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Effects of light and darkness on cell deaths in damaged retinal ganglion cells of the carp retina

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Abstract. Effects of light and darkness on the apoptosis of retinal ganglion cells (RGCs) in young carp were measured by TUNEL method after transection of the optic nerve. Following the operation, the fish were kept under one of four regimens; constant darkness (DD), constant light (LL), 12 hr light and 12 hr dark (LD) and 3 hr of flickering light followed by 21 hr in the dark (FL). On day 3, the highest ratio of apoptotic RGCs was seen under conditions of DD, followed by LL, LD, and FL. On day 6, the percentages of apoptotic RGCs were lower under every experimental condition than what they had been earlier on day 3, but the same ranking order was maintained. Immunohistochemically it could be shown that phosphorylated ERKs were more intensively localized in FL rather than DD retinas. The results suggest that illumination regimens, and in particular cyclic diurnal light/dark changes, have an influence on the degree of apoptosis of damaged RGCs, and that inhibition of apoptosis is correlated with the higher expression of phosphorylated ERKs.

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INTRODUCTION

In mammalian eyes, most of the retinal ganglion cells undergo apoptosis after axotomy of optic nerve fibres (Berkelaar et al. 1994). In fish, on the other hand, it has been reported that cell death does not usually occur even if some photoreceptor cells are destroyed through injury (Marotte et al. 1979). There is, however, the possibility that techniques at the time of the quoted study might have been insufficiently sensitive to detect a cell loss in the RGC population.

Recent studies have revealed that the structure and function of retinal cells is influenced by the nature of the light stimulation (Powers et al. 1988, Umino et al. 1991, Dong and McReynolds 1992, Harsanyi et al. 1996). As a matter of fact, when optic nerve fibres were transected, RGCs were unable to send their sensory information to the central nervous system (CNS), and the animals then went blind. The RGC is, of course, not by itself sensitive to light, but will continue to receive signals generated by stimulation with light from photoreceptor cells via neurons such as bipolar, horizontal and amacrine cells even after the optic nerve fibres have been severed. It is, therefore, reasonable to suppose that the extent of RGC deaths, initiated by axotomy, may be influenced by light and dark conditions post-operation.

In this research, based on experiments with young adult carp, it was first established by TUNEL method, whether RGC apoptosis occurred naturally in normal, intact eyes. Next, after transections of the optic nerves, the operated fish were kept under one of four different light and dark regimes. Flickering lights were included because of the neurochemical effects attributed to them, for example on the retina of white perch (Umino et al. 1991), mud puppy (Dong and McReynolds 1992), and goldfish (Harsanyi et al. 1996). Flickering but not sustained light had been reported to increase the release of dopamine, perhaps from interplexiform cells. Apoptotic cell deaths of the RGCs were detected by TUNEL on days 3 and 6 post-operation and quantitatively assessed.

The depolarization of the RGC membrane potential, caused by light through a series of responses from photoreceptor cells via secondary and other neurons, induces changes in the activities of intracellular messengers like cyclic adenosine monophosphate (c-AMP) and mitogen activated protein kinases (MAPK), both implicated in directly influencing the survival or death of RGCs (Iuvone et al. 1991, Hanson et al. 1998, Meyer-Franke et al. 1998). Since activating extracellular signal-regulated protein kinases (ERKs) and MAPK were found to inhibit neuronal apoptosis (Xia et al. 1995, Guillonneau et al. 1998), the present experiments involve immunohistochemical determinations of phosphorylated ERKs in FL and DD retinas, which gave the lowest and highest apoptotic ratios, respectively.

METHODS

Operation and light conditions

Young, 8 - 10 month old carps, (*Cyprinus carpio* Linne), with body lengths of 8 - 10 cm, were used for the experiments. Before surgical operations took place all fish were acclimated for at least 10 days under an artificial light/dark regimen (12 hr L/D) at a temperature of ca. 20 - 25°C. The light source was a 15 W fluorescent lamp of 1,000 - 1,500 lux, approximately equivalent to 65 - 90 $\mu W\ cm^{-2} s^{-1}$ positioned 10 cm above the aquarium. Spectral filters were not used. Intact control retinas were obtained from fish kept under the condition mentioned above.

The surgical procedures were the same as those previously reported by Dezawa et al. (1998). In brief, fish were anaesthetized by immersion in a 0.066% solution of MS-222 (meta-aminobenzoic acid ethylester) (Sigma, St. Louis, MO). The left optic nerve was exposed and completely transected through the dural slit at a distance of 2 mm from the back of the eye. The fish were kept under one of the following four light-dark diurnal regimes; 1) constant darkness (DD), 2) constant light (LL), 3) 12 hr light and 12 hr dark (LD), or 4) 3 hr of flickering light (at 3 Hz), followed by 21 hr of darkness (FL). In all cases in which exposures to light were required, the light source was the same as mentioned above and, once again, placed 10 cm above the surface of the water. Light intensity and spectral distribution in the aquarium water were the same as described before (Dezawa et al. 1998). Operated fish were kept individually in separate aquaria, but 3-5 individuals were examined for each experimental condition.

TUNEL staining

Experimental fish were anaesthetized (through an overdose solution of MS-222) on days 3 and 6, and had their retinas removed. At least three and maximally five fish were used for each condition on each of the two observation days, (i.e. 3 and 6 days post-operation). The reti-

nas were fixed overnight by immersion in 4% paraformaldehyde/0.1 M phosphate-buffered saline (PBS) (pH 7.4), and sections were cut with a cryostat. A central area of the retina (3 mm in radius from the disk), except for the disk itself, was isolated and subjected to further analysis. TUNEL staining was performed by an *in situ* apoptosis detection kit (Trevigen, Gaithersburg, MD). In brief, sections were preincubated in equilibration buffer (provided by the kit) for 30 minutes at room temperature and were then treated with terminal deoxytransferase (TdT) and biotinylated nucleotide for 45 minutes at 37°C. After washing, labelled nuclei were detected by streptavidin-fluorescein isothiocyanate (FITC). The sections were finally stained with 4,6-diamidino-2-phenyl indole (DAPI, Polysciences Inc. Warrington, PA) to label all the nuclei. The detection of non-specific positivity was performed by omission of TdT. The degree of apoptotic cells in the ganglion cell layer was expressed through the ratios of TUNEL positive cells in relation to the total number of nuclei stained with DAPI. Statistical analyses (ANOVA with pairwise comparison by the Bonferroni method) were performed between the different conditions.

Immunofluorescence microscopy

Fish were killed on day 3 post-operation by immersion in an overdose solution of MS-222. The retina was dissected out, and immersed overnight in 4% paraformaldehyde in 0.1 M PBS (pH 7.4). In the case of DD fish, these operations were performed in the dark. The central area of the retina with a 3 mm radius from the disk, was used for the analysis. Untreated intact retinas were also fixed in the same solution as controls. Retinas were sectioned vertically into 14 μm thick sections with a cryostat. For the primary antibodies, the polyclonal rabbit anti-phospho-ERK antibody to recognize specifically the dually phosphorylated form of ERK1 (p44) and ERK2 (p42) (Promega, Madison, WI) was used at 1:800 dilution. Sections were blocked with 0.1 M PBS containing 5% goat serum and 3% bovine serum albumin for 3 hours. The sections were incubated overnight with the above primary antibodies at 4°C, and then for one hour at room temperature with biotin-conjugated rabbit IgG (1:200; Vector, Burlingame, CA). The procedure was followed by TSA-Direct kit (NEN-Life Science Products, Boston, MA) analysis, combined with FITC-labelled streptavidin, according to the manufacturer's instructions. Specificity controls were obtained by omitting the primary antibodies. Sections were counter-stained with propidium iodide, and observed with a confocal laser scanning microscope (Radians 2000, BioRad Laboratories, Hertfordshire, UK).

RESULTS

Comparison of apoptotic ratios under four light-dark regimens

Non-specific positivity was not detected in the TdT-omitted control stainings. Under DAPI staining, the ganglion cells form an almost monocellular layer, composed of round and clear nuclei. All normal nuclei were stained blue with DAPI, but those of apoptotic cells were identified because of their light blue-green colour due to double staining with DAPI and TUNEL (Fig. 1). The intact retina showed a ratio of 3.6 ± 0.6 % of apoptotic cells, suggesting that cells in the ganglion cell layer die naturally in the retina of the young carp (Fig. 1A).

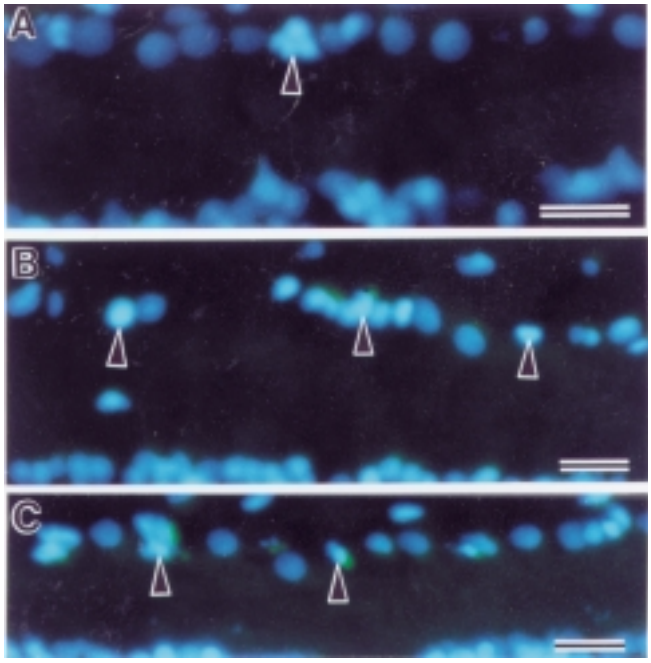

Fig. 1. TUNEL staining of ganglion cell layer from control (A), DD (B) and FL fish (C) at day 3. Among the DAPI stained blue nuclei, apoptotic cells were identified on the basis of their blue-green colour caused by double DAPI and TUNEL staining (some cells are indicated by arrowheads). In DD, more apoptotic cells were observed in the ganglion cell layer than in the FL fish. Scale bars = 20 μm.

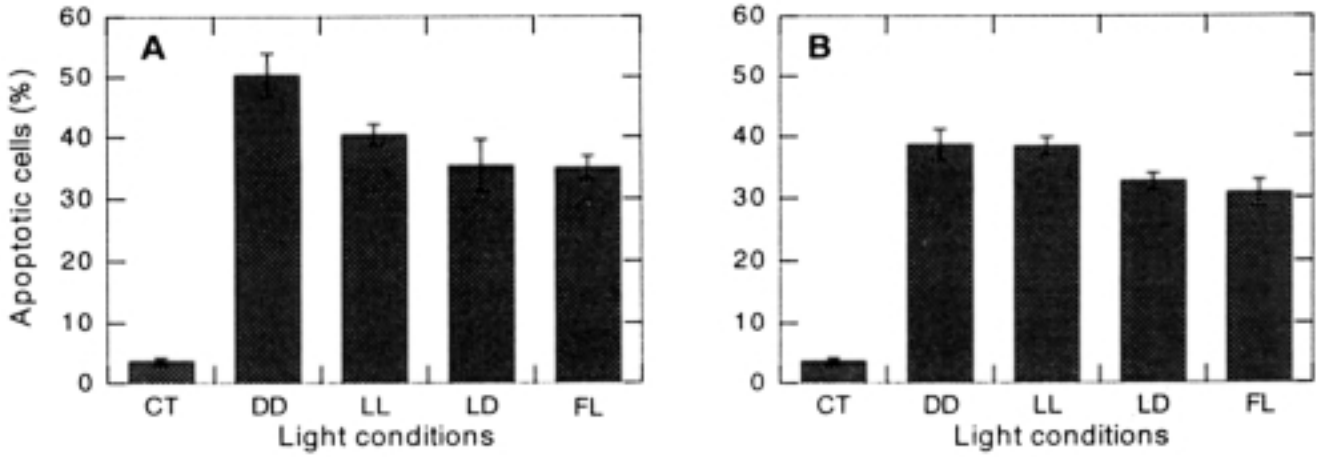

Fig. 2. Apoptotic ratios (%) of RGCs of control fish (CT) and specimens kept under four different light-dark regimes (i.e. DD, LL, LD and FL) on day 3 (A) and day 6 (B) post-operation.

The highest ratio, on day 3, of 50.3 ± 3.7 % of apoptotic cells was recorded in fish having undergone DD treatment. This was followed by fish from the LL (40.4 ± 1.7%) and LD groups (35.4 ± 4.3%). The lowest percentage of RGCs undergoing apoptosis came from fish of the FL group (35.1 ± 1.9%) (Figs. 1B, 1C, 2A). All of the ratios were highly significant (P<0.01) when compared with those of the intact controls. A highly significant difference was also present between DD - FL, and significant differences (0.01<P< 0.05) occurred between DD - LL, DD -LD, and LL - FL, but no significant differences were seen between LL - LD and LD - FL.

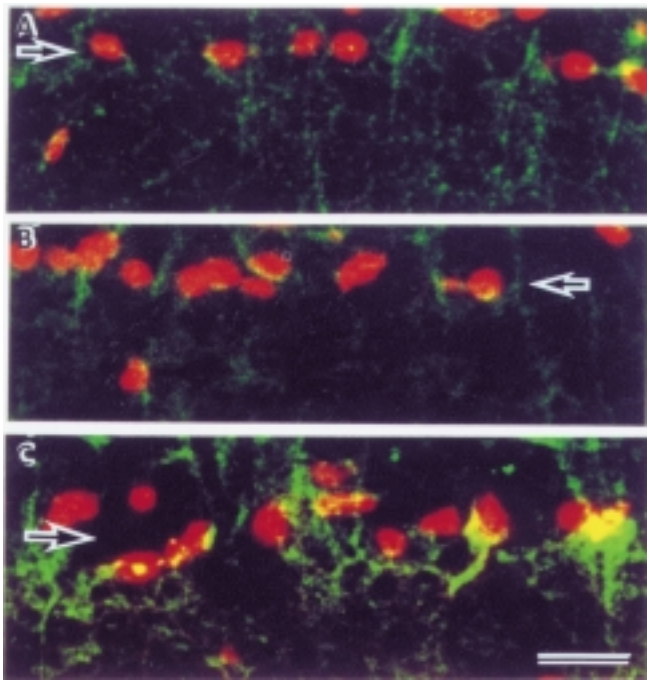

Fig. 3. Immunohistochemistry of phospho-ERK in control (A), DD (B) and FL fish (C) on day 3. Arrows indicate approximate position of ganglion cell layer (nuclei in this layer exhibit red coloration). FL fish exhibit stronger phospho-ERK activity (green coloration) than either control or DD fish. Scale bar = 20 μm.

On day 6, the ratios of apoptotic to non-apoptotic cells at each light regime were lower than those of day 3, respectively, maintaining, however, the same ranking order, namely, DD (38.7 ± 2.5%), LL (38.4 ± 1.4%), LD (32.7 ± 1.3%) and FL (30.8 ± 2.2%) (Fig. 2B). There were highly significant differences between controls and all regimens, between DD -FL, LL - LD and LL - FL, and a significant difference was also seen between DD and LD. However, no significant differences were detected between DD - LL and LD - FL.

Immunofluorescence microscopy

Non-specific immunoreactivity was not detected in the specificity controls. We investigated the activation state of ERKs by immunohistochemistry, using anti--phospho-ERK antibodies. In the intact control retina no obvious positivity to phospho-ERK was seen in the ganglion cell layer (Fig. 3A).

Under DD conditions (day 3), a weak phospho-ERK--positivity was noticeable in the ganglion cell layer (Fig. 3B). The pattern of immunoreactivity obtained was almost identical to that observed in the intact retinas (Fig. 3A). In FL fish (day 3), however, the number of cells with strongly stained perikarya increased in the

ganglion cell layer. Cellular processes of the inner plexiform layer, probably corresponding to Müller cell processes, were also immunoreactive for phospho-ERKs (Fig. 3C).

DISCUSSION

Although predominantly monocellular in nature, the ganglion cell layer of the vertebrate eye consists not exclusively of RGCs, but also of other kinds of cells like, for instance, displaced amacrine cells. In the frog retina only 87% of total number of cells were reported to be actually RGCs (Beazley et al. 1986). The exact number of the RGCs in the young carp retina was not determined in our experiments, but considering that at least some cells other than RGCs were included in the count, the true ratios of apoptotic to total number of RGCs must have been even a little higher than those obtained. On the other hand, the majority of the TUNEL positive reactions in the ganglion cell layer is likely to have come from RGCs, because the latter were directly injured by the axotomy (Beazley et al. 1986).

In this study of young carp, 3.6 ± 0.6 % of the RGCs of the intact retina were apoptotic. Perhaps not surprisingly, since the fish retina continues to grow throughout a fish's life span (Easter et al. 1981), a similar result was reported from the intact adult goldfish retina (Vallani et al. 1997, Johns 1997). Higher proliferations and cell deaths of particularly retinal cells were seen near the peripheral regions of the retina. Identical results were reported from the frog retina (Hollyfield 1971, Jacobson 1976, Jenkins and Straznicky 1986). On the basis of these results on lower vertebrates, it may be assumed that apoptotic cell death of RGCs occurs naturally in the eyes of young carp.

There is no longer any doubt that exposure to light can directly or indirectly influence the morphology and survival of retinal cells in fish eyes (Vihtelic and Hyde 2000). Retinas of goldfish, for example, reacted morphologically to prolonged exposures of weak stimulation with light by producing wavy deformations of the retinal layers and functionally by significantly decreasing the retinal cells' responsiveness to light (Powers et al. 1988). As to the neurochemical effects of flickering lights, for example on the retina of white perch (Umino et al. 1991), mud puppy (Dong and McReynolds 1992), or goldfish (Harsanyi et al. 1996), it was reported that flickering but not sustained light increased the release of dopamine, perhaps from interplexiform cells. Consequently, RGC activity may be directly influenced by neurotransmitters such as dopamine released by an exposure to flickering light even after optic nerve fibre transections. "Flashes of light" have even been reported to be effective in the survival and regeneration of RGCs in the cat retina after peripheral nerve transplantation, but an explanation for the survival was not provided (Watanabe et al. 1999).

We noticed that both LL and DD on day 3 showed similar apoptotic ratios of 40.4 and 50.3 %, respectively, and these were significantly higher than those of LD and FL eyes. This finding suggests that oscillations of light and dark phases are important for the survival of damaged RGCs, following optic nerve transection. The apoptotic ratios of FL fish eyes (35.1 % on day 3 and 30.8 % on day 6) were the lowest of all, indicating that quick and repetitive "on" and "off" oscillations of the stimulating light must have been quite effective in helping RGCs to survive, even if the period of exposure to the flickering lights was only 3 hr/day.

In 1998, we provided data on the effects of light-dark conditions on the regeneration of severed optic nerve fibres of carp eyes (Dezawa et al. 1998). In that paper carp optic nerves were severed and the operated specimens were kept under four different light-dark regimens similar to this paper. It was shown then that the regenerations of optic fibres under LD and FL were significantly more successful than those of LL and DD fish. Taken together, the results of the previous and present experiments demonstrate that optimal physiological conditions to decrease the death of RGCs and to promote optic nerve regeneration occur only when light and darkness are diurnally alternating.

Membrane depolarizations of the RGCs caused either by stimulation with light (Imaki et al. 1997) or by an artificial injection of potassium (Iuvone et al. 1991) increase c-AMP concentrations in the cytoplasm of these cells. There are reports claiming that an increase in c-AMP helps nerve cells to evade cell death (Hanson et al. 1998, Meyer-Franke et al. 1998). Furthermore, it is known that c-AMP activates ERKs in the RGCs via protein kinase A (Robertson et al. 1999), and also that ERK levels are increasing in the RGCs by stimulation with light (Imaki et al. 1997). Activated ERKs may activate c-AMP response element binding proteins (CREB), a process which is then thought to lead to the expression of many genes controlling differentiation and proliferation of cells (Robertson et al. 1999). ERKs are known to possess antagonistic functions to Jun N-terminal kinase (JNK) and p38 in apoptosis, and it has been reported that for the induction of apoptosis in neuronal cells an activation of

both JNK and p38 and a concurrent inhibition of ERKs are critical (Xia et al. 1995). In fact, Guillonneau (1998) and Liu et al. (1998) both concluded that multi-activations of cellular factors by ERKs are critically related to the survival and death of retinal cells.

The notion, therefore, that light is effective (if not essential) for the survival of the RGCs seems reasonable. In fact, the present study has revealed that DD-fish showed the highest apoptotic ratio (in other words, the worst survival) of all four conditions tested. If, on the other hand, the eyes were stimulated with flickering lights as with the FL-fish, membrane depolarizations of RGCs would occur repetitively, initiating significant increases of c-AMP and ERK levels in the RGCs, thus providing the latter with potent 'survival weapons'. On the basis of our result, i.e. that immunoreactivity of activated ERKs was more intense in RGCs of FL retinas and less so in DD retinas, we may conclude that an increase in activated ERKs can lead to an inhibition of cell deaths and that, conversely, a reduced activation of ERKs, as seen under the DD regime, can promote cell deaths.

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