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Taurine trophic modulation of goldfish retinal outgrowth and its interaction with the optic tectum

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Summary. Goldfish retinal explant outgrowth in the presence of fetal calf serum is stimulated by taurine. In the absence of it, but with glucose in the medium, length of neurites is still elevated by the amino acid. Using the medium in the presence of glucose, but in the absence of fetal calf serum, we explored the effect of optic tectum medium from cultures of them coming from goldfish without crush of the optic nerve or 3, 5, 10, 14 and 20 days after crush. Retinal explants, intact or from goldfish with crush of the optic nerve 10 days prior to starting the culture, were employed in order to measure the possible effect of optic tectum media and the interaction with taurine. In other type of experiments the optic nerve was crushed 1, 2, 4, 7 and 10 days before dissection of the optic tectum, and then co-cultured with intact or 10 days post-crush retinal explants. Optic tectum media produced a time-dependent effect on outgrowth in lesioned retinas with a maximum effect around 5 days after the lesion for the corresponding optic tectum. Taurine, 4 mM, did not further affect the outgrowth in the presence of optic tectum media, but did significantly increase length of neurites either in intact or in post-lesion retinas. Co-culture of optic tectum at different days post-lesion and retinas at 10 days post-lesion increased the outgrowth around 4 days post-lesion, in a preparation resulting in mutual effects of both types of tissues. The addition of taurine in these conditions did not further increase outgrowth, rather inhibited it according to the time after lesion of optic nerve corresponding to the co-cultured optic tectum. The effect of taurine was concentration-dependent, since 0.2 mM was more effective than 2 or 4 mM in the presence of optic tectum with lesion of 2 days. These results demonstrate the time-course of the regeneration processes in the visual system of goldfish, indicating the crucial periods after crush in which the tectum could produce stimulation and later decrease or no effect on outgrowth from the retina. In addition, they are evidences of the interaction between taurine and optic tectum production of time-produced specific agents. The mechanisms underlying these effects are closely related to calcium, as it was demonstrated by the addition of extracellular or intracellular chelators to the medium, which inhibited the effects of the optic tectum and the trophic properties of taurine in this system. The inhibitor of taurine transport, guanidoethylsulfonate, also decreased the stimulatory effects of the optic tectum and of taurine, indicating an interaction of substances produced by the tectum with taurine, and an effect of taurine mediated through its entrance to the cells. Overall, retinal explants outgrowth in the absence of fetal calf serum, the interaction of agents of the optic tectum and taurine modulates outgrowth from the retina, and these effects are mediated by calcium levels and by the levels of intracellular taurine.

Keywords: Co-culture – Optic nerve crush – Optic tectum – Retinal outgrowth – Taurine

Introduction

Previous studies indicate that post-crushed optic nerve or post-axotomized results in differential regeneration of goldfish retinal explants (Cubillos et al., 2000), in addition, the medium in the absence of fetal calf serum (FCS) differentially affect outgrowth from explants cultured in the presence of FCS, and taurine stimulates it in both conditions (Cubillos et al., 2002). Moreover, conditioned media from aggregates or explants of retinal cells increase survival of ganglion cells of newborn rats (de Araujo and Linden, 1990). It seems that firing from retinal cells increases survival of rat superior colliculus, probably by the release of trophic agents by afferent fibers (Galli-Resta et al., 1993), indicating retino-fugal and retino-petal pathways, as demonstrated for transmitters systems (Lima and Urbina, 1998).

Target-independent processes also occur. For instance, structures such as retina and visual midbrain seem to be determinant during development of chicken tectal neuron, which occurs in an independent manner from the retina, and are dependent once synaptic contacts are made (Luksch and Poll, 2002).

Brain derived neurotrophic factor and neurotrophins 4/5 constitute anterograde survival molecules during development in the superior colliculus of the rat (Spalding et al., 2002). In addition, the activation of protein kinase C increases survival of ganglion cells in culture (dos Santos and de Araujo, 2000). It has been known that trophic

factors influencing visual system formation or its regeneration are produced in the retina, the target, and the optic nerve, involving neurons and glial cells (Cubillos et al., 2002; Schwalb et al., 1995).

Since the optic tectum is affected by the lesion of the optic nerve, due to interruption of the communication with the retina, in the present report we intended to understand the time-dependent effects due to the lack of proper interaction between both structures with the aim of detecting possible influences of substances produced by the target of the retina. Thus, medium from cultured optic tectum following different days after crush of the optic nerve was added to retinal explants, and the co-culture of optic tectum and retina was done for evaluating outgrowth and the possible interaction with taurine trophic effect. In addition, the effect of calcium and the relevance of intracellular taurine were also evaluated.

Materials and methods

Animals and retinal cultures

The optic nerve of goldfish (*Carassius auratus*) 5–6 cm was crushed with fine forceps at around 1 mm behind the emersion from the eye. The optic tectum was dissected according with Beltramo et al. (1994), and was obtained intact (NLOT) or at various days after the crush (LOT): 1, 3, 5, 10, 14 or 20 days. The tectum was plated in Leibovitz medium, L-15 with 0.1 mg/ml of gentamicin and 10 mM *N*-2-hydroethylpiperazine-*N*′-[2-ethanesulphonic acid (HEPES), and after 3 days the medium was obtained, filtered and used for culturing the retina. In other type of experiments, in which the tectum was obtained for simultaneous culture with retinal explants, the time after lesion was: 1, 2, 4, 7 and 10 days. All cultures were performed in the absence of FCS and in the presence of 0.25 mM glucose, as predetermined (Cubillos et al., 2002). Retinas used, either for cultures in the presence of optic tectum media or for co-cultures, were intact (NLR) or lesioned by crush of the optic nerve 10 days before plating (LR).

Taurine and optic tectum

Taurine was added to the cultures of retinal explants in concentration of 4 mM for studies with optic tectum media (Lima et al., 1988), and in concentrations of 0.2, 2 and 4 mM for co-cultures of optic tectum and retina (Cubillos et al., 2002). All resections of the retina were done 10 days after crushing the optic nerve. Evaluations of outgrowth were done at 5 or 10 days after plating, as indicated.

Effect of chelating calcium or blocking transport of taurine

In some experiments, chelating agents of extracellular or intracellular calcium, ethylenglycol-bis(β-amino-ethyl ether)-*N*,*N*′-tetraacetic acid (EGTA, 1 mM) or 1,2-bis(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid methyl ester (BAPTA-AM, 0.1 μM) (Lima et al., 1993), were used in order to determine the implication of this ion on optic tectum and taurine action over the outgrowth from retinal explants. Guanidoethylsulfonate (GES), inhibitor of taurine transporter, was added to the medium in some cultures of optic tectum and retinal explants in concentrations of 0.1, 1, 10 and 50 mM. GES, 10 mM, was also added to retinal-optic tectum co-cultures in the absence or in the presence of 4 mM taurine.

Evaluation of outgrowth

Outgrowth was determined by neurite length and density (Cubillos et al., 2002; Lima et al., 1988) by the program SigmaScanPro (Jandel Scientific). Length was only reported, since it corresponded with the results for the density of neurites.

Statistical analysis

Each value is the mean ± standard error of the mean. Analysis of variance was performed for group of results and Students' *t*-test for specific comparisons. Significance was considered if $P < 0.05$.

Results

Media of cultured optic tectum and the outgrowth of retinal explants with or without optic nerve lesion maintained in vitro in the absence or in the presence of taurine

The outgrowth from NLR or LR in the presence of media from NLOT or LOT at various days after lesion of the optic nerve cultured for 3 days was measured 5 days after plating the retinal explants. The results in Fig. 1 present a small emission of neurites by the NLR in comparison with the LR in basal conditions, and a stimulatory effect of the outgrowth from NLR by the medium of LOT which optic nerve was crushed 3 days after culturing it alone (empty bar number 3 in *x* axis). LOT either did not affect out-

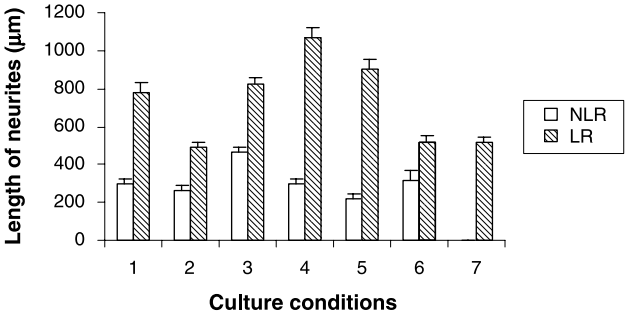

Fig. 1. Outgrowth from goldfish retinal explants expressed as length of neurites from retinas cultured for 5 days, corresponding to goldfish without (NLR) or with crushing of the optic nerve 10 days before plating (LR). ANOVA for NLR, $F_{(5,99)} = 10.50$, $P < 0.001$, and for LR, $F_{(5,343)} = 20.91$, $P < 0.001$. Culture conditions: *1* retinas; *2* retinas + medium from tectum cultured for 3 days corresponding to goldfish non-lesioned (NLOT); *3–7* retinas + medium from tectum cultured for 3 days which optic nerve was crushed 3, 5, 10, 14 and 20 days (LOT), respectively, before plating them. $P < 0.05$ for all cases between NLR and LR. NLR condition 3, $P < 0.05$ respecting 1. LR conditions 2, 4, 5, 6, and 7, $P < 0.05$ respecting 1

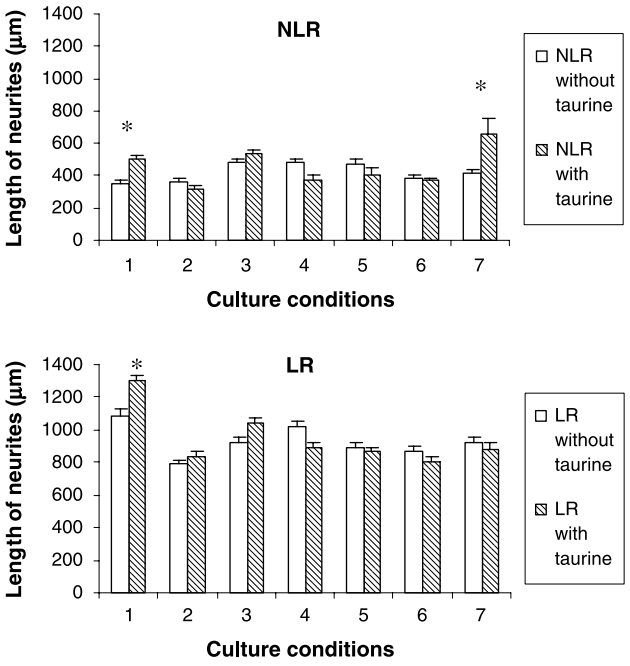

Fig. 2. Outgrowth from goldfish retinal explants expressed as length of neurites from retinas cultured for 5 days in the absence or in the presence of 4 mM taurine, corresponding to goldfish, **NLR** without lesion or **LR** with crushing of the optic nerve 10 days before plating. ANOVA for NLR, $F_{(5,993)} = 6.44$, $P < 0.001$, and for LR, $F_{(5,569)} = 11.35$, $P < 0.001$. Culture conditions: *1* retinas; *2* retinas + medium from tectum cultured for 3 days corresponding to goldfish non-lesioned (NLOT); *3–7* retinas + medium from tectum cultured for 3 days which optic nerve was crushed 3, 5, 10, 14 and 20 days (LOT), respectively, before plating them. $^{*}P < 0.05$ between without and with taurine

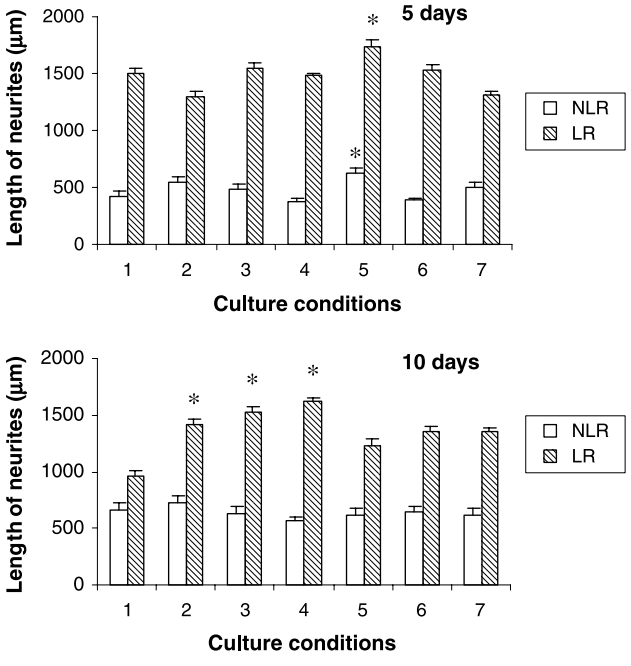

Fig. 3. Outgrowth from goldfish postcrushed retinal explants expressed as length of neurites in co-culture with optic tectum, corresponding to retinas of goldfish without lesion (NLR) or with lesion (LR) of the optic nerve. **A** 5 days or **B** 10 days after plating. ANOVA for 5 days in culture, RNL, $F_{(6,317)} = 4.58$, $P < 0.001$, and LN, $F_{(6,338)} = 11.19$, $P < 0.001$. ANOVA for 10 days in culture, RNL, $F_{(6,327)} = 0.88$, $P = 0.513$, and LN, $F_{(6,348)} = 24.29$, $P < 0.001$. Culture conditions: *1* retinas; *2* retinas co-cultured with intact optic tectum (NLOT); *3–7* retinas co-cultured with optic tectum corresponding to goldfish which optic nerve was crushed 1, 2, 4, 7 and 10 days (LOT), respectively, before plating them. $^{*}P < 0.05$ respecting retinas alone or retinas in the presence of NLOT

growth or inhibited it, as shown by 20 days after crushing the nerve. Respecting LR, there was a bell-shaped curve, first with an inhibitory effect of the medium of NLOT, increase at 5 days post-lesion, and a decrease at days 14th and 20th.

In the presence of 4 mM taurine for NLR or for LR (Fig. 2, NLR and LR) the main results were the smaller outgrowth in NLR, the increase of outgrowth obtained by taurine in both conditions, the elevation of outgrowth at 20 days in the presence of taurine for NLR, and the evident interaction between elements of the optic tectum media and taurine without further increase in the presence of the amino acid.

Co-culture of optic tectum and retinal explants with or without optic nerve lesion maintained in vitro in the absence or in the presence of taurine

The presence of NLOT or LOT at various days after the lesion with NLR in the same flask produced a small increase in outgrowth observed by the 4 days of LOT in culture (number 5 in *x* axis), although outgrowth was very modest (Fig. 3). The lesion of the optic nerve for the cultured retina (LR) produced a considerable increase in outgrowth with a significant small increase of neurite length in the retinas co-cultured with optic tectum from animals with lesions done 4 days prior to plating (LOT). In addition, NLOT did not affect outgrowth from NLR after 5 days, but did increase outgrowth from LR at 10 days in culture. The presence of optic tectum NLOT, and LOT 1 and 2 days post-lesion (numbers 2, 3 and 4 in *x* axis) of the optic nerve further increased the outgrowth from LR (Fig. 3). Thus, at this period of co-culture, there was a clear increase of outgrowth between 1 and 2 days after lesion of the optic nerve of fish from which tectum was obtained.

As shown in Fig. 4, NLR with NLOT or with LOT were not affected by the addition of taurine, but in the case of LR the coexistence of optic tectum and taurine impaired the outgrowth, giving a bell-shaped response.

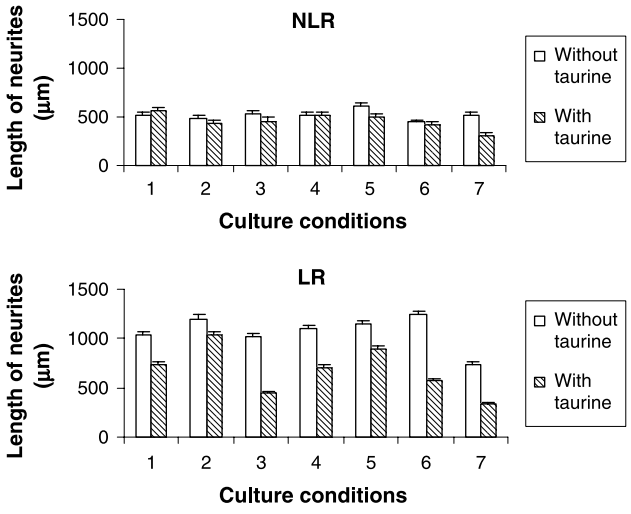

Fig. 4. Outgrowth from goldfish retinal explants expressed as length of neurites from retinas co-cultured with optic tectum for 5 days in the absence or in the presence of 4 mM taurine, corresponding to goldfish **NLR** without lesion or **LR** with crushing of the optic nerve 10 days before plating. ANOVA for NLR, without taurine $F_{(5,1141)} = 3.75$, $P < 0.01$, and with taurine, $F_{(5,454)} = 4.53$, $P < 0.001$. ANOVA for LR without taurine $F_{(5,1795)} = 28.75$, $P < 0.001$, and with taurine, $F_{(5,1871)} = 78.82$, $P < 0.001$. Culture conditions: *1* retinas; *2* retinas co-cultured with intact optic tectum (NLOT); *3–7* retinas co-cultured with optic tectum corresponding to goldfish which optic nerve was crushed 1, 2, 4, 7 and 10 days (LOT), respectively, before plating them. $^*P < 0.05$ respecting retinas alone or retinas in the presence of NLOT

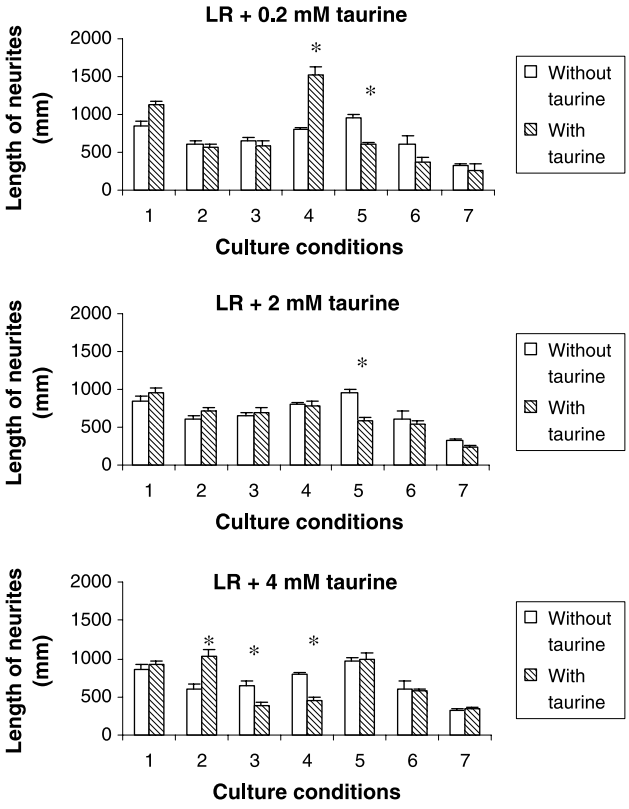

Fig. 5. Outgrowth from post-crushed goldfish retinal explants (LR) expressed as length of neurites from retinas co-cultured with optic tectum for 5 days in the absence or in the presence of 0.2, 2 and 4 mM taurine. ANOVA for LR + 0.2 mM taurine: without taurine $F_{(6,205)} = 9.34$, $P < 0.01$, and with taurine, $F_{(6,207)} = 29.86$, $P < 0.001$; for LR + 2 mM taurine: without taurine $F_{(6,205)} = 9.12$, $P < 0.001$, and with taurine $F_{(6,264)} = 21.05$, $P < 0.001$; and for LR + 4 mM taurine: without taurine $F_{(6,205)} = 9.12$, $P < 0.001$, and with taurine, $F_{(6,238)} = 37.15$, $P < 0.001$. Culture conditions: *1* retinas; *2* retinas co-cultured with intact optic tectum (NLOT); *3–7* retinas co-cultured with optic tectum corresponding to goldfish which optic nerve was crushed 1, 2, 4, 7 and 10 days (LOT), respectively, before plating them. $^*P < 0.05$ respecting retinas without and with taurine

Co-culture of optic tectum and retinal explants with optic nerve lesion maintained in vitro in the absence or in the presence of 0.2, 2 and 4 mM taurine

As demonstrated in previous reports, lower doses of taurine were more effective than the optimal previously reported for cultures in medium supplemented with FCS. In these set of experiments it is clear that the effect of variable concentrations of taurine in the absence of FCS differentially modulate the outgrowth from the explants (Fig. 5). It seems that around 2–4 days after lesion of the optic nerve, the factors and other elements produced by the optic tectum in co-culture with the retina determined the mutual action and the final effect. Higher concentrations of taurine could be inhibitory in the presence of more stimulatory conditions of optic tectum. In brief, the final effect depended on the concentration of taurine and of the time after lesion of the optic nerve corresponding to tectum in co-culture.

Co-culture of optic tectum and retinal explants with optic nerve lesion maintained in vitro in the absence or in the presence of calcium chelators, and 0.2 or 4 mM taurine

In order to explore the role of calcium in these processes, experiments were performed in the presence of EGTA or BAPTA. As shown in Fig. 6, EGTA reduced the effect of optic tectum in the absence of taurine, without effect at the basal level or in the presence of LOT 10 days (number 7 in *x* axis). In the presence of taurine, either 0.2 or 4 mM, EGTA reduced the effect of the combination of optic tectum in co-culture and taurine (Fig. 6). Similar results were obtained with BAPTA.

Co-culture of optic tectum and retinal explants with optic nerve lesion maintained in vitro in the absence or in the presence of 0.1, 1, 10 and 50 mM guanidoethylsulfonate and 0.2 or 4 mM taurine

The effect of GES on retinal outgrowth in this type of experiments did not produce a reduction of the effect of

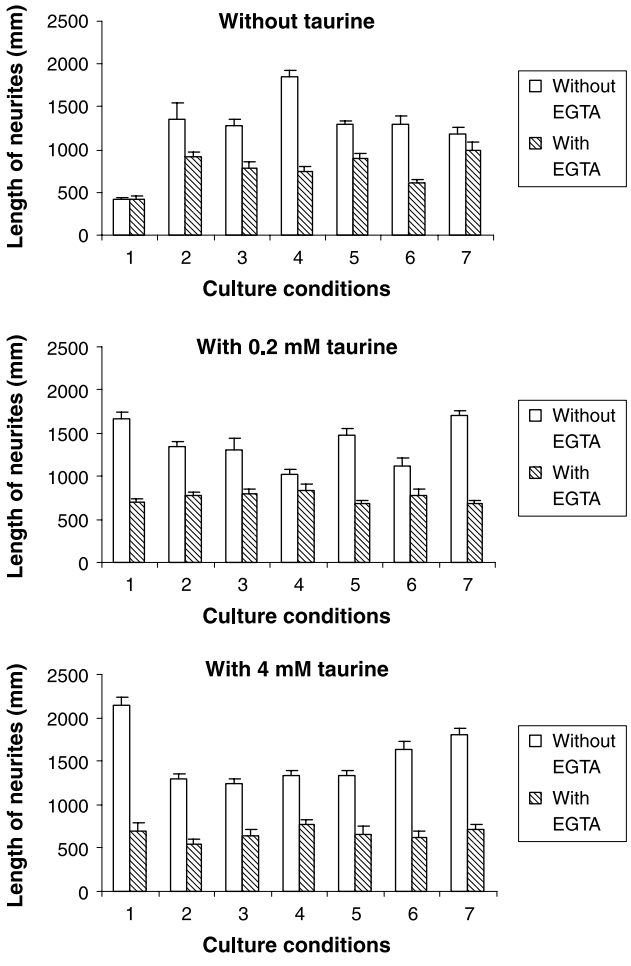

Fig. 6. Outgrowth from post-crushed goldfish retinal explants (LR) expressed as length of neurites from retinas co-cultured with optic tectum for 5 days in the absence or in the presence of 1 mM EGTA and 0.2, and 4 mM taurine. ANOVA for without taurine: without EGTA, $F_{(6,288)} = 40.23$, $P < 0.01$, and with EGTA, $F_{(6,312)} = 10.11$, $P < 0.001$; for with 0.2 mM taurine: without EGTA, $F_{(6,273)} = 8.59$, $P < 0.001$, and with EGTA, $F_{(6,324)} = 1.22$, $P = 0.29$; and for 4 mM taurine: without EGTA, $F_{(6,318)} = 20.45$, $P < 0.001$, and with EGTA, $F_{(6,209)} = 1.56$, $P = 0.16$ Culture conditions: *1* retinas; *2* retinas co-cultured with intact optic tectum (NLOT); *3–7* retinas co-cultured with optic tectum corresponding to goldfish which optic nerve was crushed 1, 2, 4, 7 and 10 days (LOT), respectively, before plating them. $P < 0.05$ between without or with EGTA

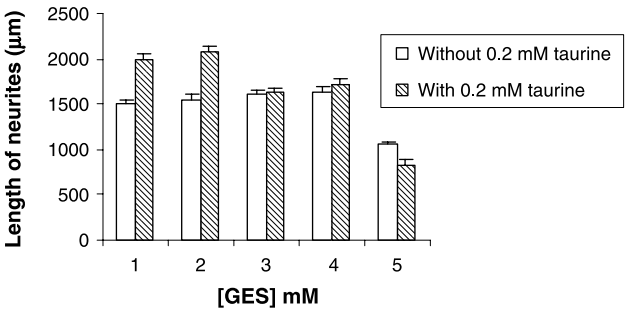

Fig. 7. Outgrowth from post-crushed goldfish retinal explants (LR) expressed as length of neurites from retinas cultured for 5 days in the absence or in the presence of 0.2 mM taurine. ANOVA for without taurine, $F_{(4,245)} = 18.53$, $P < 0.001$, and with taurine, $F_{(4,245)} = 72.54$, $P < 0.001$. Culture conditions: *1* without GES; *2–5* with GES 0.1, 1, 10 and 50 mM, respectively. $P < 0.05$ between with or without taurine in *1* and *2*

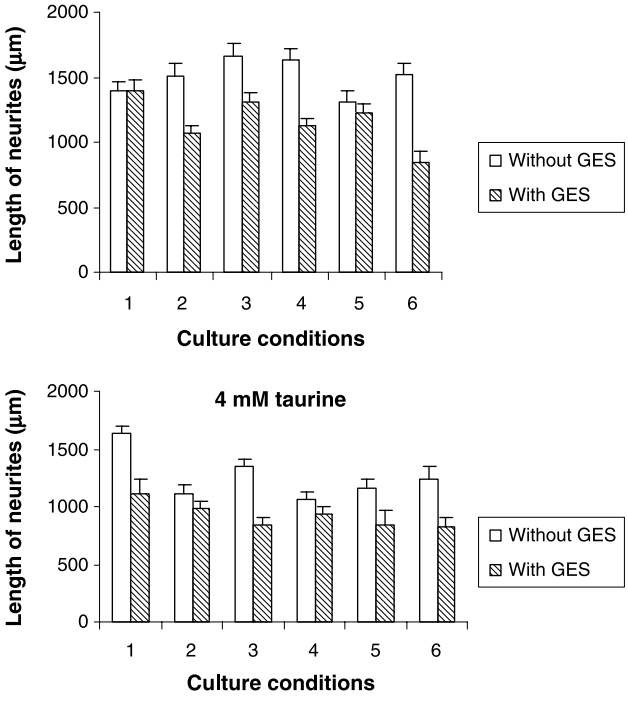

Fig. 8. Outgrowth from post-crushed goldfish retinal explants (LR) expressed as length of neurites from retinas co-cultured with optic tectum for 5 days in the absence or in the presence of GES, and 4 mM taurine. ANOVA for without taurine: without GES, $F_{(5,285)} = 4.01$, $P < 0.01$, and with GES, $F_{(5,285)} = 5.28$, $P < 0.001$; and for with 4 mM taurine: without GES, $F_{(5,294)} = 6.73$, $P < 0.001$, and with GES, $F_{(5,267)} = 1.49$, $P = 0.19$. Culture conditions: *1* retinas; *2* retinas co-cultured with intact optic tectum (NLOT); *3–7* retinas co-cultured with optic tectum corresponding to goldfish which optic nerve was crushed 1, 2, 4, 7 and 10 days (LOT), respectively, before plating them. $P < 0.05$ without or with GES

taurine at a low concentration, although there was an impairment of the trophic action with 1 and 10 mM, and a clear reduction at 50 mM of GES (Fig. 7). In the presence or in the absence of taurine, there was a time-dependent decrease of outgrowth from the retinal explants in the presence of 10 mM GES, except in basal condition (Fig. 8). In the presence of 4 mM taurine, even in the absence of optic tectum in the culture, GES also produced a reduction of the outgrowth from retinal explants.

Discussion

Conditioned media prepared with retinal cells from newborn rats or chick embryos increases survival of rat retinal explants post-optic axotomy (Rehen et al., 1993), indicating that trophic factors produced by the retina of distinct

species could modulate outgrowth. Using congenitally anophthalmic mice as recipients for retinal transplants, it was demonstrated that tectal cells produce factors in response to grafts, and innervation of the superior colliculus stimulates the tectum in order to produce chemotropic factors for maintaining correct innervations (Hankin and Lund, 1990). In addition, visual system neurons maintain trophic interactions independent of impulse activity, as it was shown in the rat by using tetrodotoxin, enucleation and metabolic stage in the mature rat (Thurlow and Cooper, 1988). Although refinement of the projections implicates activity and synaptic contacts, in other report was shown that tetrodotoxin does not affect retinotopic position in the goldfish (Johnson et al., 1999). However, chemical communication is determinant for the final results of optic nerve regeneration.

The present results indicate that the intact optic tectum, that means the tectum of goldfish without lesion of the optic nerve, continuously produces factors that could affect the outgrowth from the goldfish retina, mainly stimulating outgrowth in co-culture, and producing inhibition if medium from optic tectum of variable times post-lesion was added to the retinal explants. Interestingly, the effect of medium from optic tectum various days after crush on outgrowth from lesioned retinas showed a bell-shaped response with a maximal effect around 5 days. This is not surprising, since around those days the whole system, including retina and optic nerve, is activated by the lesion. Although there are differences in outgrowth depending on the time after lesion of the corresponding tectum from which media was obtained, the addition of taurine did not further increased the regeneration, indicating a saturable effect in these specific conditions.

A number of molecules have been identified to be related with the establishment of a correct retino-tectal pathway. Among them are factors from the optic nerve of the goldfish, called AF-1 and AF-2, producing nerve regeneration in vivo (Schwalb et al., 1995). In addition, TAG-1, a glycosylphosphatidyl inositol-anchored protein, plays a role in the retinotopic organization and regeneration of retino-tectal pathway (Lang et al., 2001). In the goldfish, ganglion cells project to a continuous retinotopic array (Wang and Meyer, 2000). The expression of axonal growth-associated phosphoprotein, GAP-43, increases after axotomy in retinal ganglion cells and optic nerve of *Rana pipiens*, a process modulated by basic fibroblast growth factor (Soto et al., 2003). The retino-tectal topography and its recovery during optic nerve regeneration is modulated by ephrin-A2 in the goldfish, which is expressed in resident neurons and not in new cells (King et al., 2004), and has been also implicated in retino-tectal map formation (Rodger et al., 2000). In addition, a heparin sulfate proteoglycan, present in the anterior optic tectum is necessary for axonal outgrowth (Su and Elam, 2003). Also, studies in the goldfish using autoradiographic deoxyglucose method show that visual stimulation increases metabolic activity, an elevation blocked by ouabain injection (Melzer and Powers, 2001).

The interaction of retina and optic tectum in vitro is nicely observed in the experiments in which intact retina or retina with previous lesion of nerves were co-cultured for 10 days with optic tectum at different days times post-crush. This bidirectional communication indicates that there is also a time-dependent bell-shaped effect related to the condition of the optic tectum, intact or at specific days after the lesion. These evidences are guide for choosing determined periods of time in which the optic tectum of goldfish could be tested as stimulatory of outgrowth from different species retina. In vivo retino-petal and retino-fugal pathways are crucial for regeneration of the retina. For instance, ^{125}I-labeled nerve growth factor is retrogradely transported from the optic nerve, but not from the optic tectum to the retina of goldfish (Yip and Johnson, 1983). These authors quote that nerve growth factor stimulates regeneration of goldfish optic nerve when applied in the site of the lesion and is also accumulated by retinal ganglion cells.

The coordination of time-dependent effects has been demonstrated in a series of processes influenced by trophic agents during the regeneration of the optic nerve of goldfish (Lima et al., 1988; Neuman et al., 1983). For instance, X-radiation produces a promoting effect at early stages of regeneration and a reducing effect later (Neuman et al., 1983). Critical times for each step process of goldfish optic nerve regeneration have been demonstrated in several experiments. Moreover, the use of colchicine, given intraocular, indicates a vulnerable period during which microtubules are essential for nerve outgrowth (Dybowski et al., 1999), also gefiltin, a neuronal intermediate filament protein, is crucial for this process (Niloff et al., 1998).

The conjoint effects of the amino acid taurine and optic tectum further indicates that there are critical times and concentrations. Amino acids present a characteristic topographical distribution pattern in the pigeon tectal layers, with decrease of glutamate and aspartate after contralateral retinal ablation (Fonnum and Henke, 1982). Taurine concentration increases and that of glutamate decreases in the goldfish retina, after crush of the optic nerve (Guerra et al., 1998; Lima et al., 1998; Nusetti et al., 2005), also the transport of taurine to the optic tectum still occurs after crushing the optic nerve (Guerra et al., 2000). The variations

of individual amino acid levels cannot be considered to take place independently, since, for instance, modifications in taurine could cause changes in glutamatic acid in nervous tissues (van Gelder and Drujan, 1978). Interestingly, the superior colliculus of the rabbit decreases the release of glutamate after enucleation (Sandberg and Corazzi, 1983).

The mechanisms by which the observed results take place seem to be related to the presence of calcium in the medium and to its level into the cells, as previously demonstrated only for taurine (Lima et al., 1993), also the trophic effect of the optic tectum needs calcium for acting as that. Finally, the entrance of taurine was inhibited with GES, which presence in the medium affected outgrowth indicating that taurine trophic effect is mediated through intracellular mechanisms and that some of the effects of the tectum might include the entrance of endogenous taurine to the cells.

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