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Neurites outgrowth and amino acids levels in goldfish retina under hypo-osmotic or hyper-osmotic conditions

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ABSTRACT

Amino acids are known to play relevant roles as osmolytes in various tissues, including the retina. Taurine is one of these active molecules. In addition, taurine stimulates outgrowth from the goldfish retina by mechanisms that include extracellular matrix, calcium fluxes and protein phosphorylation. The present report aims to explore the effect of medium osmolarity on goldfish retinal outgrowth and the possible modifications produced by changing eye osmolarity on amino acid levels in the retina. Goldfish retinal explants were obtained 10 days after crush of the optic nerve and cultured under iso-, hypo- or hyper-osmotic conditions. Hypo-osmotic medium was prepared by diluting the solutions 10% twice, preserving fetal calf serum concentration. Hyper-osmotic medium was done by adding 50 or 100 mM urea or mannitol. Evaluation of length and density of neurites was performed 5 days after plating. Outgrowth was reduced in hypo- and in hyper-osmotic conditions. Taurine, 4 mM, increased length and density of neurites in iso-osmotic, and produced stimulatory effects under both hyper-osmotic conditions. The *in vivo* modification of osmolarity by intraocular injection of water or 100 mM urea modified levels of free amino acids in the retina. Taurine and aspartate retinal levels increased in a time-dependent manner after hypo- and hyper-osmotic solution injections. Serine, threonine, arginine, γ-aminobutyric acid, alanine and tyrosine were elevated in hyper-osmotic conditions. Outgrowth *in vitro*, after *in vivo* osmolarity changes, was higher in the absence of taurine, but did not increase in the presence of the amino acid. The fact that certain outgrowth took place in these conditions support that the impairment was not due to tissue damage. Rather, the effects might be related to the cascade of kinase events described during osmolarity variations. The time course under these conditions produced adjustments in ganglion cells probably related to taurine transporter, and phosphorylation of specific proteins.

1. Introduction

Several intracellular osmotic molecules play roles in the maintenance of cell volume, either inorganic or organic osmolytes (Lang et al., 1998). Amino acids are well known osmolytes in the retina (Pasantes-Morales et al., 1999) and in the brain (Li and Olson, 2008). A variety of signals are involved in osmoregulation, in which certain kinases, such as mitogen activated protein kinases (MAPK) and phosphatidylinositol-3′-kinase (PI3K), and levels of intracellular calcium function in the signalling pathway (Hoffman and Pedersen, 2006; Lambert, 2004). Also osmoresistant efflux of taurine seems to be mediated through *N*-methyl-D-aspartate receptors (Rodríguez-Navarro et al., 2009). In addition, the participation of taurine transport for preserving concentrations of the amino acid is regulated by osmolarity in certain types of kidney cells (Han et al., 2006).

Taurine is a sulphur amino acid present in high concentration in the retina, whereas plays roles as antioxidant, regulator of calcium fluxes, modulator of phosphorylation of certain proteins, stimulator of axonal outgrowth, and osmoregulator, among others (Huxtable, 1989; Lima et al., 1988; Lombardini and Props, 1996). The mechanisms by which taurine exerts its trophic role in the goldfish retina have been related to the type of substrate where explants have been cultured (Lima et al., 1989a), to calcium fluxes (Lima et al., 1993), and to protein phosphorylation (Lima and Cubillos, 1998). Ganglion cells volume homeostasis might be determinant in the outgrowth stimulated by taurine. These retinal cells possess taurine transporters allowing the axonal movement of the amino acid to the optic tectum (Guerra et al., 2000), and its presence has been demonstrated by immunocytochemistry (Nusetti et al., 2009). Interestingly, retinal taurine transporter is osmotically regulated *in vivo* in the rat (Morimura et al., 1997), and β-alanine, inhibitor of taurine transporter, affects trophic action of taurine in a greater extent in normo- than in hypo-osmotic conditions, indicating modification of the transporter in relation to outgrowth due to change in osmolarity (Cubillán et al., 2009).

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According to the above data, concerning the influences of calcium fluxes and protein phosphorylation on retinal outgrowth, and with the aim to better understanding the mechanisms by which taurine affect neuritic outgrowth from the retina (Lima et al., 1988, 2001; Lima, 1999), a series of experiments were performed changing the culture medium of explants. Moreover, the *in vivo* effect of hypo-osmotic and hyper-osmotic solutions was used to evaluate possible changes in amino acids content in the retina, as well as the result of posterior culturing for determining their roles on outgrowth.

2. Materials and methods

2.1. Animals, retinal cultures and treatments

Retinas of goldfish (*Carassius auratus*), 5–6 cm in length, anesthetized in 0.05% tricaine, were dissected 10 days after crush of the optic nerve with fine forceps. Squares of 500 μm (5–6 per retina) were prepared by sectioning the retina with a McIlwain tissue chopper and placed, 10–14 fragments (two retinas from the same fish), on poly-L-lysine pre-coated tissue flasks (25 mm^2). The total number of animals per experiment was 12–18, 3 for each condition. The nutrient medium was Leibovitz, L-15 (free of taurine), 3 ml per dish (Sigma) with 0.1 mg/ml of gentamicin and 20 mM of (*N*-2-hydroethyl]piperazine-*N*′-2-ethanesulfonic acid) (HEPES) (Landreth and Agranoff, 1979; Lima et al., 1988, 1989a). Hypo-osmotic media were done by dilution of 10% with distilled water at 24 and 72 h in culture (Cubillán et al., 2009). Hyper-osmotic media were prepared with 50 or 100 mM urea (López-Domínguez et al., 2007) or mannitol (El-Sherbeny et al., 2004). It is considered that internal concentration of urea reaches 1/3 on external one (Brühmann and Hanke, 1980), and that transport of [^{14}C]urea takes place in the retina (Gratton et al., 1993). Some experiments were performed combining the hypo-osmotic media and adding 50 or 100 mM urea or mannitol, in order to explore the possible counteraction of both conditions. Taurine, 4 mM, as medium optimal concentration (dose-dependent effect is bell-shaped with maximum trophic effects between 3 and 5 mM), was added to some cultured retinal explants (Lima et al., 1988, 1989a). For media with urea, 100 mM was chosen due to its basal effect on outgrowth, and for mannitol we considered to test both concentrations while controls remained the same. Density of neurites was evaluated at 5 days after plating, using a predetermined scale (Lima et al., 1988, 1989a), and length (μm) was measured by using the program SigmaScanPro (Jandel). Treatment *in vivo* was done by intraocular injection of 10 μl of isotonic saline solution, distilled water, or 100 mM urea twice, on day 1 and day 3. Retinas were dissected at 5 and 7 days after the first administration. In experiments for *in vitro* continuation the injections were done on days 1 and 3, the optic nerve was crushed on day 1, cultures performed 10 days after the lesion, and evaluation of outgrowth at 5 days in culture. The animal treatment protocols were approved by Institute Ethical Committee following international rules.

2.2. Amino acids analysis

Amino acids were determined by HPLC with fluorescence detection employing a modified method (Lima et al., 1989b). The HPLC system consisted in a Waters 2690 Separations Module and a Shimadzu RF-551 fluorescence detector. A Supelco LC-18 column 4.6 mm × 100 mm, 5 μm was employed for amino acid separation. Immediately before injection, 100 μl of the supernatants was derivatized with 100 μl of a mixture of 25 mg *o*-phthalaldehyde, 500 μl MeOH, 25 μl β-mercaptoethanol (1 g/ml) and 4.5 ml 0.4 M potassium buffer pH 10.4. Aliquots of the derivatized preparation were injected to the chromatographic system. The column was eluted with increasing concentrations of acetonitrile in a 50 mM pH 6.4 sodium phosphate buffer, with 5% acetonitrile and 0.1% tetrahydrofuran. The main step gradient used was 0–10 min, 95% of buffer solution, 10–55 min 70%, 55–59 min 20%, 59–62 min 98%. Amino acids were quantified by the method of the external standard and concentrations were expressed in nmol/mg retinal protein (Lowry et al., 1951).

2.3. Statistical analysis

Results are expressed as mean ± standard error of the mean, one-way analysis of variance was performed followed by Tukey–Kramer Multiple Comparisons Test for evaluating results (GraphPad InStat 3). Statistical significance was considered if $P < 0.05$.

3. Results

3.1. Effects of anisosmotic solutions on neuritic outgrowth

Length of neurites was significantly reduced in hypo-osmotic and in hyper-osmotic with 100 mM urea respecting outgrowth in iso-osmotic media. Outgrowth in hypo-osmotic media was significantly lower than in hyper-osmotic with 50 mM urea, but similar to that in hyper-osmotic with 100 mM urea. The dilution with water of hyper-osmotic medium and 50 mM urea, reduced outgrowth respecting hyper-osmotic with 50 mM urea alone. The same treatment with 100 mM urea did not differ from urea alone. A significant, but small difference was obtained between hypo-osmotic conditions plus urea 50 and hypo-osmotic media with 100 mM urea. Density of neurites was significantly smaller in hyper-osmotic conditions with 50 mM or 100 mM urea, and in hypo-osmotic plus 50 or 100 mM urea respecting iso-osmotic medium. Density in hyper-osmotic 100 mM urea media was lower respecting hypo-osmotic and hyper-osmotic condition with 50 mM urea (Fig. 1). Hyper-osmotic solutions made with the addition of mannitol, 50 or 100 mM, significantly reduced outgrowth, and differ from results with urea, being more potent in decreasing length of neurites, in affecting density of them, and in counteracting the effect of diluting the medium. Mannitol, 50 or 100 mM, significantly diminished outgrowth from the explants, and diluting the media did not modify this effect. Density of neurites was affected in all conditions tested (Fig. 2). Representative explants cultured in iso-, hypo-, and hyper-osmotic (mannitol 50 mM) conditions are shown in Fig. 3.

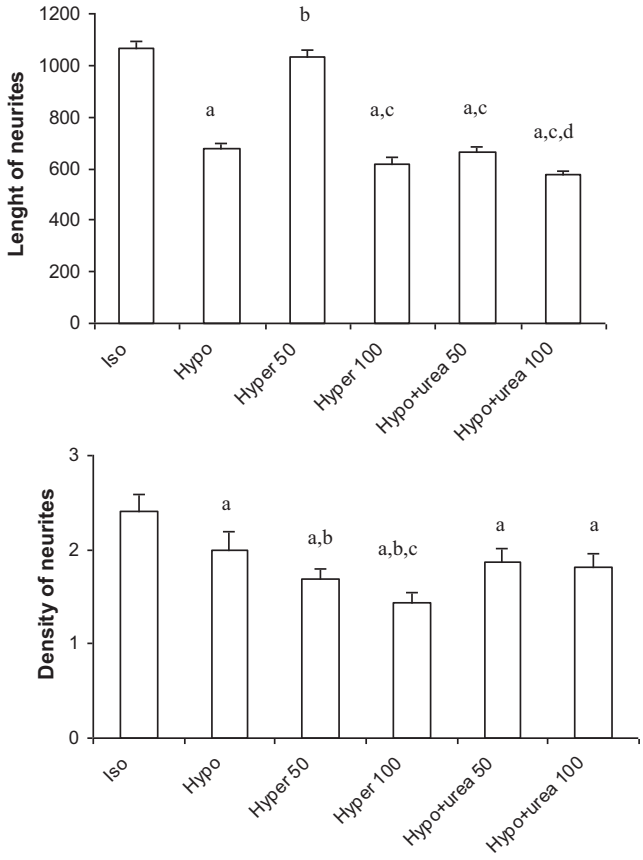

Fig. 1. Length in μm ($n = 70–197$), $F_{(5,824)} = 90.015$ $P < 0.0001$; and density of neurites according to predetermined scale ($n = 21–25$), $F_{(5,149)} = 3.011$ $P = 0.0577$, of retinal explants cultured 10 days after crush of the optic nerve and evaluated 5 days later. Hypo-osmotic condition was done by diluting the media 10% twice. Hyper-osmotic conditions were done with 50 or 100 mM urea. [a]$P < 0.001$ respecting Iso; [b]$P < 0.001$ respecting Hypo; [c]$P < 0.001$ respecting Hyper 50 mM; [d]$P < 0.05$ respecting Hypo + urea 50.

3.2. Effects of urea on neuritic outgrowth in the presence of taurine

Taurine significantly increased the length of neurites in iso-osmotic medium. There was a reduction of outgrowth in the