Amino Acids (1999) 17: 323–334

Amino
Acids

Enhanced taurine release in cultured cerebellar granule cells in cell-damaging conditions

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Accepted June 8, 1999

Summary. The release of taurine from cultured cerebellar granule neurons was studied in different cell-damaging conditions, including hypoxia, hypoglycemia, ischemia, oxidative stress and in the presence of free radicals. The effects of both ionotropic and metabotropic glutamate receptor agonists on the release were likewise investigated. The release of [^{3}H]taurine from the glutamatergic granule cells was increased by K^+ (50 mM) and veratridine (0.1 mM), the effect of veratridine being the greater. Hypoxia and ischemia produced an initial increase in release compared to normoxia but resulted in a diminished response to K^+. Hypoglycemia, oxidative stress and free radicals enhanced taurine release, and subsequent K^+ treatment exhibited a correspondingly greater stimulation. A common feature of taurine release in all the above conditions was a slow response to the stimulus evoked by K^+ and particularly to that evoked by veratridine. All ionotropic glutamate receptor agonists potentiated taurine release, but only the action of kainate seemed to be receptor-mediated. Metabotropic receptor agonists of group I slightly stimulated the release. The prolonged taurine release seen in both normoxia and cell-damaging conditions may be of importance in maintaining homeostasis in the cerebellum and reducing excitability for a longer period than other neuroprotective mechanisms.

Keywords: Amino acids – Taurine release – Cerebellar granule cells – Cell-damaging conditions – Glutamate receptors – Veratridine – Potassium stimulation

Abbreviations: AIDA: (RS)-1-aminoindan-1,5-dicarboxylate; AMPA: 2-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione; DCG IV: (2S,2′R,3′R)-2-(2′,3′-dicarboxycyclopropyl)glycine; DHPG: (S)-3,5-dihydroxyphenylglycine; EGLU: (2S)-2-ethylglutamate; L-AP3: L(+)-2-amino-3-phosphonopropionate; L-AP4: L(+)-2-amino-4-phosphonobutyrate; L-SOP: O-phospho-L-serine; NBQX: 6-nitro-7-sulphamoyl[f]quinoxaline-2,3-dione; NMDA: N-methyl-D-aspartate; *trans*-ACPD: (1S,3S)-1-aminocyclopentane-1,3-dicarboxylate.

Introduction

The inhibitory amino acid taurine has an important role in osmoregulation in brain cells (Oja and Saransaari, 1996a; Pasantes-Morales and Schousboe, 1997). In addition, this sulphur-containing compound acts as a neuromodulator. This function is well documented in the cerebellum, where it depresses the firing of cerebellar neurons (see Oja and Kontro, 1983; Huxtable, 1992) and modulates neurotransmitter functions (Namima et al., 1983; Wahl et al., 1994). Taurine-like immunoreactivity has been found in several types of cerebellar cells, including Purkinje, stellate and granule cells and also astrocytes (Chan-Palay et al., 1982; Madsen et al., 1985). Taurine is released from cerebellar slices and synaptosomal preparations by depolarizing stimuli (Bernardi et al., 1984; Kontro and Oja, 1989; Kubo et al., 1992). Further recent evidence implies that taurine could be neuroprotective, counteracting excitability and excitotoxicity in nervous tissue (see Saransaari and Oja, 1997a).

We have recently demonstrated a markedly enhanced release of both endogenous and preloaded labeled taurine from hippocampal slices in several cell-damaging conditions (Saransaari and Oja, 1997b,c; 1998a). These conditions also evoke taurine release from cultured cerebral cortical astrocytes (Saransaari and Oja, 1999a), but the possible contribution of neurons to taurine release is not known. Now the release of [^{3}H]taurine from cultured cerebellar granule neurons was studied under the same experimental conditions, i.e., in hypoxia, hypoglycemia, ischemia, oxidative stress and the presence of free radicals. In order to differentiate whether possible alterations in the release merely result from leakage through damaged cell membranes or from compromised control of ionic fluxes, both basal and depolarization-evoked taurine releases were subjected to study. The glutamatergic granule cells are known to take up taurine by means of a high-affinity transport system (Holopainen et al., 1987) and to release taurine in response to depolarizing stimuli (Holopainen et al., 1989; Schousboe and Pasantes-Morales, 1989). For this reason the effects of ionotropic and metabotropic glutamate receptor agonists and antagonists on taurine release were also investigated.

Materials and methods

Material

[1,2-^{3}H]Taurine (specific activity 1.07 PBq/mol) was purchased from Amersham International, Bristol, U.K. Tissue culture media and fetal calf serum were obtained from Gibco/Biocult Lab., Ltd., Scotland, and tissue culture dishes and flasks from Nunc A/S, Denmark. Glutamate receptor agonists and antagonists were from Tocris Cookson, Bristol, U.K.

Cell culture

Cultures of cerebellar granule cells were prepared as described in detail elsewhere (Holopainen et al., 1987). Briefly, the cerebella were cut into pieces and dissociated by mild trypsinization followed by trituration in deoxyribonuclease solution containing a trypsin inhibitor. The cells were suspended in enriched Dulbecco's medium and cultured

on dishes previously coated with poly-L-lysine. The medium contained fetal calf serum (10%), K^+ (25 mM), glucose (30 mM), p-aminobenzoate (7 μM), insulin (100 mU/l), GABA (50 μM) and antibiotics (gentamicin). After 48 h in culture, 10 μM cytosine-1-beta-D-arabinofuranoside was added for 24 h. The cultures were kept at 37°C in humidified air containing 5% CO_2 for 7–9 days before efflux experiments. These primary cultures of cerebellar granule cells have been shown by immunocytochemistry to contain more than 97% neurons and less than 3% glia, fibroblasts and endothelial cells. Approximately 92% of neuronal cells have been morphologically identified as granule cells, while most of the remaining neurons appear to be GABAergic interneurons (Currie, 1980).

Release experiments

The release experiments were carried out as earlier described in detail by Holopainen and Kontro (1988). The granule cells were preincubated for 5 min at 37°C in standard Krebs-Ringer-Hepes-glucose medium (pH 7.4) and thereafter loaded with [^{3}H]taurine (10 μM) for 15 min. The release of radioactivity accumulated by the cells was monitored by medium changes at 2-min intervals and analyzed generally in three phases: (1) spontaneous release into standard medium (20–30 min, basal phase), (2) release induced by modified medium (30–40 min, stimulation phase), and (3) spontaneous release into standard medium (40–50 min, recovery phase). Several other experiments were of longer duration. In them, the stimulation phase was delayed and lasted 20 min (40–60 min) with the recovery phase from 60 to 80 min. Finally, the cells were detached in NaOH (0.4 M) and homogenized. The radioactivities of the collected 2-min fractions and that remaining in the cells at the end of experiments were determined as described elsewhere (Holopainen et al., 1987).

Neural cell damage was induced by modified experimental conditions applied from the beginning of the release experiments onwards. In hypoglycemia, glucose was omitted from the medium and in hypoxia the medium was bubbled with N_2 gas for 1 h before and then during experiments. In ischemia, the glucose-free medium was bubbled with N_2 gas (Taylor et al., 1995). Lipid peroxidation (Wills, 1969) (oxidative stress) was induced by $FeSO_4$ (7.5 μM) together with ascorbate (1.5 mM) (Agostinho et al., 1994). Free radical production was achieved by exposure to hydrogen peroxide (0.01%) (Pelmar, 1995).

The efflux rate constants for the above three experimental phases, designated k_1, k_2 and k_3, were analyzed as in Holopainen et al. (1985). The intervals used in the calculations were 24–30 min, 34–40 min and 44–50 min, respectively. Only the calculated k_1 and k_2 are usually given. The statistical significance of differences between the experimental groups was evaluated by analysis of variance using the critical values published by Owen (1962) for multiple comparisons between several experiment means and one control.

Results

The release of [^{3}H]taurine from the cultured cerebellar granule cells was enhanced by K^+ (50 mM) and veratridine (0.1 mM) (Table 1, Fig. 1A). Veratridine stimulation was significantly greater than K^+ stimulation, still gradually increasing during the poststimulation recovery phase (Fig. 1A). Maximal stimulation by K^+ was also attained after withdrawal of the stimulus. At the onset of K^+ stimulation there was invariably in normoxia (Fig. 1A) and hypoxia (Fig. 1B) a small initial enhancement in taurine release which was markedly accentuated in ischemia (Fig. 1C).

The ionotropic glutamate receptor agonists kainate, N-methyl-D-aspartate (NMDA) and 2-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) (all 1.0 mM) potentiated taurine release (Fig. 2). Of the respective

Table 1. Taurine release from cultured cerebellar granule cells under cell-damaging conditions

Experimental Conditions	Efflux rate constants ($\times 10^{-3}$ min^{-1}) ± SEM	
	k_1 (24–30 min)	k_2 (34–40 min)
Normoxia	6.37 ± 0.19 (56)	4.87 ± 0.11 (6)
+ K^+		5.86 ± 0.16 (5)
+ veratridine		7.28 ± 0.24 (5)
Hypoglycemia	8.98 ± 0.41** (12)	5.33 ± 0.15* (4)
+ K^+		9.04 ± 0.93** (4)
+ veratridine		8.40 ± 0.54 (4)
Free radicals	10.71 ± 0.41** (12)	6.60 ± 0.34** (4)
+ K^+		7.54 ± 0.43** (4)
+ veratridine		8.69 ± 0.32** (4)
Oxidative stress	7.73 ± 0.36** (12)	5.57 ± 0.07* (4)
+ K^+		7.29 ± 0.16** (4)
+ veratridine		8.51 ± 0.50* (4)

The cells were cultured for 4 days with [^{3}H]taurine (10 μM). Thereafter the release of labeled taurine was monitored in Krebs-Ringer-Hepes-glucose medium (pH 7.4) for 50 min, from 30 to 40 min with K^+ (50 mM) or veratridine (0.1 mM). The cell-damaging conditions were applied from the beginning of incubations. Number of independent experiments in parenthesis. In normoxia K^+ and veratridine significantly ($p < 0.01$) stimulated taurine release. Significance of differences from the corresponding condition in normoxia: *$p < 0.05$, **$p < 0.01$.

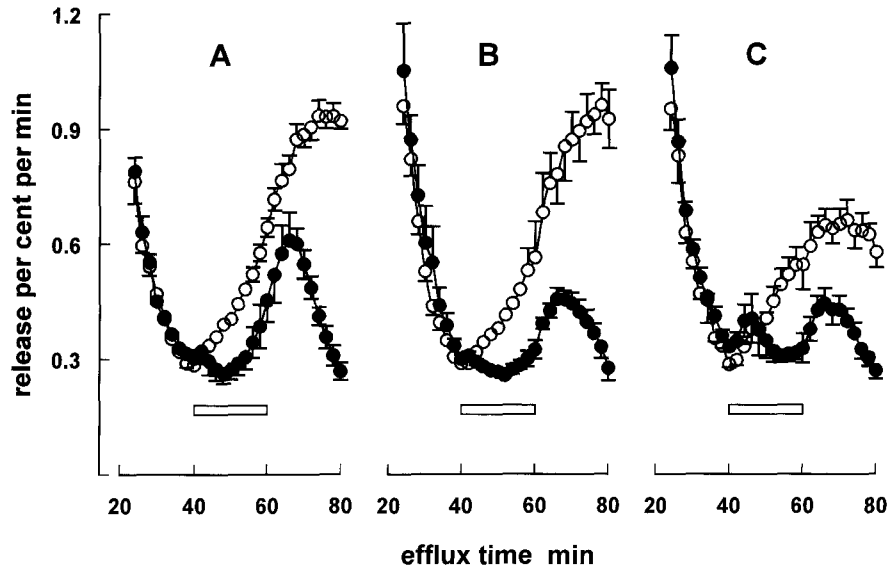

Fig. 1. Time-course of taurine release from cultured cerebellar granule cells in normoxia (**A**), hypoxia (**B**) and ischemia (**C**). Veratridine (0.1 mM) (-●●-) or K^+ (50 mM) (-○○-) was present from 40 to 60 min, as indicated by the bar. The results are mean values (± SEM) of 4 independent experiments

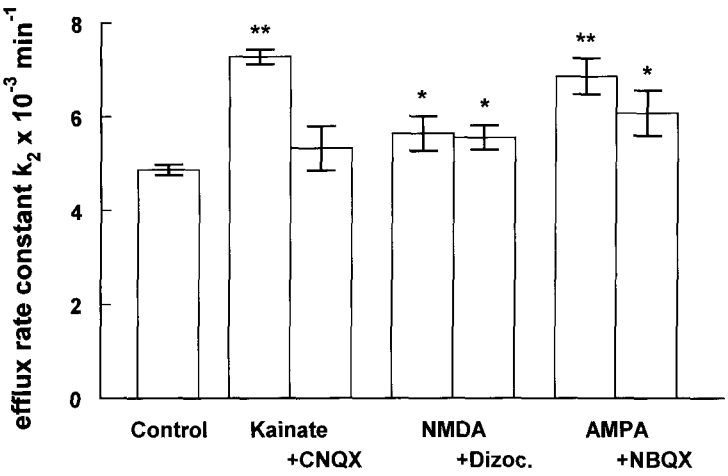

Fig. 2. Effects of ionotropic glutamate receptor agonists (1.0 mM) and antagonists (0.1 mM) on taurine release from cultured cerebellar granule cells. The results are efflux rate constants k_2 for the stimulation phase (24–30 min). The mean values (± SEM) of 4–8 independent experiments are shown. Significance of differences from the control: $*p < 0.05$, $**p < 0.01$

antagonists, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (0.1 mM) abolished the stimulation but dizocilpine and 6-nitro-7-sulphamoylbenzo-[f]quinoxaline-2,3-dione (NBQX) (0.1 mM) had no significant effects. At the 0.1-mM concentration kainate and AMPA also slightly but significantly potentiated taurine release (data not shown).

Of the metabotropic glutamate agonists quisqualate (1.0 mM) markedly stimulated the release, as did the agonists of group I receptors (1S,3S)-1-aminocyclopentane-1,3-dicarboxylate (trans-ACPD) and (S)-3,5-dihydroxyphenylglycine (DHPG) (both 0.1 mM) (Fig. 3). Their effects were not markedly influenced by the respective antagonists L(+)-2-amino-3-phosphonopropionate (L-AP3) and (RS)-1-aminoindan-1,5-dicarboxylate (AIDA). The agonists of group II and III metabotropic receptors, (2S,2′R,3′R)-2-(2′,3′-dicarboxycyclopropyl)glycine (DCG IV), L-(+)-2-amino-4-phosphonobutyrate (L-AP4) and O-phospho-L-serine (L-SOP) had no effects on taurine release (Fig. 3).

The cell-damaging conditions were applied from the beginning of incubations with exposure to K^+ or veratridine for 10 or 20 min. In all cases the initial release was increased (Figs. 1 and 4), more markedly in hypoglycemia, oxidative stress and the presence of free radicals than under oxygen-deficient conditions. Moreover, in the former experiments the enhancement lasted longer (k_2) (Table 1, Fig. 4). K^+ stimulation still evoked more release under all other conditions except hypoxia and ischemia. Veratridine stimulation was greater in oxidative stress and free radical-containing medium and smaller in ischemia (Table 1, Fig. 1). Both K^+ and veratridine stimulations outlasted the stimulus applied, veratridine-evoked stimulations not being attenuated to the basal level within 20 min after stimulus withdrawal (Fig. 1).

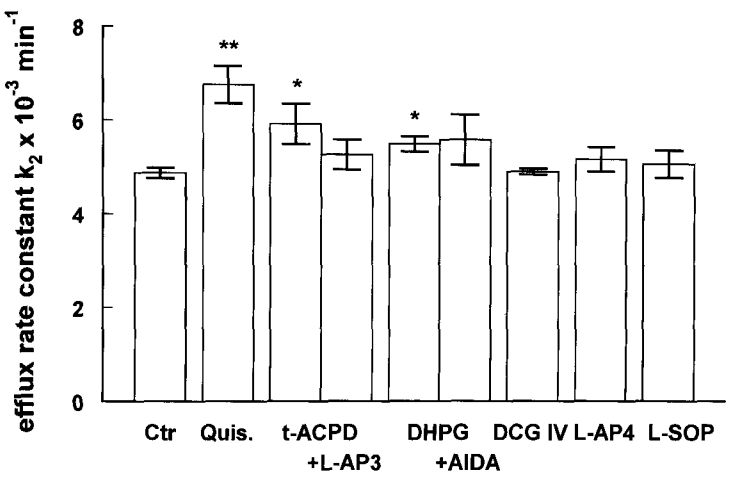

Fig. 3. Effects of metabotropic glutamate receptor agonists and antagonists (all 0.1 mM) on taurine release from cultured cerebellar granule cells. The results are efflux rate constants k_2 for the stimulation phase (24–30 min). The mean values (±SEM) of 4–8 independent experiments are shown. Significance of differences from the control: $*p < 0.05$, $**p < 0.01$

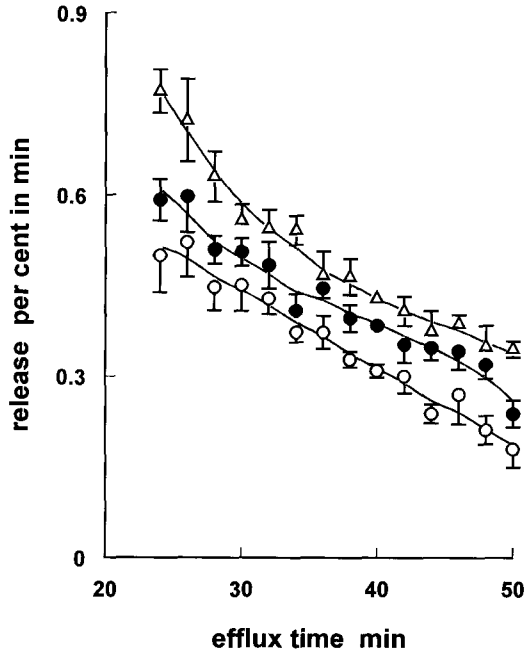

Fig. 4. Time-course of taurine release from cultured cerebellar granule cells in normoxia (-○-) and oxidative stress (-●-) and in the presence of free radicals (-△-). These cell-damaging conditions were applied from the beginning of incubations. The results are mean values (±SEM) of 4–6 independent experiments

Discussion

The release of taurine from cerebellar granule cells has been studied mainly in the context of its osmoregulatory function. In hypoosmotic solutions cerebellar granule neurons exhibit a regulatory volume decrease subsequent to swelling (Pasantes-Morales et al., 1993), resulting in an efflux of both organic and inorganic osmolytes through channel-like sites. Taurine release is indeed evoked by a reduction in the osmolarity of incubation media. The K^+-stimulated release is Cl^--dependent and inhibited by an increase in medium osmolarity, suggesting that it may be likewise associated with cell swelling (Schousboe et al., 1990). The hypoosmolarity-induced taurine release from cerebellar granule cells is mediated by diffusion, not by the transport systems (Schousboe et al., 1991), which are also known to operate in membranes of these cells (Holopainen et al., 1987). The identity of pores or leaky channels in the swelling-activated taurine release is still not established. Stretch-sensitive chloride channels have been held as possible candidates but other, possibly yet unidentified, channel-forming protein molecules cannot be excluded (Pasantes-Morales and Schousboe, 1997).

There is a considerable body of evidence indicating a functional role for taurine other than as an osmolyte in these cerebellar neurons. The efficient high-affinity transport system in granule neurons has properties characteristic of transmitter uptake, being specific and Na^+- and energy-dependent (Holopainen et al., 1987). Electrophysiological studies have indicated the presence of both GABA- and glycine-activated Cl^- channels in granule neurons (Robello et al., 1993; Kaneda et al., 1995; Brickley et al., 1996; Virginio and Cherubini, 1997). Taurine has also been shown in these cells to activate large Cl^- conductance channels blocked by both the $GABA_A$ receptor antagonist bicuculline and the glycine antagonist strychnine (Zhu and Vicine, 1997; Zhu et al., 1998). Moreover, the binding of strychnine to cerebellar granule cell membranes is inhibited by taurine (Elster et al., 1998). Taurine also, similarly to the glycine and GABA agonist isoguvacine, reduces the K^+-evoked release of D-aspartate in granule neurons (Wahl et al., 1994).

One prominent feature of K^+-stimulated taurine release from different brain preparations, namely the slow time course (Saransaari and Oja, 1992), is also evident in cerebellar granule cells (Holopainen et al., 1989; Schousboe and Pasantes-Morales, 1989; Schousboe et al., 1990). This propensity has been held as evidence against the neurotransmitter role of taurine (Schousboe and Pasantes-Morales, 1989). Taurine release has been shown to be at least partially Ca^{2+}-dependent (Holopainen et al., 1989; Philibert et al., 1989), whereas the regulatory volume decrease and osmolyte fluxes are independent of extracellular Ca^{2+} (Morán et al., 1997). The veratridine-evoked taurine release was in granule cells particularly prolonged, outlasting the stimulus in both normoxia and hypoxia. Veratridine is a depolarizing agent opening both neuronal and astrocytic Na^+ channels, the density of channels being greater in neurons than in astrocytes (Nowak et al., 1987). The increased taurine release even during the recovery phase may result from the fact that veratridine keeps Na^+ channels open (Nowak et al., 1987).

Cerebellar granule cells possess both ionotropic and metabotropic glutamate receptors (Chuang et al., 1992; Aronica et al., 1993; Bessho et al., 1993; 1994; Holopainen et al., 1994; Santi et al., 1994). All ionotropic agonists stimulate taurine release in brain slices (Magnusson et al., 1991; Saransaari and Oja, 1991; 1997d), but NMDA is the most effective. The kainate-stimulated release was now inhibited by the kainate antagonist CNQX in cerebellar granule neurons, which would point to the involvement of receptor sites. The non-NMDA receptor agonists kainate and AMPA also induce the release of D-[^{3}H]aspartate (Gallo et al., 1990) and endogenous glutamate and – to a lesser degree – other amino acids, including taurine and glycine, by a Ca^{2+}-independent and receptor-mediated mechanism (Levi et al., 1991). The metabotropic glutamate receptors seem to play a minor role in taurine release in granule cells, though here the unspecific ligand of iono- and metabotropic receptors, quisqualate, and agonists of group I receptors slightly enhanced the release. These effects were not, however, influenced by the respective antagonists, which would indicate nonspecific actions. On the other hand, we have shown that taurine release is regulated by group I metabotropic receptors in hippocampal slices prepared from developing mice (Saransaari and Oja, 1999b).

Under our short-term cell-damaging conditions the increase in taurine release was rather small when compared to responses to similar insults in hippocampal slices (Saransaari and Oja 1997b,c; 1998a,b) or to hyposmotic conditions in cerebellar granule cells (Pasantes-Morales et al., 1993). Taurine release in cerebral cortical astrocytes is likewise not very pronounced under the present cell-damaging conditions, but in contrast to granule neurons the response to K^+-stimulation is then markedly accentuated (Saransaari and Oja, 1999a). Under the hypoxic conditions applied here the initial release was, however, fairly large, possibly exhausting the releasable pool of taurine, whereas in the presence of free radicals and oxidative stress more releasable taurine could have been available. The sources of release could thus have been different under different conditions. In addition to this, alterations in taurine reuptake may constitute a source of differences. To date, hypoosmotic media enhance taurine uptake into cultured cerebellar granule cells (Schousboe et al., 1991) but abolish the high-affinity uptake and inhibit the low-affinity uptake of taurine in cerebral cortical slices (Oja and Saransaari, 1996b). However, it is not known how the uptake mechanisms in granule cells respond to the present incubation conditions.

Chemical ischemia has evoked in cerebellar granule cells a rapid exocytotic vesicular release of D-aspartate, followed by a slower nonexocytic release (Pocock and Nicholls, 1998). Such a biphasic release reflects the ATP-dependency of vesicular release, which has also been thought to be Ca^{2+}-dependent (Szatkowski and Attwell, 1994). The slight biphasic nature of K^+-evoked taurine release from cultured cerebellar granule cells was now clearly augmented in ischemia. The reason of it is obscure but may be associated with the possible fast depletion of ATP stores in the cells and the decline of exocytotic component of release.

In general, it seems to us that the complex mechanisms of cell damage should be investigated with whole-tissue preparations, e.g., brain slices, rather

than with isolated cells (Obrenovitch and Urenjak, 1997). Furthermore, it should be kept in mind that ischemic responses are markedly dependent on the vulnerability of the brain area subjected to study (Pulsinelli, 1985). The prolonged taurine release outlasting the applied stimuli in cerebellar granule neurons under both normoxic and cell-damaging conditions may be of importance for the maintenance of homeostasis in the cerebellum. As an inhibitory amino acid taurine could induce hyperpolarization and reduce cell excitability over a longer time-span than other possible neuroprotective mechanisms. Taurine also increases Cl^- conductance and affects Ca^{2+} fluxes in nervous tissue (Saransaari and Oja, 1997a), being thus able to contribute to the preservation of cell integrity under damaging conditions.

Acknowledgements

The skillful technical assistance of Mrs Oili Pääkkönen and Mrs Sari Luokkala and the financial support of the Medical Research Fund of Tampere University Hospital and the Academy of Finland are gratefully acknowledged.

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Received May 7, 1999