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PACAP-Mediated Neuroprotection of Neurochemically Identified Cell Types in MSG-Induced Retinal Degeneration

Tamas Atlasz · Krisztina Szabadfi · Peter Kiss · Norbert Babai · Zsombor Koszegi · Andrea Tamas · Dora Reglodi · Robert Gabriel

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Abstract Pituitary adenylate cyclase-activating polypeptide (PACAP) is neuroprotective in animal models of different brain pathologies and injuries, including cerebral ischemia, Parkinson's disease, and different types of retinal degenerations. We have previously shown that PACAP is protective against monosodium glutamate (MSG)-induced retinal degeneration, where PACAP-treated retinas has more retained structure and PACAP induces anti-apoptotic while it inhibits pro-apoptotic signaling pathways. The aim of the present study was to investigate cell-type specific effects of PACAP in MSG-induced retinal degeneration by means of immunohistochemistry. Rat pups received MSG (2 mg/g b.w.) applied on postnatal days 1, 5, and 9. PACAP (100 pmol in 5 μl saline) was injected into the right vitreous body, while the left eye received only saline. Retinas were processed for immunocytochemistry after 3 weeks. Immunolabeling was determined for vesicular glutamate transporter 1, tyrosine hydroxylase, calretinin, calbindin, parvalbumin, and vesicular γ-aminobutyric acid (GABA) transporter. In the MSG-treated retinas, the cell bodies and processes in the inner nuclear, inner plexiform, and ganglion cell layers displayed less immunoreactivity for all antisera. Apart from photoreceptors, only one major retinal cell type examined in this study; the calbindin-immunoreactive horizontal cell seemed not to be affected by MSG application. After simultaneous application of MSG and PACAP, staining of retinas was similar to that of normal eyes, with no significant alterations in immunoreactive patterns. These findings further support the neuroprotective function of PACAP in MSG-induced retinal degeneration.

Keywords Excitotoxicity · Neuropeptide · MSG · PACAP · Retinoprotection

T. Atlasz · K. Szabadfi · N. Babai · Z. Koszegi · R. Gabriel
Department of Experimental Zoology and Neurobiology,
University of Pecs,
Pecs, Hungary

P. Kiss · A. Tamas · D. Reglodi (✉)
Department of Anatomy, University of Pecs,
Szigeti str. 12,
7624 Pecs, Hungary
e-mail: dora.reglodi@aok.pte.hu

Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a member of the growing family of neurotrophic and neuroprotective factors playing important roles during neuronal development and protection against different types of injuries, like cerebellar and cortical development, protection against Parkinson's disease, excitotoxicity, and ischemia (Waschek 2002; Somogyvari-Vigh and Reglodi 2004; Shioda et al. 2006; Falluel-Morel et al. 2007). PACAP and its receptors are present in the eye (Seki et al. 2000a, b; Vereczki et al. 2006), and various different effects on inflammatory processes, cytokine production, vascular supply, and circadian functions have already been demonstrated (Nilsson 1994; Jozsa et al. 2001; Hannibal and Fahrenkrug 2004; Nakatani et al. 2006).

Recent studies have shown that PACAP has trophic effects during retinal development, e.g., it is suggested to be a major endogenous component in defining tyrosine hydroxylase (TH) phenotype in the retina (Bagnoli et al. 2003; Fahrenkrug et al. 2004; Borba et al. 2005). Similarly to other neuronal tissues, in the last few years, we have provided evidence that PACAP is also protective in vivo in the retina, against toxic injury induced by monosodium glutamate (MSG) and ischemic injury induced by bilateral

carotid artery occlusion (Tamas et al. 2004; Babai et al. 2005, 2006; Kiss et al. 2006; Atlasz et al. 2007). Apart from the in vivo models, PACAP also has neuroprotective effects retinal in cultures, for example, in glutamate-induced cellular toxicity (Shoge et al. 1999). Currently available data on the protective effects of PACAP strongly imply that PACAP may have clinical importance in retinoprotection (Staines 2008). We have also shown that these in vivo protective effects of PACAP are associated with inhibition of pro-apoptotic signaling pathways and stimulation of anti-apoptotic signaling molecules (Racz et al. 2006a, b, c, 2007).

The aim of the present study was to further investigate the effects of PACAP treatment in some well-defined cell types after MSG-induced retinal degeneration in neonatal rats. Therefore, the expression of glutamate transporter 1 (VGLUT1), vesicular GABA transporter (VGAT), TH, calbindin, calretinin, and parvalbumin was determined by immunohistochemistry, as these markers are found in neurochemically and morphologically identified retinal cell populations.

Materials and Methods

Newborn Wistar rats were housed under light/dark cycles of 12:12 h. Animal housing, care, and application of experimental procedures were in accordance with institutional guidelines under approved protocols (no: BA02/2000–20/2006, University of Pecs). Rats (altogether $n=22$) were injected s.c. with 2 mg/g b.w. MSG ($n=16$) three times, on postnatal days (PD) 1, 5, and 9. PACAP38 (100 pmol in 5 μl saline solution corresponding to 20 micromolar solution) was injected into the vitreous body of the right eye with a Hamilton syringe after each MSG injection. The same volume of saline was injected into the other eye of the same animals. The dose of PACAP38 was based on previous observations where this dose was proven to be effective in optic nerve transection and MSG toxicity (Seki et al. 2003; Tamas et al. 2004). A separate group of animals did not receive any treatment and served as normal control group ($n=6$). After 3 weeks, animals were killed with an overdose of anesthetic (120 mg/kg pentobarbital, Nembutal, Sanofi-Phylaxia, Hungary); the eyes were immediately dissected in ice-cold phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (PB, pH 7.4) for 1 h at room temperature. Tissue was then washed in 0.1 M PB and cryoprotected in 15% sucrose for 30 min, 20% sucrose in PBS for 1 h, and 30% sucrose overnight at 4°C. For cryostat sectioning, retinas were embedded in tissue-freezing medium (Tissue-Tek, OCT Compound, Sakura Finetech, NL), cut in a cryostat (Leica, Nussloch, Germany) at 14–16 μm radial sections. Sections were mounted on chrome–alum–gelatin-coated subbed slides and stored at −20°C until use. At least 48 sections/eye were examined. Retinal sections were rinsed in PBS, permeabilized by incubation for 5 min in 0.1% Triton X-100 in PBS and incubated with 0.1% bovine serum albumin, 1% normal goat serum, and 0.1% Na-azide in PBS for 1 h to minimize nonspecific labeling. Sections were incubated with the primary monoclonal or polyclonal antibody for overnight at room temperature. We used the following antibodies: anti-VGLUT1, anti-VGAT, anti-TH, anti-parvalbumin, anti-calbindin, and anti-calretinin (Table 1). After several washes in PBS, sections were incubated for 2 h at 37°C in the dark with the corresponding secondary antibodies (Table 1). Sections were then washed in PBS and were coverslipped using Vectashield (Vector Laboratories, Inc., Burlingame, CA). For control experiments, primary antisera were omitted, and after the protocol, specific cellular staining was not found. Digital photographs were taken with a Nikon Eclipse 80i microscope equipped with a cooled CCD camera. Images were taken with the Spot software package. Photographs were further processed with the Adobe Photoshop 7.0 program. Images were adjusted for contrast only, aligned, arranged, and

Table 1 List of antibodies to label specific cells in rat retina

Antigen	VGLUT 1	VGAT	TH	CALR	CALB	PARV
Primary antibody	Policlonal-rabbit	Policlonal-rabbit	Monoclonal-mouse	Policlonal-rabbit	Monoclonal-mouse	Monoclonal-mouse
Source	Sigma	Sigma	Chemicon	Chemicon	Sigma	Sigma
Primary antibody dilution	1:500	1:500	1:1,000	1:1,000	1:1,000	1:1,000
Secondary antibody	Anti-rabbit Texas Red IgG	Anti-mouse FITC IgG	Anti-mouse FITC IgG	Anti-mouse FITC IgG	Anti-mouse FITC IgG	Anti-mouse FITC IgG
Source	Jackson	Jackson	Jackson	Jackson	Jackson	Jackson
Secondary antibody dilution	1:100	1:100	1:100	1:100	1:100	1:100

labeled using the functions of the above program. Images were evaluated by an examiner blinded to the experimental treatment.

Results

In rat retina, VGLUT 1 has been described in the outer plexiform layer (OPL) and throughout the inner plexiform layer (IPL), consistently with the expected synaptic localization of the protein (Gong et al. 2006). In our normal control preparations, VGLUT-1-immunopositive structures were also present in the OPL and IPL (Fig. 1a). VGLUT 1 staining in the OPL and IPL of the rat retina shows the terminals of photoreceptors and bipolar cells, respectively. Retinal tissue from animals treated with MSG showed severe degeneration compared to control retinas (Fig. 1b). Much of the IPL disappeared, and the inner nuclear layer (INL) and ganglion cell layer (GCL) were intermingled. One can see substantial reduction in the size of the terminals of bipolar cells in the IPL. Intraocular PACAP38 treatment after MSG application led to a nearly intact appearance of VGLUT 1 immunoreactivity in retinal structures if animals received PACAP into the vitreous of the right eye at all three times of MSG application. In this case, a substantial protective effect could be observed; the bipolar cell terminals in the IPL remained well discernible (Fig. 1c).

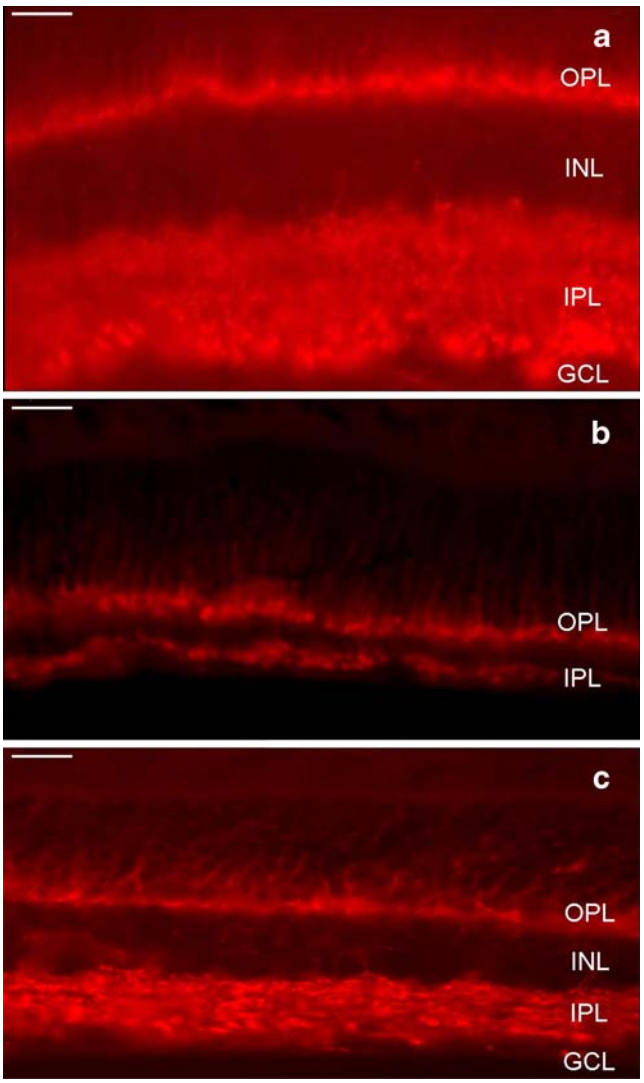

Figure 1 VGLUT 1 immunoreactivity (scale bar, 20μm). **a** VGLUT 1 staining in the OPL and IPL of the rat retina. Terminals of photoreceptors and bipolar cells as shown on the control slide. **b** After three times MSG treatment, damage to the bipolar cell terminals in the IPL is obvious. **c** PACAP treatment resulted in retained VGLUT1 immunoreactivity

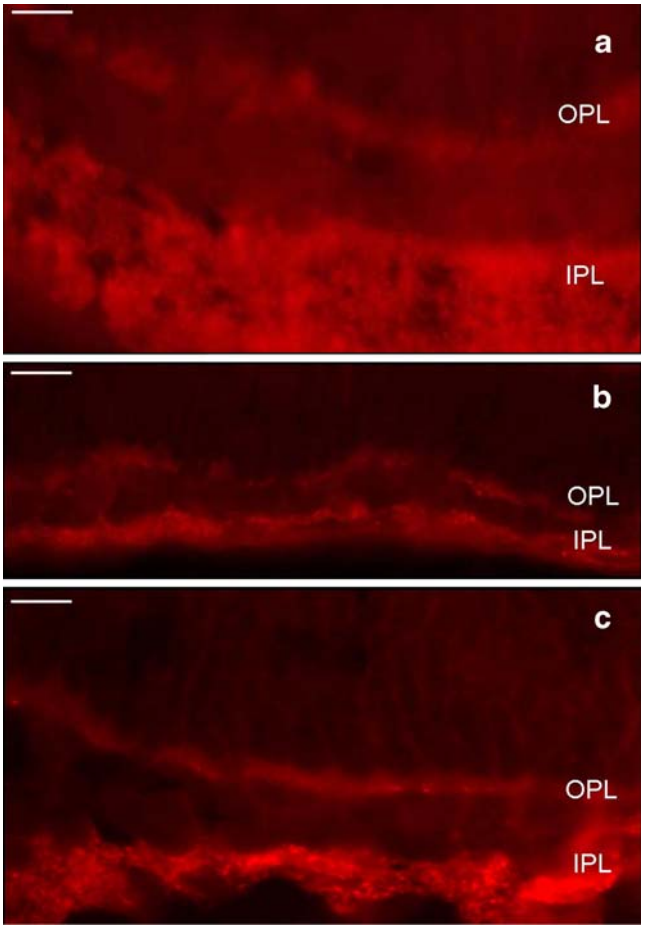

Figure 2 VGAT immunoreactivity (scale bar, 20μm). **a** Immunofluorescent staining obtained with VGAT antibodies. **b** After MSG application the strength of reactivity was reduced. **c** PACAP diminished the harmful effects of MSG

As shown in Fig. 2a, we were able to verify the cellular localization of the VGAT in the OPL and IPL (Contini and Raviola 2003) in control retinas. Strong VGAT immunoreactivity could be detected in the IPL (Fig. 2a). After three times MSG application, the strength of reactivity of VGAT-positive structures was reduced. The entire inner retina, especially the IPL, was only faintly labeled (Fig. 2b).

PACAP treatment significantly ameliorated the toxic effects of MSG (Fig. 2c).

TH immunoreactivity was present in a wide-field amacrine cell-type in the INL (Vaney 1990). In our control preparation, a few labeled amacrine cells were seen in the inner nuclear layer (Fig. 3a) whose number decreased upon MSG treatment (Fig. 3b). Figure 3c shows that PACAP treatment retained the TH-positive amacrine cells in the INL. No difference in the intensity of TH labeling in cells was observed in treated retinas when compared to controls.

As it was shown earlier, Ca-binding proteins calbindin (Röhrenbeck et al. 1987), calretinin (Gabriel and Witkovsky 1998), and parvalbumin (Wässle et al. 1993) are abundant in various types of retinal cells. These results could be reproduced in our normal control retinas. Calbindin immunoreactivity was found in the cell bodies and processes of the horizontal cells (Fig. 4a). MSG treatment seemed to generate only a small alteration in the intensity of the immunoreactivity (Fig. 4b). After PACAP application, immunoreactivity was similar to the control level (Fig. 4c).

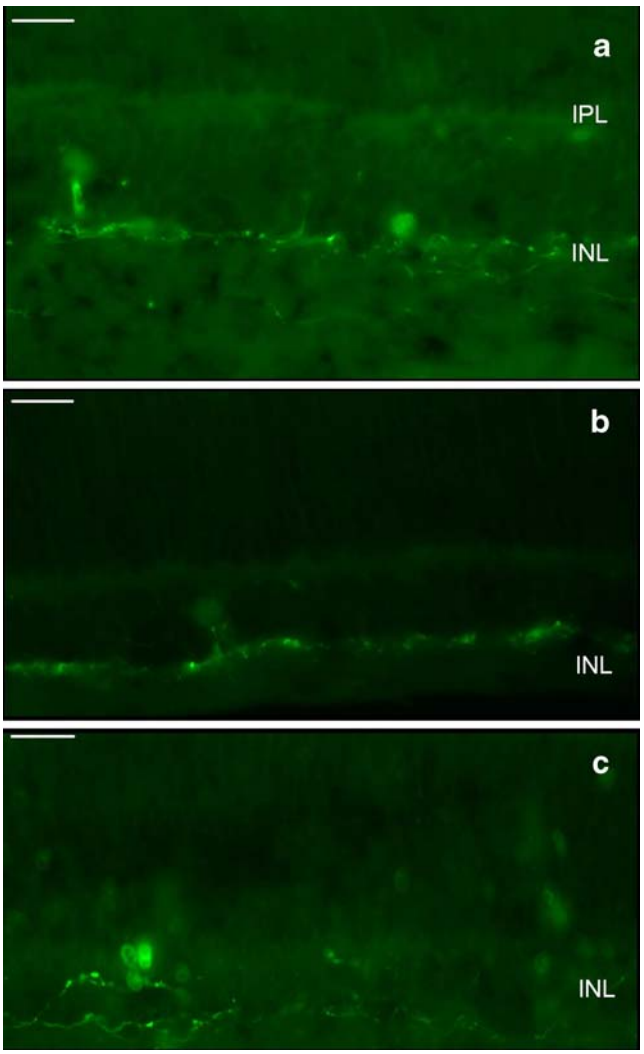

Figure 3 TH immunoreactivity (scale bar: 20μm). **a** TH-immunostained retina showing the distribution of TH-positive structures in the INL. **b** Number of TH positive amacrine cells in the INL decreased upon MSG treatment. **c** Applying PACAP, the number of the cells remained unchanged compared to the control

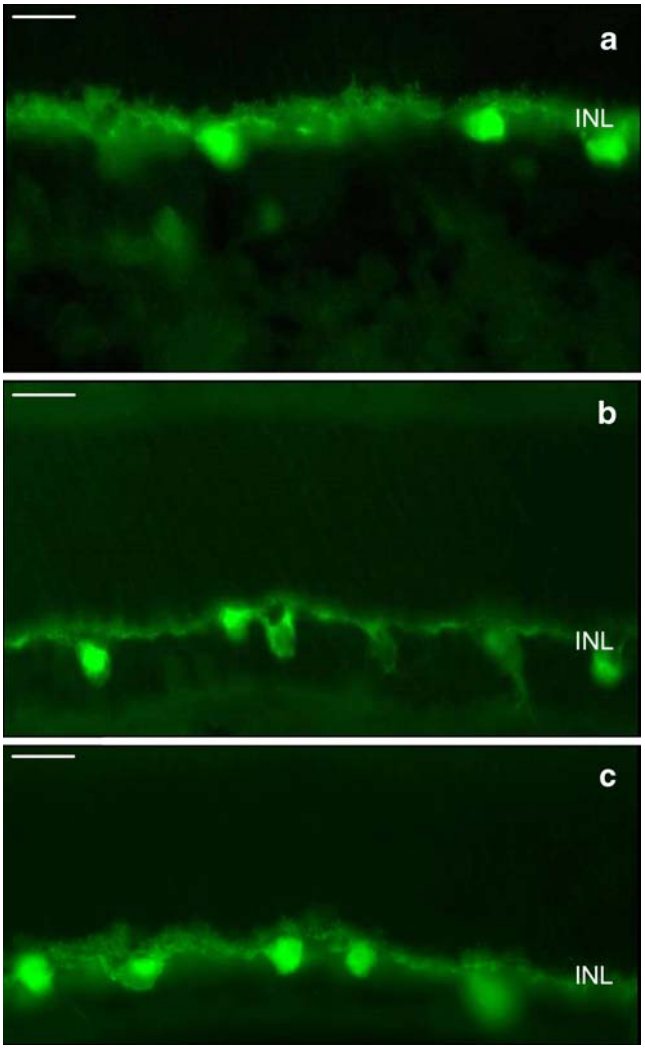

Figure 4 Calbindin immunoreactivity (scale bar, 20μm). **a** In control conditions the calbindin was present in cell bodies and processes of the horizontal cells. **b** MSG-induced only minimal changes in calbindin staining. **c** PACAP treatment restored the original pattern of immunoreactivity

High intensity of calretinin immunoreactivity was displayed by subpopulations of inner retinal cell classes, especially ganglion and amacrine cells (Fig. 5a). MSG application induced caused the fusion of INL, IPL, and GCL and also the number of the labeled retinal cells was reduced. The highest level of immunoreactivity was shown by cells in this fused layer (Fig. 5b), but this reactivity was weaker than that of the control tissue. PACAP treatment resulted in most cases not only in a protection of the retinal layers but also the density of the immunoreactive cells was similar to that of the untreated control tissue (Fig. 5c).

Parvalbumin immunoreactivity was identified in the population of AII glycinergic amacrine cells and in a few

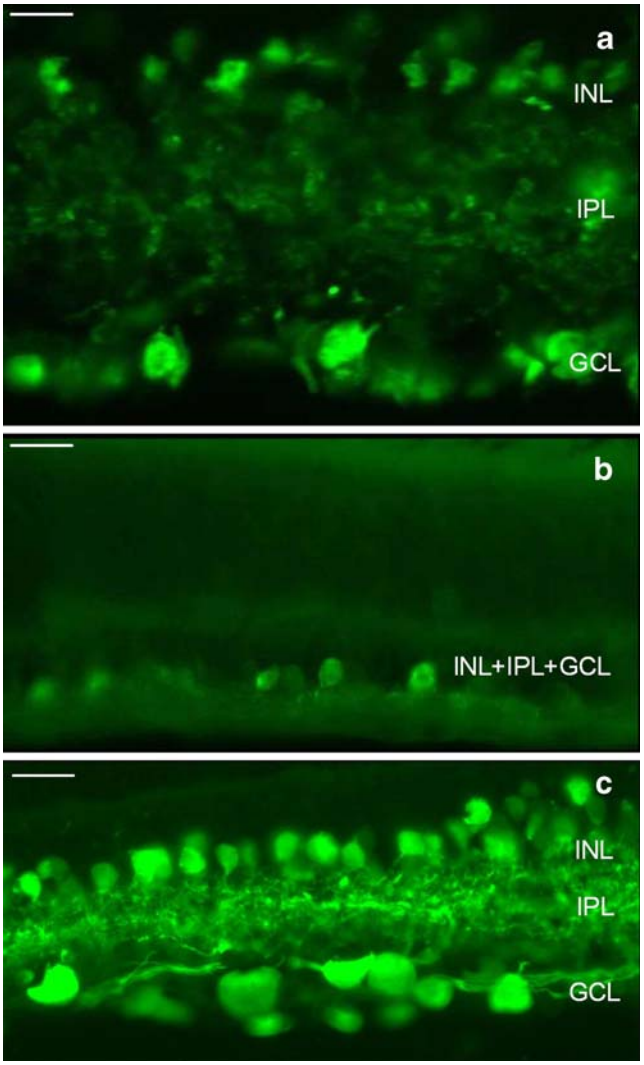

Figure 5 Calretinin immunoreactivity (scale bar, 20μm). **a** In control slides, calretinin is present in ganglion and amacrine cells. **b** MSG treatment caused alterations: immunoreavtivity was weaker and the number of stained cells decreased. **c** PACAP treatment counteracted the MSG-induced changes in calretinin-immunolabeling. The number of the labeled cells and the strength of staining in neurons was close to those in controls

ganglion cells. We noted systematic differences in the intensity of the immunoreactivity between the control and the MSG-treated retinal cells (Fig. 6a,b). After MSG and PACAP treatment, the structure was similar to the controls, but the IPL was thinner than in the case of the control tissue (Fig. 6c). The number of the labeled cells in the INL did not seem to be lower than in control specimens (Fig. 6a,c).

Discussion

In this study, we were able to identify a number of neurochemical markers characteristic to specific cell types in the retina, which were sensitive to MSG treatment but reactive to neuroprotection provided by PACAP38 (Tables 2 and 3). Apart from photoreceptors, only one major retinal cell type examined in this study; the calbindin-immunoreactive horizontal cells, did not seem to be affected by MSG application.

Vesicular transporters regulate the uptake and type of neurotransmitter sequestered into synaptic vesicles and the amount and type of neurotransmitter released (Pothos et al. 2000). Recently, vesicular transporters for the primary excitatory neurotransmitter glutamate and the major inhibitory neurotransmitter GABA have been identified as VGLUT 1 and VGAT, respectively (McIntire et al. 1997; Bellocchio et al. 2000; Takamori et al. 2000).

The principal glutamatergic neurons, photoreceptors, and the bipolar cells show VGLUT 1 immunoreactivity in their terminals (Johnson et al. 2003), detected in the OPL and throughout the laminas of the IPL (Gong et al. 2006). We found that MSG destroyed many of the bipolar cells because of the presence of ionotropic glutamate receptors on their dendrites (Thoreson and Witkovsky 1999) and caused reduction in the thickness of the IPL. Significant neuroprotection occurred in the IPL after PACAP38 treatment. The remainder bipolar cell terminals in the MSG-

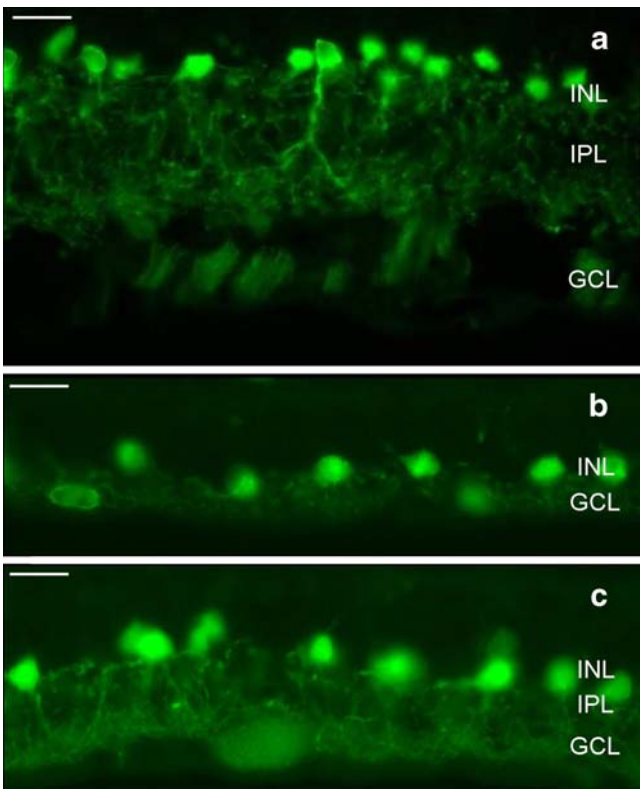

Figure 6 Parvalbumin immunoreactivity (scale bar, 20μm). **a** In control conditions, the calcium binding protein, parvalbumin was present in AII amacrine cells. **b** MSG treatment caused alterations in the labeling intensity and also the number of stained cells were reduced. **c** PACAP treatment counteracted the MSG-induced changes in parvalbumin immunolabeling. Conversely, the number of the labeled cells and the strength of stained neurons increased compared to the MSG-treated specimens

Table 2 Number of eyecups and tissue sections under different experimental conditions

	Control	MSG	MSG and PACAP	Total
Number of eyecups	12	16	16	44
Number of tissue sections	576	768	768	2,112

treated material probably belong to the ON-type bipolar cells, which bear MGluR6 on their dendrites (Nakajima et al. 1993).

VGAT was localized to the OPL and IPL, with weak immunostaining of cell bodies in the INL and GCL (Haverkamp et al. 2000; Cueva et al. 2002). Many of the bipolar inputs to amacrine cells were eliminated by MSG treatment because ionotropic glutamate receptors are present on OFF-bipolar cells. In addition, several amacrine cell types contain ionotropic glutamate receptors; therefore, both cell populations are prone to excitotoxic damage.

TH-positive dopaminergic amacrine cells are involved in several retinal functions; they are vital for development, light adaptation, and many other functions (Vaney 1990; Gustincich et al. 2004; Tkatchenko et al. 2006; Vugler et al. 2007). These cells are known to be supported by PACAP38 during development (Borba et al. 2005), and therefore, it is not surprising that the same substance may provide protection against damaging agents. Our present results are in accordance with previous observations showing the protective effects of PACAP on dopaminergic neurons (Takei et al. 1998; Reglodi et al. 2004). The maintenance of dopaminergic cells maybe essential for the survival of the retinal cells since dopamine is known to be a trophic factor itself to several retinal neurons (Witkovsky and Dearry 1991).

The presence of calcium-binding proteins is typical in retinal cells. Calbindin is a good marker for horizontal cells (Röhrenbeck et al. 1987; Hamano et al. 1990); calretinin was detected in several ganglion cell types and in some amacrine cells (Pasteels et al. 1990; Gabriel and Witkovsky 1998). Parvalbumin immunoreactivity was identified within a subpopulation of ganglion cells and in AII amacrine cells (Sanna et al. 1990; Wässle et al. 1993).

Calbindin immunoreactivity in horizontal cells did not change much after the MSG treatment because photoreceptors remained intact. Horizontal cells contain calcium-impermeable glutamate receptors (Brandstätter 2002) and also calcium-buffer proteins (Röhrenbeck et al. 1987); therefore, they are well-protected against depolarization-induced calcium influx and intracellular-free calcium rise (Brandstätter 2002).

Calretinin is present in several retinal cell types, including ganglion and amacrine cells. Many of these cells disappear when MSG is applied to the developing retina; however, even under these condition, they are relatively resistant to damage. Even more surprisingly, their relative density seems to be equal or sometimes even higher then normal when the tissue is treated with PACAP38 after MSG. This may be due to the fact that cells without calretinin are more sensitive to MSG and cannot be rescued by PACAP. As we have described earlier (Babai et al. 2005), the PACAP-mediated rescue does not provide total protection under the experimental conditions we used in this study; therefore, our assumption will be examined in the future with a more precise quantitative approach, which takes into account the density of retinal neurons at identical retinal eccentricities.

Parvalbumin plays a role in calcium metabolism of the AII amacrine cells, which are constituents of the rod-pathway. After MSG treatment, the dendritic arbor of these cells shrunk, partly because they probably lost one of their major synaptic partners, the cone OFF-bipolar cells. Also their output targets may have diminished in number because many of the ganglion cell, including those of the large alpha-type cells, probably degenerated due to the MSG-treatment. Protection provided by PACAP restores a dendritic tree, which resembles that of the untreated tissue.

In conclusion, PACAP can provide effective protection to many retinal neuron types, primarily for those, which possess PACAP receptors and do not contain ionotropic glutamate receptors; however, it maybe extremely effective if these cells also contain calcium-binding proteins. Further experiments are needed to identity the cells which match all three above criteria.

Table 3 Characteristics of immunolabeling under different experimental conditions

Treatments	Antibodies											
	Anti-VGLUT 1		Anti-VGAT		Anti-TH		Anti-calbindin		Anti-calretinin		Anti-parvalbumin	
	I	A	I	A	I	A	I	A	I	A	I	A
Control	+++	+++	++	++	++	++	++	+	+++	++	++	++
MSG	+	+	+	+	+	+	+	+	+	+	+	+
MSG and PACAP	++	++	++	++	+	++	++	+	+++	++	++	++

Number of "+" are proportional with labeling strength.
I intensity, *A* amount of structures labeled

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