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Taurine, glutamate and GABA modulate the outgrowth from goldfish retinal explants and its concentrations are affected by the crush of the optic nerve

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Summary. The amino acid taurine plays an important trophic role during development and regeneration of the central nervous system. Other amino acid systems, such as those for glutamate and gamma-aminobutyric acid (GABA), are modified during the same physiological and pathological processes. After crushing the optic nerve, goldfish retinal explants were plated in the absence and in the presence of different amino acids and amino acid receptor agonists. The length and the density of the neurites were measured at 5 days in culture. Taurine increased the length and the density of neurites. Glutamate and glycine increased them at low concentration, but were inhibitors at higher concentration. The combination of N-methyl-D-aspartate (NMDA) and glycine produced a greater inhibitory effect than NMDA alone. NMDA or alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) added simultaneously with taurine impaired the stimulatory effect of the latter. GABA stimulated the emission of neurites in a concentration dependent manner. Hypotaurine also elevated the length of neurites, but cysteinesulfinic acid did not produce a significant effect. The concentrations of taurine, glutamate and GABA were determined by HPLC with fluorescent detection in the retina of goldfish at various days post-crushing the optic nerve. The levels of taurine were significantly increased at 48 h after the crush, and were elevated up to 20 days. Glutamate level decreased after the lesion of the optic nerve and was still low at 20 days. GABA concentration was not significantly different from the control. The interaction of these amino acids during the regenerative period, especially the balance between taurine and glutamate, may be a determinant in restoring vision after the crush.

Keywords: Amino acids – GABA – Glutamate – Outgrowth – Regeneration – Retina – Taurine

Abbreviations: AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid; GABA, gamma-aminobutyric acid; NMDA, N-methyl-D-aspartate

Introduction

Neurotransmitters, in addition to their role as classical synaptic agents, are involved in the regulation of development, neuronal cytoarchitecture and neuronal connectivity (Lauder, 1993; Lipton and Kater, 1989; Schwartz, 1990). The accumulated studies support that these molecules may operate in order to produce an optimal level of intracellular calcium to maintain viability and outgrowth (Kater et al., 1988, Lipton and Kater, 1989; Wong, 1995).

Excitatory amino acids can act as neurotrophic or as neurotoxic agents (Lipton and Kater, 1989). The importance of glutamate and ionotropic glutamate receptors during development of the retina has been pointed out for the rabbit (Wong, 1995), and the rat (Ishikawa et al., 1996; Nichol et al., 1995). In addition, the activation of metabotropic glutamate receptors produces retinal and optic nerve atrophy in the adult rat, and also initiates toxicity in the developing retina (Fix et al., 1995). Modulation of the glutamate system after the crush of the optic nerve occurs in the goldfish, in which the expression of N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) glutamate receptors are modified in ganglion cells (Hieber and Goldman, 1995).

In the developing rat brain, the response to NMDA is enhanced and neurotoxicity is greater than in the adult brain (Johnston, 1994), probably as a regulation of total cell number. It has been shown that glutamate released from the entorhinal cortical explants inhibits outgrowth and promotes synaptogenesis in co-cultured young hippocampal pyramidal neurons (Mattson et al., 1988a). Moreover, localized brief applications of glutamate can stimulate the formation of new protrussion or small processes along dendrites of older hippocampal neurons *in vitro* (Patterson, 1988). It is interesting to note that in young hippocampal cultures non-NMDA receptors exert an effect on outgrowth, whereas in older cultures NMDA receptors are involved (Mattson et al., 1988b). According to the participation of glutamate receptors in the expression of neurites is the evidence that specific NMDA receptor antagonists block the stimulation of outgrowth from cerebellar granule cell neurons (Pearce et al., 1987). On the other hand, glutamate produces cell death at high doses in the rat hippocampus (Mattson et al., 1988a; Rothman et al.,1987), in the mouse cortex (Choi et al., 1987), and in rat retinal ganglion cells (Hahn et al., 1988). Both NMDA and non-NMDA receptors are related to synaptogenesis and plasticity (Constantine-Paton et al., 1990; McDonald and Johnston, 1990), but also make specific brain areas susceptible to excitotoxicity (McDonald and Johnston, 1990).

Gamma-aminobutyric acid (GABA) neurons appear early in the development of the embryonic central nervous system, including the retina (Lauder et al., 1986; Versaux-Botteri et al., 1994). Also, $GABA_A$ receptors are expressed in coincidence with its elevation in the brain (Cobas et al., 1991). The trophic effect of GABA in cerebellar granule cells *in vitro* includes promotion of neurite outgrowth, synaptogenesis and formation of low-affinity GABA receptors (Meier et al., 1991). In addition, the activation of $GABA_A$ receptors in the hippocampus leads to excitatory effects during the early post-natal

period and blockade of these receptors with bicuculline results in a reduction of outgrowth from cultured hippocampal neurons (Barbin et al., 1993).

Nutritional deficiency of the sulfur amino acid taurine produces irreversible degeneration in the retina of kittens (Hayes et al., 1975; Sturman et al., 1985a,b). It also leads to functional and structural changes in the central nervous system of the cat (Schmidt et al., 1975), and the rat (Pasantes-Morales et al., 1983). Drug induced taurine depletion leads to a loss of optic nerve axons in rats (Lake, 1988), which is possibly related to the observation that the addition of taurine increases the outgrowth of the goldfish retina (Lima et al., 1988; 1993). The latter model has been very useful in understanding the mechanisms and factors involved in the regeneration of the central nervous system (Landreth and Agranoff, 1976; Lima et al., 1988, 1992), especially ganglion cells (Matus et al., 1997).

The aims of the present work were: 1) to study the effect of glutamate, glycine and GABA, as well as the glutamate receptor agonists, NMDA and AMPA, on the outgrowth of the goldfish retina in culture, and 2) to evaluate the possible modifications of retinal concentrations of taurine, glutamate and GABA *in vivo* after crushing the optic nerve, in order to provide an evidence of the endogenous role of these amino acids during the regenerative process.

Material and methods

Animals

Goldfish (*Carassius auratus*), 4–6 cm in length were adapted to darkness for 30 min and anesthetized in 0.05% tricaine (Sigma) prior to dissection of the retina. The lesion of the optic nerve was performed 10–14 days before explantation, by pulling the eye forward of the orbit and crushing the nerve with fine forceps (Lima et al., 1988).

Preparation of explants and culture conditions

The retina of fish was dissected, placed in a small volume of culture medium, and chopped in a McIlwain tissue chopper into 500 μm squares (Lima et al., 1988). Explants were placed (10–15 per dish) in poly-L-lysine (Sigma) precoated culture flasks. Nutrient medium (2 ml per dish) was Leibovitz (L-15, Gibco), 20 mM HEPES, 10% fetal calf serum, and 0.1% w/v gentamicin (Sigma). Cytosine arabinose (Sigma, 2 mM) was added 24 h after plating. The post-crush explants were cultured in the absence or in the presence of several amino acids and agonists of ionotropic glutamate receptors. The amino acids (Sigma) were added in mM as follows: taurine 4; glutamate, glycine, and GABA all were examined at 0.05, 0.1, 1 and 4; hypotaurine and cysteinesulfinic acid were examined at 4. Glutamate receptor agonists, NMDA or AMPA, were added in concentrations of 8 nM or 0.05 mM. In some experiments outgrowth was measured with the combination of amino acids or amino acids and glutamate receptor agonists.

Evaluation of outgrowth

The outgrowth of postcrush retinal explants was determined after 5 days in culture. The length of the neurites (20–30 per explant or less, if the number of neurites was lower) was measured in μm with an ocular micrometer. The neurite to be measured was evaluated from the border of the explant and was followed up to the maximal length reached, which could include the fusion of fibers also emitted by the explants. The number of fibers per explants was defined as the density of neurites in a scale of 0–4.

Determination of amino acids

The amino acids were determined by high performance liquid chromatography using a fluorescent detector (Lima et al., 1989a). The system consisted of a Model 2150 solvent delivery pump, a Model 2152 LC controller, a Model 2157 auto-sampler injector (LKB, Broma, Sweden), and a Spectroflow 980 fluorescence detector (Kratos Analytical). Output from the detector was recorded with a Model 2220 recording integrator. The column used was LC-18 (4.6 × 100 mm, 5 μm) (Supelco). Each retina was homogenized in 300 μl distilled water (0.85 ± 0.08 mg of protein/ml) and precipitated with sulfosalicylic acid 20%. After centrifugation at 35,000 g for 20 min, 75 μl of the supernatant were taken for derivatization. The reaction was carried out by addition of 125 μl of 0.4 M potassium borate buffer pH 10.4 and 100 μl of N,N-dimethyl-1-naphthylamine-5-sulfonic acid chloride solution (0.5 mg/ml, Sigma, in acetonitrile, Merck) followed by incubation at 65°C for 10 min. The reaction was stopped by placing the tubes on ice, and after centrifugation at 30,000 g for 2 min, aliquots of supernatant were injected into the chromatographic system. The solutions used to create the gradient mobile phase were: Buffer A, 50 mM sodium phosphate pH 6.3, 0.3% tetra-hydrofurane and 5% acetonitrile, and Buffer B, acetonitrile. The main gradient in percentage of Buffer B was: 0–25 min 15%; 25–45 min 20; 45–60 min 30; 60–70 min 50; 70–75 min 90; 75–78 min 90; 78–80 min 0. Taurine, glutamate, and GABA were quantified by the method of external standard and expressed in nmol/mg of protein. Protein was determined by the method of Lowry et al. (1951). Stock solutions of amino acids (Sigma, 1 mg/ml) were prepared in 0.5 N HCl and stored at −20°C. The concentration of the amino acids was determined at various days after crushing the optic nerve.

Statistical analysis

Each value is expressed as mean ± standard error. Two-tailed analysis of variance (ANOVA) was used for comparisons. The probabilities of the differences between means were derived from the ANOVA (Barlow, 1983).

Results

Effect of amino acids on outgrowth from retinal explants

The length of the neurites in control explants and in the presence of taurine or glutamate at various concentrations is represented in Fig. 2. There was no outgrowth of explants of retinas when the optic nerve was not crushed. Taurine, 4 mM, stimulated the outgrowth, increasing both length (Fig. 1 and 2) and density (Table 1) of the neurites. Glutamate increased the length of neurites at concentrations of 0.05 and 0.1 mM, but decreased it when 4 mM was used (Fig. 2). The density of neurites was also modified by glutamate, 0.05, 0.1 and 1 mM (Table 1).

Glycine, in concentrations of 0.05, 0.1 and 1 mM, also increased the length of fibers (Fig. 3) and decreased the density (Table 1), but at 4 mM it decreased the length without modifying density. GABA increased the length of neurites at low concentrations and did not significantly change it at higher concentrations (Fig. 4). This amino acid decreased the density of neurites at low concentrations, but not at higher (Table 1). Hypotaurine increased the length of the fibers (Fig. 5) without significantly altering the density (Table 1), while cysteinesulfinic acid did not have any effect.

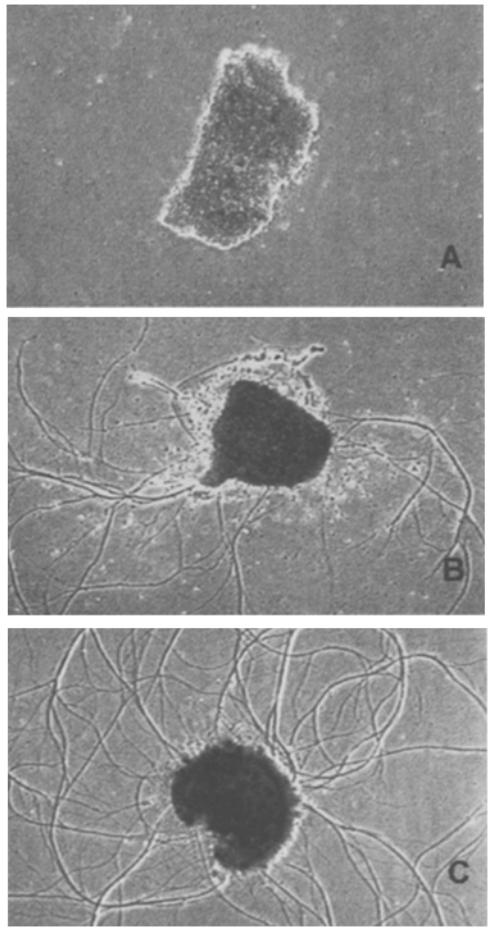

Fig. 1. Photomicrographs of goldfish retinal explants at 5 days in culture **A**) without lesion of the optic nerve, **B**) post-crush explant in the absence of taurine, and **C**) post-crush explant in the presence of 4 mM taurine. 1.5 cm = 500 μm

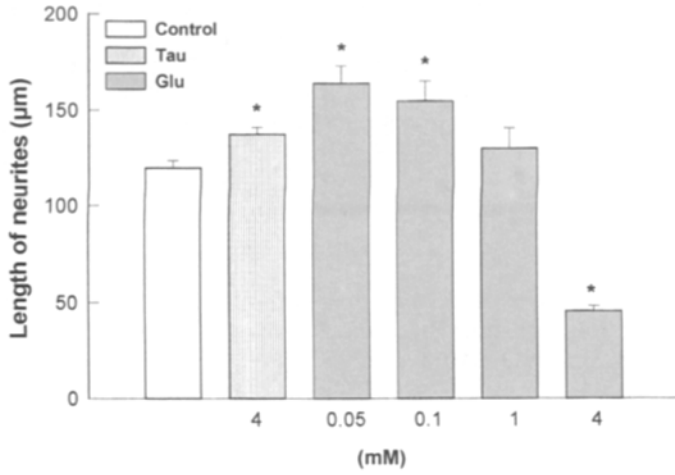

Fig. 2. Length of neurites from postcrush retinal explants after 5 days in culture in the absence (control) and in the presence of taurine 4 mM, or glutamate 0.05, 0.1, 1 and 4 mM. Each value is mean ± SEM. ANOVA $F_{(4,321)} = 86.47$ $p < 0.0001$. * $p < 0.01$ with respect to control

Table 1. Effect of amino acids on neurite density from goldfish retinal explants

Treatment	(mM)	Neurite density
Control		2.52 ± 0.05
Taurine	4	3.20 ± 0.05*
Glutamate	0.05	2.25 ± 0.06*
	0.1	1.93 ± 0.11*
	1	1.92 ± 0.11*
	4	2.43 ± 0.07
Glycine	0.05	2.28 ± 0.07*
	0.1	1.61 ± 0.07*
	1	1.72 ± 0.09*
	4	2.61 ± 0.05
GABA	0.05	2.22 ± 0.07*
	0.1	1.62 ± 0.07*
	1	1.75 ± 0.07*
	4	2.66 ± 0.07
Hypotaurine	4	2.81 ± 0.14
Cysteine sulfinic acid	4	2.85 ± 0.11

Each values is mean ± SEM. n = 25 − 111. *$p < 0.05$ with respect to control.

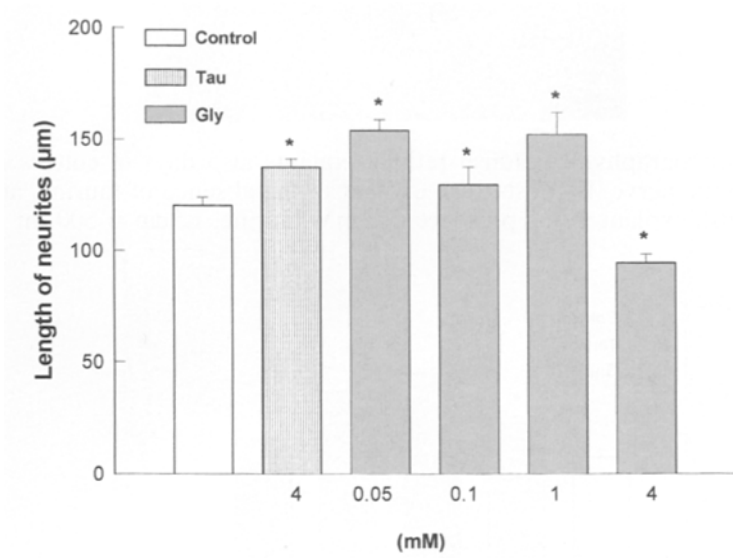

Fig. 3. Length of neurites from postcrush retinal explants after 5 days in culture in the absence (control) and in the presence of taurine 4 mM, or glycine 0.05, 0.1, 1 and 4 mM. Each value is mean ± SEM. ANOVA $F_{(4,443)} = 20.50$ $p < 0.0001$. * $p < 0.01$ with respect to control

Effect of glutamate receptor agonists on outgrowth from retinal explants

The addition of NMDA, 8 nM or 0.05 mM, did not significantly modify the length of the neurites from the explants (Fig. 6), but did reduce the density (Table 2). The length and density of neurites (Fig. 6, Table 2) were decreased

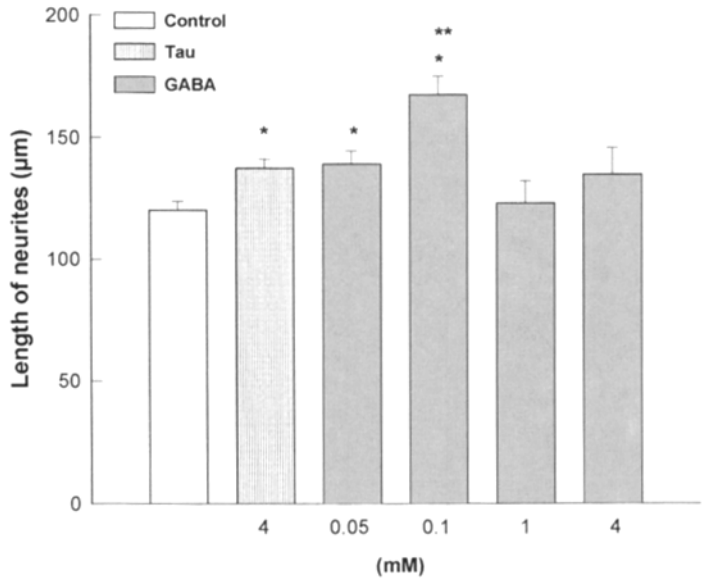

Fig. 4. Length of neurites from postcrush retinal explants after 5 days in culture in the absence (control) and in the presence of taurine 4 mM, or GABA, 0.05, 0.1 1 and 4 mM. Each value is mean ± SEM. ANOVA $F_{(4,388)} = 4.68$ $p < 0.0001$. * $p < 0.01$ with respect to control, ** $p < 0.01$ with respect to taurine or GABA 0.05 mM

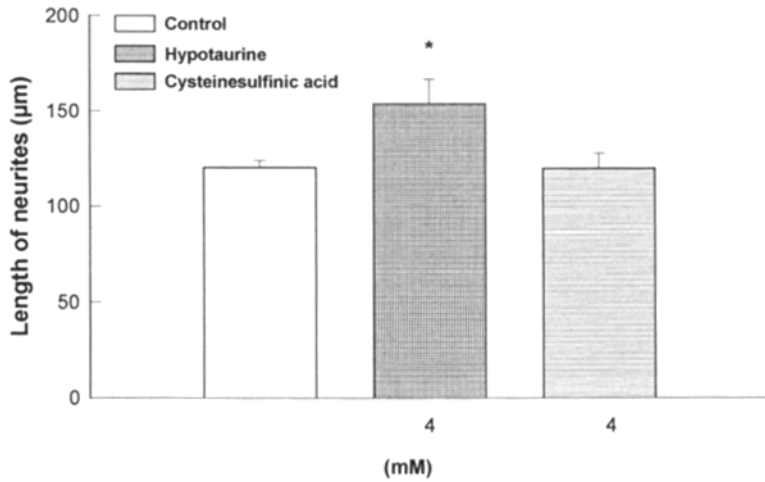

Fig. 5. Length of neurites from postcrush retinal explants after 5 days in culture in the absence (control) and in the presence of hypotaurine or cysteinsulfinic acid, 4 mM. Each value is mean ± SEM. * $p < 0.001$ with respect to control

by the simultaneous presence of NMDA and taurine or NMDA and glycine, relative to taurine or glycine alone, respectively. AMPA, 8 nM or 0.05 mM, did not significantly change the length of the fibers (Fig. 7), but reduced the density at higher concentrations (Table 2).

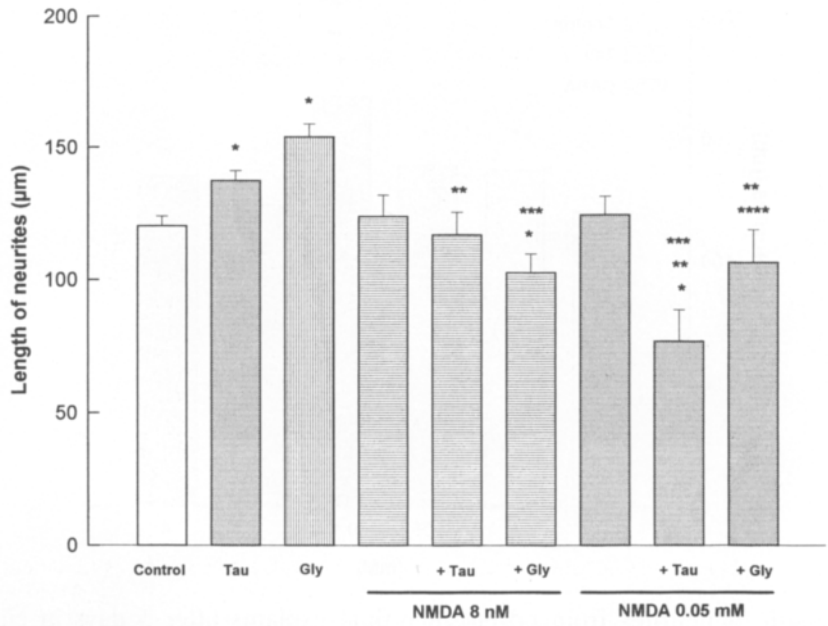

Fig. 6. Length of neurites from postcrush retinal explants after 5 days in culture in the absence (control) and in the presence of taurine 4 mM. NMDA 8 nM or 0.05 mM, glycine 0.05 mM or the combination of NMDA 8 nM or 0.05 mM and taurine 4 mM or NMDA 8 nM or 0.05 nM and glycine 0.05 mM. Each value is mean ± SEM. ANOVA *NMDA 8nM*: NMDA and taurine $F_{(3,365)} = 8.54$ $p < 0.0001$; NMDA and glycine $F_{(3,318)} = 8.42$ $p < 0.0001$. * $p < 0.01$ with respect to control, ** $p < 0.01$ with respect to NMDA, *** $p < 0.01$ with respect to taurine, **** $p < 0.01$ with respect to glycine. ANOVA *NMDA 0.05mM*: NMDA and taurine $F_{(3,346)} = 3.67$ $p < 0.05$; NMDA and glycine $F_{(3,281)} = 11.91$ $p < 0.0001$. * $p < 0.01$ with respect to control, ** $p < 0.05$ with respect to taurine, *** $p < 0.01$ with respect to glycine

Table 2. Effect of glutamate receptor agonists on neurite density from goldfish retinal explants

Treatment		Neurite density
Control		2.52 ± 0.05
Taurine	4 mM	3.20 ± 0.05*
NMDA	8 nM	1.96 ± 0.10*
	+Taurine 4 mM	1.84 ± 0.20**
	+Glycine 0.05 mM	1.32 ± 0.09***
NMDA	0.05 mM	2.09 ± 0.09*
	+Taurine 4 mM	1.23 ± 0.09**
	+Glycine 0.05mM	1.84 ± 0.11***
AMPA	8 nM	2.42 ± 0.09
	+Taurine 4 mM	2.42 ± 0.09**
	0.05 mM	1.10 ± 0.06*

Each value is mean ± SEM. n = 20 − 62. *$p < 0.05$ with respect to control; **$p < 0.05$ with respect to taurine; ***$p < 0.05$ with respect to glycine.

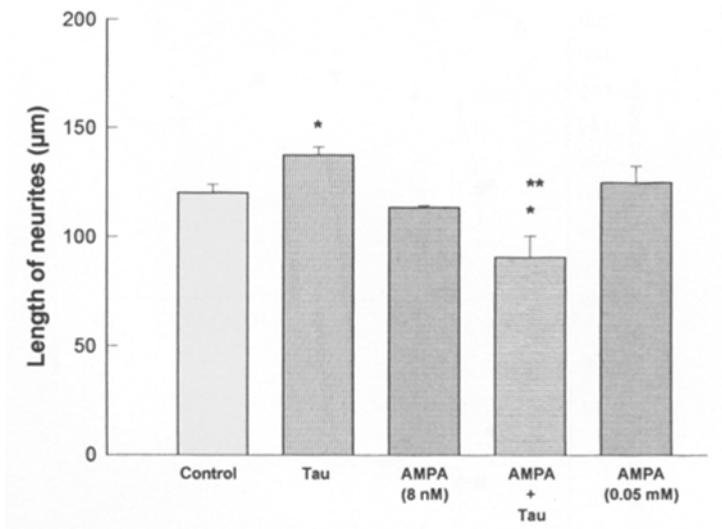

Fig. 7. Length of neurites from postcrush retinal explants after 5 days in culture in the absence (control) and in the presence of taurine 4 mM, AMPA 8 nM or 0.05 mM, and the combanation of AMPA 8 nM and taurine 4 mM Each value is mean ± SEM. ANOVA $F_{(4,340)} = 6.73$ $p < 0.0001$. * $p < 0.01$ with respect to control, ** $p < 0.05$ with respect to taurine

Taurine, glutamate and gamma-aminobutyric acid levels in the retina after crushing the optic nerve

Taurine concentration increased within 48 h in the retina of goldfish after crushing the optic nerve, and the elevated levels were maintained up to 20 days after the lesion (Fig. 8A). Glutamate concentration decreased significantly 5 days after the lesion, and remained lower than the control even 20 days after the lesion (Fig. 8B). GABA concentration was not significantly affected by the lesion (Fig. 8C).

Discussion

As it was previously demonstrated (Lima et al., 1988) the length and the density of neurites from goldfish retinal explants were increased by taurine, a process that is partially mediated through an increase in calcium flux (Lima et al., 1993) without affecting cell proliferation (Matus et al., 1997). The effect of taurine was reported in the present work in order to be compared with the possible effects of the other amino acids under study. Length and density of neurites, instead of nerve growth index (the product of the two parameters) were reported since they can be independently affected (Matus et al., 1997). The taurine precursor, hypotaurine, undergoes oxidation in the retina of the rat and the goldfish (Obregón and Lima, 1996). That is probably the reason hypotaurine also stimulates the outgrowth of goldfish retinal explants. However, cysteinesulfinic acid did not significantly modify the emission of neurites. This effect could be related to the regulatory roles of cysteinesulfinic acid

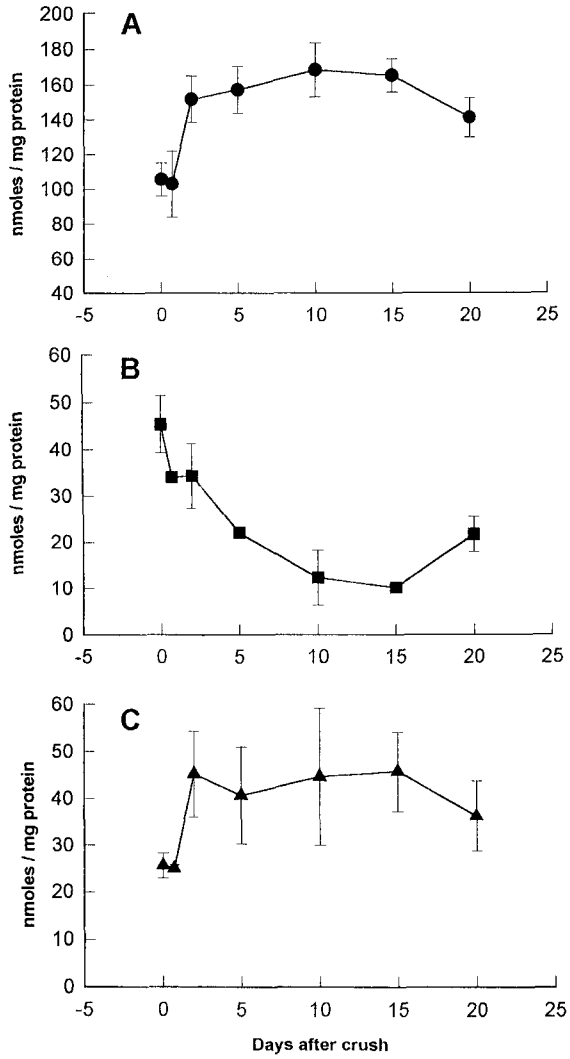

Fig. 8. Concentrations of taurine (**A**), glutamate (**B**) and GABA (**C**) in the retina of goldfish without lesion of the optic nerve and various days after the crush. Each value is mean ± SEM. ANOVA taurine $F_{(6,91)} = 3.49$ $p < 0.005$; glutamate $F_{(6,91)} = 5.84$ $p < 0.001$, GABA $F_{(6,98)} = 0.36$

decarboxylase and cysteine dioxygenase in the production of taurine in the central nervous system (Bagley et al., 1995; de la Rosa and Stipanuk, 1985).

Endogenous glutamate appearance in embryonic rabbit retina and its action through non-NMDA and NMDA receptors is involved in development (Wong, 1995). The concentration of this amino acid is high at birth and remains elevated during the postnatal period, while glutamine and GABA gradually increase in the developing retina of the rat (Ishikawa et al., 1996). Also in the rat, 60% of retinal ganglion cells die during the first postnatal week in culture studies, while only 10% survive in serum free

medium, in which glutamate increases survival up to 70% through non-NMDA and NMDA receptors (Nichol et al., 1995). Depolarization is essential for maintenance and outgrowth of ganglion cells (Araki et al., 1995), and in cultures of spinal cord neurons (Brenneman et al., 1990). Postnatal treatment of rats with the metabotropic glutamate receptor agonist, D,L-2-amino-3-phosphonopropionate, produces retinal and optic nerve atrophy in adult rats, and initiates progressing retinal toxicity (Fix et al., 1995). After crushing the optic nerve, the expression of NMDA receptors decreases and then increases in ganglion cells when their axons are forming stable connections in the optic tectum of the goldfish, but AMPA-like receptors decrease (Hieber and Goldman, 1995). In the present report, low concentrations of glutamate added to explants increase outgrowth, but higher concentrations decrease it. This might indicate that the excitatory stage induced by the presence of glutamate facilitates the regeneration, but an overstimulation of the cells by the excitatory amino acid may impair the emission of neurites from the retina. Glycine displays a behaviour similar to glutamate when given alone, it also potentiates the effect of NMDA in decreasing outgrowth, indicating its role as a positive allosteric modulator of NMDA receptors, a relationship that is not affected in young animals with respect to adulthood (Boje and Skolnick, 1992), and might persist during degeneration. However, the stimulatory effect of glycine when added alone could be the result of its interaction with strychnine-sensitive receptors, a point that needs further investigation.

In other studies including different areas of the central nervous system the role of glutamate in development and regeneration has been described. Application of AMPA into guinea-pig cochleas results in a destruction of all postsynaptic endings of the auditory nerve, and in an up-regulation of NMDA and metabotropic glutamate receptors in the primary auditory neurons (Puel et al., 1995). After injury of the cortex, NMDA receptors decrease in the homolateral striatum and increase in recovered rats (Vago and Marshall, 1996). The modification of the glutamate system is also demonstrated for the transporter, since in axotomized hypoglosal motoneurons of the rat glutamate uptake is up-regulated, and may increase the resistance of cells against the accumulation of glutamate during the process of nerve regeneration (Kiryu et al., 1995).

Not only NMDA, but kainate promotes survival of cerebellar granule cell neurons in an additive manner (Balázs et al., 1990a,b). Our results indicate that both NMDA and non-NMDA rectors might be implicated in the regeneration of the retina, because even if NMDA did not have any effect by itself the simultaneous addition of this agonist and glycine reduced the outgrowth, as it is well known that the amino acid positively modulates NMDA receptors (Lodge and Collingridge, 1991). Interestingly, when the explants were cultured in the presence of taurine the addition of NMDA decreased the outgrowth, resulting in an even lower neurite length than that from control explants. It could be that the trophic effect of taurine, partially mediated by an increase in calcium influx, could be not only blocked, but reversed by a further increase in the intracellular calcium concentration through the activation of NMDA receptors. The fact that glutamate could inhibit outgrowth, and not

NMDA or AMPA by themselves, suggests that glutamate metabotropic receptors could be involved.

The GABA-concentration dependency curve, like that previously reported for taurine (Lima et al., 1988; 1989b), was bell-shaped. The role of GABA in outgrowth and development, has been indicated in several models, for example the treatment with diazepam elevates the growth rate in Tetrahymena (Darvas et al., 1985). Also the opening of chloride channels by GABA triggers methamorphosis in abalone larvae and honey comb worm (Lauder, 1993). In the rat, kainate lesions of the GABAergic horizontal cells in developing retinas produce a modification of the structure of the outer plexiform layer (Messersmith and Redburn, 1990).

In addition to the demonstration of the *in vitro* role of excitatory and inhibitory amino acids, the *in vivo* regulation of the amino acid concentrations indicate their function in regeneration. Taurine increased and glutamate decreased in the post-crush retina with a similar opposite shape. This reaction of the retina is probably correlated with what has been reported for glutamate and GABA in other systems. In regenerating newt retinal cells, neurons responding to GABA and glycine receptors increase and those responding to NMDA and AMPA decrease in number (Chiba and Saito, 1995). Modification in the expression of receptors has been also reported, such as down-regulation of NMDA receptor gene expression in the goldfish retina during optic nerve regeneration (Ueda and Heiber, 1995). It has been reported that GABA prevents the growth suppressive effects of glutamate on cultured hippocampal neurons by reducing calcium influx (Mattson and Hauser, 1991). Moreover, astrocytes participate in this relationship, since they express non-NMDA receptors and GABA is released from them in response to agonists (Berger et al., 1992; Levi and Patrizio, 1992). However, in this report the modifications in GABA concentration produced by the lesion of the optic nerve were not significant. The modulation of specific enzymes involved in the formation of these amino acids, and the overall interaction between their metabolic systems need to be investigated. Different embryonic forms of glutamic acid decarboxylase, with distinct functional domains and developmental patterns are probably consistent with the ontogenic role of GABA (Akimoto et al., 1993; Behar et al., 1994; Szabo et al., 1994). Excitatory and inhibitory neurotransmitter amino acids, such as glutamate and GABA, as well as the neuromodulator taurine, are involved in regeneration and outgrowth of the goldfish retina. The interaction of these molecules will influence the final result of the processes and the successful end, which will be the restablishment of vision.

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