible enzyme that is developmentally regulated in the nervous system. Several specific roles for tTG have been described and there is evidence that it may also play a role in apoptosis (Melino and Piacentini, 1998; Tucholski and Johnson, 2002). Recent findings have demonstrated that disregulation of tTG may contribute to the pathology of

several neurodegenerative conditions (Lesort et al., 2000; Cooper et al., 2002).

Under appropriate culture conditions (i.e. depolarizing stimuli, in presence of 25 mM KCl), cerebellar granule cells express fully functional NMDA receptors during development. Therefore, they can be used to establish a correlation between the expression of such receptors and the appearance of sensitivity to neurotoxic effects of glutamate and NMDA. This study was then designed to determine the relationships between NMDA-induced changes in transglutaminase activity and developmental expression of NMDA receptors in primary cultures of granule neurons.

Material and methods

Materials

Cell culture medium and serum were obtained from Life Technologies Ltd. (Milano, Italy). Monofluorescein cadaverine was from Molecular Probes (Eugene OR, U.S.A.). Monoclonal antibody for tTG (CUB 7402) from LabVision Corp. (Fremont, CA, U.S.A.). NMDA, aprotinin, leupeptin, pepstatin and other chemicals of analytical grade were obtained from Sigma (Milano, Italy). Fluorescein isothiocyanate (FITC) antibody (IgG1) was purchased from Biogenesis Ltd (Poole, England). 5-Methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]-cyclohepten-5,10-imine hydrogen maleate (MK-801) and 1-(4′-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine) (GYKI 52466) were from Research Biochemicals, Inc. (Natick, MA, U.S.A.).

Primary cultures of cerebellar granule cells

Cerebellar granule cells were prepared from 8-day-old Wistar rat pups (Charles River Italia, Calco, Italy) as previously described (Schousboe et al., 1989; Taniwaki et al., 1997). In brief, cells were dissociated from the cerebellum by mechanical chopping, suspended in Krebs-Ringer buffered medium, treated with trypsin (50 μg/ml), followed by a trypsin inhibitor and DNase (80 μg/ml). Cells were seeded onto poly-D-lysine-coated (10 μg/ml) plates or glass coverslips at a density of 0.33×10^6 cells/cm^2. Cells were cultured in Basal Medium Eagle, supplemented with 10% fetal bovine serum, 25 mM KCl, 2 mM glutamine, 100 μg/ml gentamicin, and maintained for two weeks. Cytosine arabinoside (1 μM), a mitotis inhibitor for astrocytes, was added on the first, second and seventh day after neurons were plated.

At different times of culture, i.e. 5–6, 8–9, 13–14 days *in vitro* (DIV), cells were exposed to 100 μM NMDA in Locke's buffer, usually for 30 min at 25°C. Then, cells were washed with Locke's buffer, returned to culture-conditioned medium and put in the incubator. In some experiments, glutamate receptor antagonists were added five minutes before NMDA exposure.

Transglutaminase activity

Transglutaminase activity was measured by ELISA, using fluorescein-cadaverine as a substrate and polyclonal antibody against fluorescein isothiocyanate (Biogenesis, Poole, England) as previously reported (Ientile et al., 2002). The absorbance was measured at 490 nm using a ELISA plate reader (Sunrise, Tecan GmbH, Salzburg, Austria). tTG activity was expressed as absorbance units per 100 μg of protein per 30 min. In some experiments, values of tTG activity obtained by ELISA test were compared to those of the traditional assay measuring the incorporation of [^{3}H] putrescine into *N*, *N*′-dimethylcaseine (Lorand et al., 1972).

tTG expression

tTG expression was detected by Western blotting in cell lysates. Proteins (30 μg) were separated on 8.5% sodium dodecyl sulphate-polyacrylamide gels, and transferred to nitrocellulose membranes. Blots were incubated for 1 h with specific tTG antibody CUB 7402 (1:1000 in PBS), then for 1 h with horseradish peroxidase-conjugated IgG (1:1500 in PBS). tTG antigen was detected by chemiluminescence (ECL, Amersham Pharmacia Biotech, Buckinghamshire, England) after a 20–30 sec autoradiography film exposure.

Measurement of granule cell injury

After exposure to NMDA, cultures were analyzed at various times for evaluation of cell injury by propidium iodide DNA staining, and apoptotic nuclei counted according to Ankarcrona et al. (1995). Neuronal injury was also assessed by scanning electron microscopy with a Hitachi S800 field emission scanning electron microscope at 25 kV, and cell morphology was evaluated.

Results

All primary cultures used in these experiments were maintained under depolarizing conditions (25 mM KCl). Under these conditions, control cultures showed slight

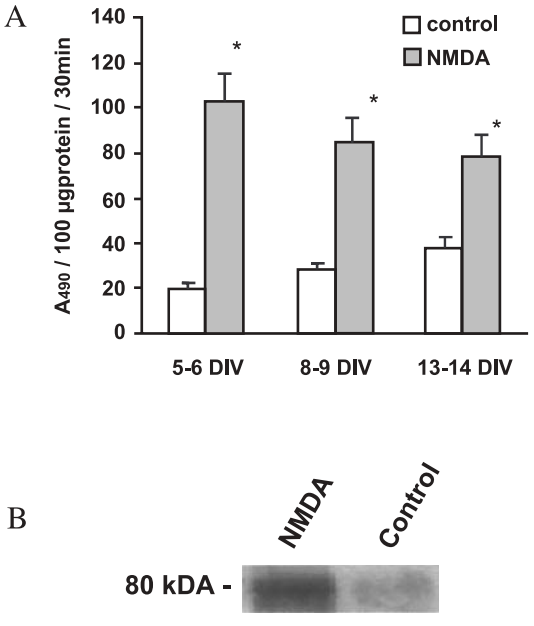

Fig. 1. NMDA-induced changes in transglutaminase activation during differentiation of cerebellar granule neurons. **A** Cells from different cultures, maintained for 5–6, 8–9, and 13–14 DIV, were incubated in presence or absence (control) of 100 μM NMDA for 30 min, in Mg^{2+}-free Locke's buffer. Following NMDA exposure, cultures were washed and incubated for 3 h in NMDA-free medium. TGase activity was then evaluated in cell lysates as described in experimental procedures. Values represent means ± SEM from three to four experiments. $^*p < 0.01$, compared with control-group (ANOVA, followed by Newman-Keuls multiple-range test. **B** Immunoblots of the expression of tTG in 5–6 DIV cell cultures in presence or absence of NMDA. Proteins (30 μg) from cell lysates were probed with the tTG monoclonal antibody CUB 7402

increases in enzyme activity during development. When neuronal cultures were incubated for 30 min in presence of NMDA (100 μM), and returned, for 3 h of incubation, to the culture-conditioned medium, significant increases in TGase activity occurred in cell lysates. Cells from different cultures, maintained for 5–6, 8–9, and 13–14 DIV, were analyzed in terms of their response to NMDA-induced changes in TGase activity. As shown in Fig. 1A, these effects were dependent on developmental stages of cell cultures. In particular, neurons cultured for 5–6 DIV responded more strongly to low concentrations of NMDA than did those at 8–9 or 13–14 DIV.

In cell lysates from 5–6 DIV NMDA-exposed cultures, after 3 h of incubation in the culture-conditioned medium, there was also an increase in tTG expression, as detected by immunolabelling with specific antibody of a 80 kDa protein band (Fig. 1B).

As reported in Fig. 2, the increase in enzyme activity, produced by exposing the cells for 30 min to 100 μM NMDA, could be completely blocked by the addition of 10 μM dizocilpine (MK-801), a selective inhibitor of the NMDA receptor. The pre-treatment of the cultures with kainate/AMPA receptor blocker, GYKI 52466 (100 μM), did not significantly reduce NMDA-induced increase in TGase activity.

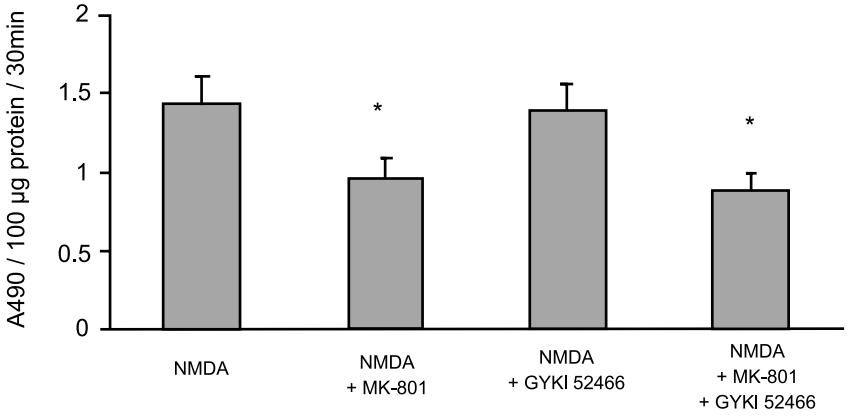

Fig. 2. Effect of NMDA on transglutaminase activity in cerebellar granule cells. Cell cultures were exposed for 30 min to NMDA (100 μM), in presence or absence of 10 μM MK-801 or 100 μM GYKI 52466, glutamate receptor antagonists, then washed and incubated for three hours in drug-free medium. The enzyme activity was measured as reported in Materials and methods. Results are mean $\pm$ SEM values from three to four experiments. $^{*}p < 0.01$ compared with 100 μM NMDA group (ANOVA, followed by Newman-Keuls multiple range test)

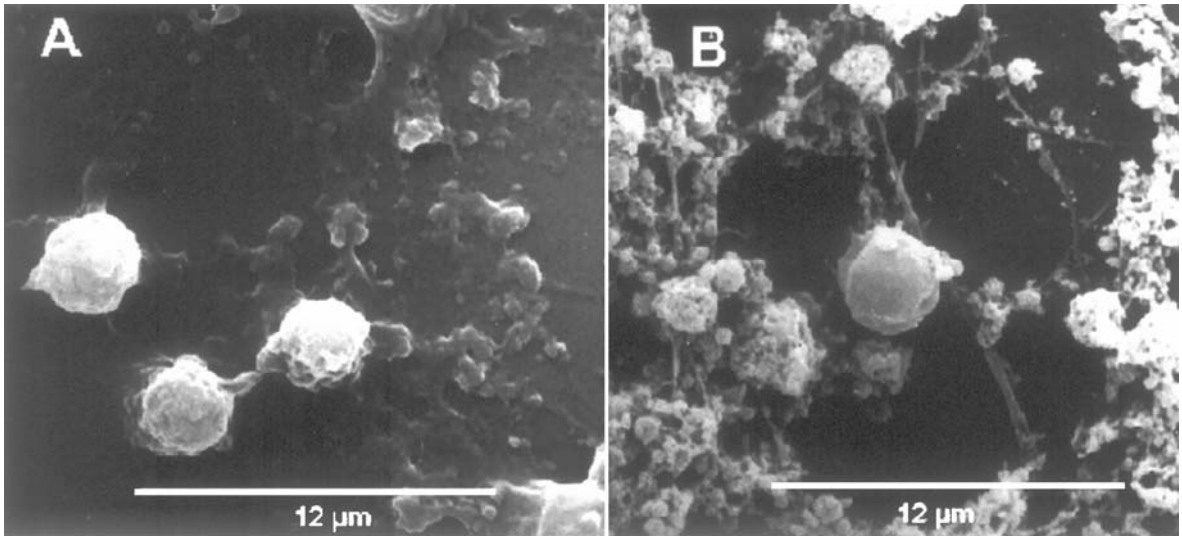

Fig. 3. Scanning electron micrograph of NMDA-exposed cerebellar granule neurons. In untreated control cultures (**A**) cerebellar neurons show plasma membrane ruffles and a well preserved morphology. In NMDA-exposed cultures (**B**) morphologic features of cell injury are evident. Granule cells were characterized by the presence of vacuoles or morula-like structures on the cell surface (original magnification $\times$ 4,000)

Table 1. Effect of NMDA on apoptotic cell death in 5–6 DIV cerebellar granule cultures

	% of apoptotic nuclei	
Time of incubation (h)	Control	NMDA
0	6.2 ± 0.6	6.3 ± 0.7
2	7.2 ± 0.7	$17.5 \pm 1.9^*$
4	6.3 ± 0.5	$27.3 \pm 2.5^*$
6	6.5 ± 0.5	$30.3 \pm 2.9^*$
8	5.5 ± 0.6	$25.2 \pm 3.1^*$
18	6.2 ± 0.6	$22.5 \pm 2.3^*$

Primary cultures (5–6 DIV) of cerebellar granule neurons were exposed for 30 min to 100 μM NMDA in Mg^{2+}-free Locke's buffer at 37°C. Cells were then washed three times with Locke's buffer and returned to the culture-conditioned medium. At the indicated times, cultures were then permeabilized, fixed and stained with propidium iodide. Apoptotic nuclei were counted by fluorescence microscopy at 40×, in 10 fields per treatment and expressed as a fraction of the total neuronal nuclei. Statistical analysis consisted of a Student's *t* test with Bonferroni correction ($^*p < 0.01$) comparing apoptotic nuclei in the presence of NMDA with those in control cultures. Data are given as means ± SEM of three independent experiments

Scanning electron microscopy analysis showed evidence of cell injury in 5–6 DIV cultures exposed to NMDA (100 μM) for 30 min, following 3 h of incubation. In particular, cerebellar granule cells were characterized by the presence of vacuoles or morula-like structures on the cell surface. A strong volume reduction was observed, with loss of cell to cell contacts and fragmentation into many smooth-surfaced cytoplasmic bodies. In contrast, control cultures showed granule cells with loculated surface folds or a delicate microvillous surface. The morphology ranged from polygonal to spindle or stellate appearance (Fig. 3).

Moreover, propidium iodide DNA staining demonstrated that the exposure of 5–6 DIV cultured granule cells for 30 min to NMDA (100 μM), followed by different periods of incubation in NMDA-free medium, caused a neuronal death characterized by chromatin condensation (not shown). The results in Table 1 demonstrate NMDA-induced apoptosis in cerebellar granule cells, with maximal response within 4 h from agonist exposure. Following times of incubation in NMDA-free medium did not produce further increases in the percentage of apoptotic nuclei in 5–6 DIV cultured neurons.

Discussion

The present work demonstrates that increases in TGase activity were involved in excitotoxin-induced response in cultured cerebellar granule cells during development under moderately depolarizing conditions (25 mM). These effects were blocked by MK-801 but not by GYKI 52466, indicating that increases in enzyme activity were likely due to a direct activation of NMDA receptors in granule cells. The response appeared to differ significantly between neurons grown for 8–9 or 13–14 DIV and those grown for 5–6 DIV. Since this phenomenon was seen in a higher percentage of the ''young'' as compared with the mature neurons, it is possible that it may be correlated in some fashion with the differential sensitivity of the ''young'' neurons to NMDA-induced toxicity.

Considering the central role of NMDA receptor activation in the excitotoxicity, it has been observed that neurons cultured under depolarizing conditions, produced by the introduction of either KCl or moderate concentrations of NMDA in the growth medium, exhibited a developmental increase in the expression of functional NMDA receptors (Resink et al., 1996; Rumbaugh and Vicini, 1999; Snell et al., 2001). Furthermore, an higher sensitivity to NMDA in granule cells, in the absence of extracellular Mg^{2+}, was reached at 5–8 DIV in comparison with granule cells older than 8 DIV (Ciardo and Meldolesi, 1991). In the experiments reported in the present paper, through all developmental stages tested, the NMDA-induced rises in TGase activity at different DIV may be due to different changes in free $[Ca^{2+}]$ in response to NMDA (Xia et al., 1995). Indeed, we previously demonstrated that increases in TGase-catalyzed cross-linking reactions are dependent on NMDA-induced Ca^{2+}-influx, since these effects were significantly reduced when cell cultures were exposed to NMDA in a medium with a nominal absence of calcium (Ientile et al., 2002).

It has been reported that NMDA-sensitive glutamate receptor stimulation produced several dose-dependent biochemical changes leading to neuronal degeneration in different experimental models. In particular, the exposure of cultured cerebellar granule cells to excitotoxic stimuli, in absence of Mg^{2+}, causes cytoplasmic Ca^{2+} deregulation and extensive cell death (Budd and Nicholls, 1996; Atlante et al., 1999). However, a causal link between intracellular calcium increases and cell death has been inferred on the basis of several lines of evidence (Tenneti and Lipton, 2000).

Our data extend recent results (Ientile et al., 2002), suggesting a relationship between doses of NMDA that evoke a prolonged elevation of $[Ca^{2+}]$ and those inducing tTG and triggering neuronal death. In fact, we also report that cerebellar granule neurons, exposed to mild concentrations of NMDA, display morphological features of cell damage, including cytoplasmic blebbing and loss of membrane extensions. This agrees with previous observations demonstrating that, depending on doses and duration of

insult, excitotoxic mechanism leads to both apoptotic cell death and necrosis (Ayata et al., 1997; Larm et al., 1997; Tenneti and Lipton, 2000; Ientile et al., 2001).

In this regard, it is well known that several proteins are modified by tTG-catalyzed cross-linking during apoptosis (Ballestar et al., 1996; Nemes et al., 1997). Although a definitive role for tTG in apoptotic cascade has not been yet established, it has been hypothesized that tTG likely contributes to morphological changes that occur during apoptosis, and may be responsible, in part, for cross-linking of substrate proteins and stabilization of protein structures. However, it is likely that also other cellular functions are modulated by tTG during apoptosis (Fesus, 1998; Melino and Piacentini, 1998).

To summarize, present results clearly point out that changes in TGase activation and tTG expression represent post-receptor activation events, that are developmentally related to the susceptibility of granule cells to the NMDA-evoked excitotoxicity.

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References

Ankarcrona M, Dypbukt JM, Bonfoco E, Zhivotovsky B, Orrenius S, Lipton SA, Nicotera P (1995) Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. Neuron 15: 961–973

Atlante A, Gagliardi S, Marra E, Calissano P, Passarella S (1999) Glutamate neurotoxicity in rat cerebellar granule cells involves cytochrome c release from mitochondria and mitochondrial shuttle impairment. J Neurochem 73: 237–246

Ayata C, Ayata G, Hara H, Matthews RT, Beal MF, Ferrante RJ, Endres M, Kim A, Christie RH, Waeber C, Huang PL, Hyman BT, Moskowitz MA (1997) Mechanisms of reduced striatal NMDA excitotoxicity in type I nitric oxide synthase knock-out mice. J Neurosci 17: 6908–6917

Ballestar E, Abad C, Franco L (1996) Core histones are glutaminyl substrates for tissue transglutaminase. J Biol Chem 271: 18817–18824

Bonfoco E, Krainc D, Ankarcrona M, Nicotera P, Lipton SA (1995) Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. Proc Natl Acad Sci USA 92: 7162–7166

Budd SL, Nicholls DG (1996) Mitochondria, calcium regulation, and acute glutamate excitotoxicity in cultured cerebellar granule cells. J Neurochem 67: 2282–2291

Ciardo A, Meldolesi J (1991) Regulation of intracellular calcium in cerebellar granule neurons: effects of depolarization and of glutamatergic and cholinergic stimulation. J Neurochem 56: 184–191

Cooper AJL, Jeitner TM, Blass JP (2002) The role of transglutaminases in neurodegenerative diseases: overview. Neurochem Int 40: 1–5

Doble A (1999) The role of excitotoxicity in neurodegenerative disease: implications for therapy. Pharmacol Ther 81: 163–221

Fesus L (1998) Transglutaminase-catalyzed protein cross-linking in the molecular program of apoptosis and its relationship to neuronal processes. Cell Mol Neurobiol 18: 683–694

Ientile R, Macaione V, Teletta M, Pedale S, Torre V, Macaione S (2001) Apoptosis and necrosis occurring in excitotoxic cell death in isolated chick embryo retina. J Neurochem 79: 71–78

Ientile R, Caccamo D, Macaione V, Torre V, Macaione S (2002) NMDA-evoked exctotoxicity increases tissue transglutaminase in cerebellar granule cells. Neuroscience 115: 723–729

Larm JA, Cheung NS, Beart PM (1997) Apoptosis induced via AMPA-selective glutamate receptors in cultured murine cortical neurons. J Neurochem 69: 617–622

Lesort M, Tucholski J, Miller ML, Johnson GVW (2000) Tissue transglutaminase: a possible role in neurodegenerative diseases. Prog Neurobiol 61: 439–463

Lipton SA (1997) Treating AIDS dementia. Science 276: 1629–1630

Loo DT, Copani A, Pike CJ, Whittemore ER, Walencewicz AJ, Cotman CW (1993) Apoptosis is induced by beta-amyloid in cultured central nervous system neurons. Proc Natl Acad Sci USA 90: 7951–7955

Lorand L, Campbell-Wilkes LK, Cooperstein L (1972) A filter paper assay for transamidating enzymes using radioactive amine substrates. Anal Biochem 50: 623–631

Melino G, Piacentini M (1998) Tissue transglutaminase in cell death: a downstream or a multifunctional upstream effector? FEBS Lett 430: 59–63

Nemes Z Jr., Adany R, Balazs M, Boross P, Fesus L (1997) Identification of cytoplasmic actin as an abundant glutaminyl substrate for tissue transglutaminase in HL-60 and U937 cells undergoing apoptosis. J Biol Chem 272: 20577–20583

Portera-Cailliau C, Hedreen JC, Price DL, Koliatsos VE (1995) Evidence for apoptotic cell death in Huntington disease and excitotoxic animal models. J Neurosci 15: 3775–3787

Resink A, Villa M, Benke D, Hidaka H, Mohler H, Balazs R (1996) Characterization of agonist-induced down-regulation of NMDA receptors in cerebellar granule cell cultures. J Neurochem 66: 369–377

Rumbaugh G, Vicini S (1999) Distinct synaptic and extrasynaptic NMDA receptors in developing cerebellar granule neurons. J Neurosci 19: 10603–10610

Schousboe A, Meier E, Drejer J, Hertz L (1989) Preparation of cultures of mouse (rat) cerebellar granule cells. In: Shahar A, DeVellis JA, Vernadakis A, Haber B (eds) A dissection and tissue culture manual for the nervous system. Alan R. Liss, New York, pp 203–206

Snell LD, Bhave SV, Tabakoff B, Hoffman PL (2001) Chronic ethanol exposure delays the 'developmental switch' of the NMDA receptor 2A and 2B subunits in cultured cerebellar granule neurons. J Neurochem 78: 396–405

Taniwaki T, Hirashima N, Becerra SP, Chader GJ, Etcheberrigaray R, Schwartz JP (1997) Pigment epithelium-derived factor protects cultured cerebellar granule cells against glutamate-induced neurotoxicity. J Neurochem 68: 26–32

Tenneti L, Lipton SA (2000) Involvement of activated caspase-3-like proteases in N-methyl-D-aspartate-induced apoptosis in cerebrocortical neurons. J Neurochem 74: 134–142

Tucholski J, Johnson GVW (2002) Tissue transglutaminase differentially modulates apoptosis in a stimuli-dependent manner. J Neurochem 81: 780–791

Xia Y, Ragan RE, Seah EE, Michaelis ML, Michaelis EK (1995) Developmental expression of N-methyl-D-aspartate (NMDA)-induced neurotoxicity, NMDA receptor function, and the NMDAR1 and glutamate-binding protein subunits in cerebellar granule cells in primary cultures. Neurochem Res 20: 617–629

Authors' address: Dr. Riccardo Ientile, Dipartimento di Scienze Biochimiche, Fisiologiche e della Nutrizione, Università di Messina, Policlinico Universitario – Via Consolare Valeria, I-98125 Messina, Italy,
Fax: (39) (090) 2938603, E-mail: ientile@unime.it