Search for the Optimal Monosodium Glutamate Treatment Schedule to Study the Neuroprotective Effects of PACAP in the Retina

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ABSTRACT: We have previously shown the protective effects of pituitary adenylate cyclase-activating polypeptide (PACAP) in monosodium glutamate (MSG)-induced retinal degeneration. In the present study, we have investigated the optimal model for examining this neuroprotective effect. One MSG treatment on postnatal (P) days 1 or 5, though produces some ultrastructural alterations, does not cause enough damage to study neuroprotection. When retinas were treated three times with MSG, the entire inner retina degenerated. Neuroprotection with PACAP was achieved with at least two treatments. Evidence suggests that PACAP provides protection against excitotoxicity, therefore, it may be a useful agent in reducing excitotoxic damage in the retina.

KEYWORDS: retina; MSG; PACAP; plexiform layers; ribbon synapses; degeneration

INTRODUCTION

Pathological increase of glutamate levels plays a key role in neuronal damage in many diseases. In the eye, several pathological conditions can be mimicked by experimentally elevating extracellular glutamate concentrations. The damaging effects of monosodium glutamate (MSG) on the retina have long been...
known. In accordance with others, we have also demonstrated that systemic treatment of neonatal rats with MSG leads to the destruction of the entire inner retina.\textsuperscript{3} Pituitary adenylate cyclase-activating polypeptide (PACAP) and its receptors are present in the retina,\textsuperscript{4,5} and the protective effects of both PACAP and VIP have been demonstrated in some retinal pathological conditions.\textsuperscript{6–8} PACAP also attenuates glutamate toxicity in the retina \textit{in vitro},\textsuperscript{9} in addition to similar effects observed in neuronal cultures.\textsuperscript{10,11} Recently, we have shown that intravitreous injection of PACAP ameliorates MSG-induced retinal degeneration in neonatal rats.\textsuperscript{3} The aims of the present study were (i) to examine the ultrastructural changes caused by MSG treatment and in correlation with these changes (ii) to find the optimal model for investigating the neuroprotective effect of PACAP in MSG-induced retinal degeneration, that is, when MSG already exerts its damaging effects but those can still be readily reversed by PACAP treatment.

**MATERIALS AND METHODS**

Newborn Wistar rats were injected s.c. with 2 mg/g bodyweight MSG.\textsuperscript{3} The following treatments were performed: one time injection on postnatal day (P) 1; one time injection at P5; and three times injection at P1, P5, and P9. In PACAP-treated pups, 100 pmol PACAP\textsuperscript{3} in 5 μL saline was injected into the vitreous of one eye with a Hamilton syringe at the time of (i) the first, (ii) the first two, or (iii) all three MSG injections.

At P21, retinas were processed for histological analysis as previously described.\textsuperscript{3} Briefly, immediately after removal, eyes were dissected in ice-cold phosphate buffer (PB) and fixed in 4% paraformaldehyde dissolved in 0.1 M PB. Sections of 2 μm were stained with toluidine blue. Samples for measurements derived from at least six tissue blocks prepared from at least three animals (n = 2–5 measurements from one tissue block). The following parameters were measured on digital photographs: cross-section of the retina from the outer limiting membrane to the inner limiting membrane; the width of the inner plexiform layer (IPL) from the bottom of perikarya of the last cell row in the inner nuclear layer (INL) to the top of perikarya in the ganglionic layer (GCL); and the number of cells/100 μm section length in the GCL. Statistical comparisons were made using the analysis of variance (ANOVA) test followed by Neuman–Keul’s posthoc analysis. Electron microscopy was performed on tissues fixed with 4% paraformaldehyde supplemented with 1% glutaraldehyde dissolved in 0.1 M PB. After washing in PB, tissue samples were treated with 1% OsO\textsubscript{4} in PB and embedded for routine electron microscopic examination.

**RESULTS AND DISCUSSION**

Compared to normal retinas (Fig. 1 A), one time MSG treatment either at P1 or P5 did not cause discernible alteration at the light microscopic level, but
FIGURE 1. Degeneration caused by MSG in the developing rat retina. ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer. (A): Control retina, black arrow shows intact ribbon synapses in the OPL (A1; PR: photoreceptor cell) and in the IPL (A2; b: bipolar cell terminal, a: amacrine cell dendrite, g: ganglion cell dendrite). (B): One time MSG treatment at P1; arrowheads: degenerative figures in the OPL; arrow: degenerating cell debris. (C): One time MSG treatment at P5; arrow: synapse in the IPL. (D): Three times MSG treatment; arrows: degenerating cell; arrowheads: ribbons in the photoreceptor cell. Inserts: light microscopic appearance of the retinas.

Some degenerative processes were apparent with electron microscope (Fig. 1 B, C). The photoreceptor cells as well as the other cellular layers were normal. Though the plexiform layers seemed to be undisturbed in the light microscope, both the outer nuclear layer (OPL) and the IPL showed signs of damage.
FIGURE 2. Neuroprotective effect of PACAP in MSG-induced degeneration; demonstration in light microscopic photographs and diagrams. (A): The INL and GCL are fused in the three times MSG-treated retina. (B): Three times MSG + two times PACAP-treated retina. The layers, including the IPL, are well visible. The ONL seems undisturbed, while
at the ultrastructural level (Fig. 1 B, C). In the OPL, several holes could be found in the tissue around the photoreceptor cell terminals and scattered in the OPL. The ribbon synapses seemed unaffected, the cytoplasm of the cells (that of both the photoreceptors and the interneurons) was normal (Fig. 1 B). Degenerating cell debris was found in the OPL, but signs of increased microglial activity were not seen (Fig. 1 B). These findings correlate well with the facts that (i) mostly the inner retinal neurons contain ionotropic glutamate receptors,\(^1\) therefore photoreceptors should remain unaffected and (ii) in our earlier study, we have shown that only the inner retina degenerates after repeated MSG treatment.\(^3\) However, it is surprising that even after one MSG treatment the OPL showed several signs of initial degenerative processes, while the IPL was almost unaffected. All types of synapses described in earlier studies were present.\(^1\) Degenerative signs were only occasionally seen. There was little difference between the animals treated with MSG at P1 (when new cells are still being generated) or P5 (when most intensive synaptogenesis occurs).

The entire IPL disappeared in retinas with three times MSG treatment and the INL and GCL seemed fused (Fig 1 D). At the ultrastructural level, no remnants of the IPL (e.g., ribbon synapses of bipolar cells and serial amacrine cell synapses) were found. Only a structurally disturbed OPL could be studied, where the photoreceptor synapses were still present (Fig. 1 D), along with numerous degenerative structures (holes, cell debris, extracellularly located filamentous material, etc). Such an extensive damage was not expected in the OPL, since only little alteration could be seen at the light microscopic level. Interestingly, in short-term excitotoxic insults the OPL and IPL seemed to be equally prone to damage.\(^1\) The explanation for this may be that in our case the animals were killed long after the actual insult, therefore, there might have been enough time for structural rearrangement.\(^1\)

In the above model, neuroprotection with PACAP could be achieved with at least two treatments at the time of the first and second MSG application (Fig. 2 A, B). One PACAP application was not enough to exert protection; three applications did not provide significantly better protection than the two times treatment (not illustrated). This qualitative picture is well reflected in the measured morphometric parameters (Fig. 2 C, D).

The other layers of the retina are reduced. Both the width of the IPL and the distance between the outer (arrowhead) and inner (double arrowhead) limiting membranes (OLM–ILM) is the smallest in the three times MSG-treated preparations. In the PACAP-treated retina, these parameters are close to the control results. (C): Measurements of the IPL width and the OLM–ILM distance. (D): Number of cells in the ganglion cells layer/100 \(\mu\)m section length. In the three times MSG-treated retina, the cell number is decreased compared to the control and the other treatment schedules. Two times PACAP treatment counteracts the damaging effect of three times MSG treatment.
In summary, one time MSG treatment, though produces some ultrastructural alterations, it does not cause enough damage to study neuroprotection in our model. At least two times MSG treatment is necessary to exert large-scale degeneration in the retina. Evidence also suggests that PACAP provides protection against excitotoxicity in the retina. This effect is likely to be mediated by PAC1 receptors coupled to high activity of cAMP production. Topic PACAP application therefore may be a possible treatment for conditions caused by excitotoxic damage in the retina.

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REFERENCES

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