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Effects of long-term spectral deprivation on the morphological organization of the outer retina of the blue acara (*Aequidens pulcher*)

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To investigate the developmental plasticity of colour vision, we reared fish with a trichromatic cone system (*Aequidens pulcher*) under three near-monochromatic lights, differentially stimulating each spectral cone type from the larval stage to the age of at least one year. Control conditions comprised white lights of two intensities. The treatments did not affect the visual pigments, but led to significant changes in cone outer segment lengths. Furthermore, in the blue-reared group the density of single cones within the retina was reduced by two-thirds after 18 months of exposure, while no changes were observed in the other groups. The connectivity of cone horizontal cells with the single cones was influenced by the intensity and spectral composition of the rearing lights: H1 cells were more sensitive to the spectral component, whereas H2 cells responded to intensity cues. In the blue-light group the dynamics of horizontal cell synaptic spinule formation and degradation were severely compromised. These observations show that long-term spectral deprivation leads to significant morphological changes at the level of photoreceptors and horizontal cells. While the reactions of photoreceptors may be interpreted mostly in terms of compensation, the functional consequences of the changes observed on the horizontal cell level remain to be determined electrophysiologically.

Keywords: spectral deprivation; cone visual pigments; cone outer segments; spectral cone types; horizontal cells; spinules

1. INTRODUCTION

The visual system offers many examples for experience-dependent processes in the development of neural circuitry. The classical, and maybe best-studied cases concern the primary visual cortex of mammals with its ocular dominance and orientation columns (see Sengpiel *et al*. (1999) for a review). The neural basis of colour vision, by contrast, has turned out to be highly resistant to artificial manipulation of the chromatic environment, at least as far as can be deduced from behavioural experiments and the development of retinal ganglion cells (summarized in Kröger *et al*. 1999). The suggestion that colour vision is more 'hard-wired' than other components of visual processing has, however, not been rigorously tested on a cell biological level.

The adaptive state of the teleost retina is indicated by morphological changes at the light and electron microscopic level. They comprise retinomotor movements of photoreceptor and retinal pigment epithelium cells, changes in the synaptic complexes of rods and cones, including the size of synaptic ribbons and the formation or retraction of spinules, as well as the electrical coupling of cone horizontal cells (see Djamgoz *et al*. (1995) for a review). Such adaptation-dependent reorganizations are likely to be manifestations of changes in sensitivity. Furthermore, some of these processes, especially the dynamics of horizontal cell spinules, have been implicated in the processing of spectral information, since they are associated with changes in chromatic feedback activity (Weiler & Wagner 1984). We therefore started our investigation of the developmental plasticity of colour vision by quantifying such morphological parameters.

In order to induce long-term plastic changes, we exposed fishes from the larval stage to the age of at least one year to near-monochromatic lights at wavelengths chosen to maximally stimulate each spectral cone type, and simultaneously deprived them of the other two spectral channels. We used a cichlid species (the blue acara, *Aequidens pulcher*), which has a trichromatic cone system and a highly ordered mosaic spatial arrangement of cones and horizontal cells, in order to simplify the assessment of functional morphology of the outer retina. In this paper, we summarize the morphological effects of this treatment and report some new results on the time-course of induced effects.

2. MATERIAL AND METHODS

Groups of fish were reared in 12 h : 12 h light/dark cycles of equi-irradiant (*ca*. 10^{12} quanta s^{-1} cm^{-2}) lights of three different wavelengths: 590 nm (red), for preferential stimulation of the long-wave-sensitive (L) larger member of the unequal double cones (DCs); 513 nm (green) for preferential stimulation of the middle-wave-sensitive (M) smaller member of the unequal DCs; and 450 nm (blue) for the preferential stimulation of the

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short-wave-sensitive (S) single cones (SCs; figure 1*b*). The stimulating lights were created with interference filters (half-maximum bandwidths: 5–10 nm); their position within the absorption spectra of the three cone types is shown in figure 1*b*. Two control groups were brought up under white light of 33 lux ('bright-white group') and 0.2 lux ('dim-white group'), the latter appearing slightly dimmer than the green monochromatic light to the human observer. Further details of the rearing conditions are described in Kröger *et al.* (1999). The various rearing regimes started after hatching; most fish were studied 12 months later. Additional intervals were used to investigate the survival of S cones (see figure 1*c*).

The absorption spectra of photoreceptor outer segments were determined with a computer-controlled dual-beam Liebman-type microspectrophotometer (cross-section of measuring beam: 2 μm; see Kröger *et al.* (1999) for details).

For the study of spinule numbers and cone type frequencies, isolated retinae were fixed in a paraform–glutaraldehyde (PA/GA) mixture, osmicated and prepared for light and electron microscopy according to routine protocols. Relative cone densities were determined in tangential 1 μm sections through the layer of cone inner segments stained with methylene blue. Confocal laser scan microscopy of 0.1 mm radial sections of PA/GA-fixed material incubated in 0.5% Lucifer Yellow (LY, Sigma, Deisenhofen, Germany) was used to determine the lengths of cone outer segments as well as the connectivities of horizontal cells and cone pedicles. Golgi-like label of single cells was obtained with LY applied to fresh retinas (Braun *et al.* 1997). Stacks of 0.5 μm optical sections were reconstructed in three dimensions, using the software of the ZEISS LSM 410 system to measure entire individual outer segments, and to establish the connectivity of cone-specific horizontal cells (CHCs).

3. RESULTS AND DISCUSSION

(a) *Photoreceptors*

(i) *Visual pigments*

A tangential section of the photoreceptor layer just distal to the external limiting membrane shows the square-mosaic-type arrangement of cones, consisting of a majority of unequal DCs with a larger L and a smaller M partner surrounding a central single cone (S, figure 1*a*). Some cases of equal, 'twin' cones with L characteristics were also observed. L DCs had peak absorbances (λ_{max}) at 570 nm, M DC partners had a λ_{max} at 530 nm, and S SCs were maximally sensitive at 453 nm (Kröger *et al.* 1999). Monochromatic rearing did not change these peak absorbances more than within the range of standard deviation. The observed variability was probably due to polymorphism, or varying amounts of porphyropsin (Kröger *et al.* 1999). We may therefore conclude that monochromatic rearing does not affect cone absorbances. This is in contrast to findings in other teleosts where external cues and seasonal migrations lead to shifts in the relative amounts of rhodopsin and porphyropsin, allowing plasticity in the spectral sensitivity (Bowmaker 1990). What drives these changes is presently unknown.

(ii) *Cone outer segment lengths*

In each of three specimens from each rearing group, 15 DC outer segments were assessed irrespective of their spectral identity. Their lengths were expressed relative to the lengths of the inner segment. No statistically significant differences were found between the dim-white group, the green, and the red rearing group. However, in the blue rearing group, the relative DC outer segment lengths were increased by *ca.* 44% with respect to this baseline ($p < 0.001$), whereas in the bright-white group, outer segments were shorter by 27% ($p < 0.001$; Kröger *et al.* 1999). Both of these effects may be interpreted in terms of compensatory mechanisms, which result in keeping the rate of photon catch constant. In the bright-white group, they constitute a process, *photostasis*, which aims at equalizing luminosity signals (Penn & Williams 1986). Similar effects have been reported previously in goldfish rods (Raymond *et al.* 1988). This is the first time that cones have been shown to react in this way. The elongation of L/M DC outer segments in blue light suggests the presence of similar mechanisms, which aim at balancing the relative sensitivities to different spectral ranges.

(iii) *Loss of single cones*

Figure 1*c* shows the time-course of S SC densities in the central retina between four and 18 months after exposure to the different lighting conditions. We had previously observed that in the one-year-old blue-light group, there was a centro-peripheral gradient of S SCs, with a full complement in the periphery next to the growth zone, and loss of up to 30% in the retinal fundus dorsal of the optic nerve head (Kröger *et al.* 1999). S SC populations in the white- and green-light groups were unaffected, whereas a certain degree of fluctuation was apparent in the red-light group (figure 1*c*). Following the fate of the S SCs for another six months shows a stable situation for these latter groups, and also demonstrates that in the blue-light group, the decrease of SC density continues steadily to a point where only about one-third of control numbers is preserved. With this trend ongoing it may be expected that two years of exposure to monochromatic blue light will completely deplete the population of S SCs in the central retina. This is in contrast to results obtained from animals reared under blue light of slightly longer wavelengths ($\lambda_{max} = 485$ nm), where the number of S cones seemed to stabilize after about one year (Kröger *et al.* 1999). The specific effectiveness of the short-wave component is further highlighted by the results of two preliminary experiments, where fish were transferred to white light from monochromatic rearing conditions after ten months. In the red-light group only two months of exposure to white light, which in contrast to the rearing light has a short-wavelength component, led to the disappearance of *ca.* 40% of the S SCs. Stimulating fish of the blue-light group with white light resulted in a reappearance of S SCs almost to control levels. This suggests that new S SCs were inserted into the cone mosaic in an orderly fashion. However, a larger and more focused study is necessary to produce statistically useful sets of data and to determine the mechanisms of cone regeneration and cell guidance.

The effects on cone outer segment lengths and S SC abundance suggest that the relative spectral sensitivity of the retina is under regulatory control. Since these processes occur over a long time span, the underlying mechanisms are probably different from those leading to colour constancy observed at the level of CHC responses (Kamermans *et al.* 1998), which results from adjustments

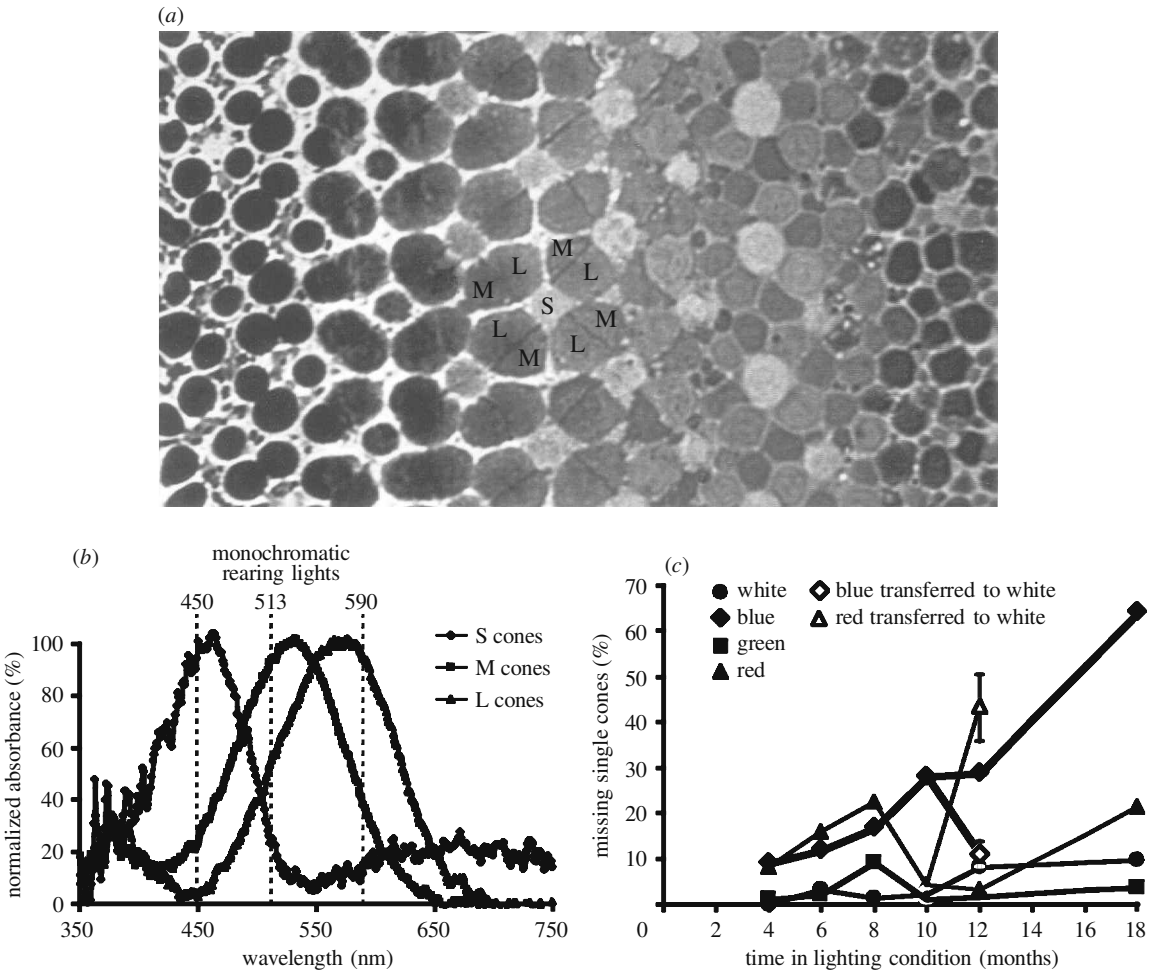

Figure 1. (*a*) Tangential section (1 µm) through the region of cone ellipsoids just distal of the external limiting membrane (right margin of micrograph). In the blue acara, most double cones consist of a long (L) and a medium (M) wave partner arranged in a square mosaic enclosing a central short-wave(S)-sensitive single cone. (Magnification × 1800.) (*b*) Position of the stimulating lights (450 nm, 513 nm and 590 nm) relative to the microspectrophotometric data of normalized absorbances (%) for the visual pigments of the three spectral cone types of the blue acara (Kröger *et al*. 1999). (*c*) Survival of S cones in the central retina under various spectral environments. Relative S SC densities were determined in each retina of two fishes per rearing group and time-point (three fishes at 18 months). In blue light (diamonds), elimination of S SCs starts at four months or earlier and seems to be ongoing at 18 months. Large fluctuations in the frequencies of SCs between time-points and specimens from the red group (triangles) may be due to transient effects with individual differences in time-course because of variable sensitivity to deprivation in the short- and middle-wavelength domains. In the white (circles) and green (squares) lights, there is no notable loss of S cones over time. The error bars (transfer experiments) show ranges of results.

of synaptic feedback and feedforward interactions between cones and CHCs. However, CHCs may also play a central role in the developmental balancing of the input from the various spectral cone types in our experiments, since they are in a strategic position to compare the relative strengths of stimulation. Their long-term message could be relayed to the photoreceptors by means of neurotrophins, which have been localized in HCs (Gao & Hollyfield 1992), and lead to apoptotic removal of SCs, a phenomenon observed in preliminary examination of our material.

(b) *Horizontal cells*

(i) *Types and connectivities*

Three types of HCs are present in the blue acara retina, one of which contacts only rods, whereas the other two are CHCs. The H1 CHC has the typical brush-like morphology with short dendrites directed towards the cone pedicles and a single axon descending into the inner nuclear layer. H1 CHCs contact all spectral cone types; the density of contacts decreases with increasing distance from the perikaryon, which is localized exactly beneath a central SC (Braun *et al*. 1997). H2 CHCs situated vitread to the former cells have larger dendritic fields. Similar to the H2 cells in cyprinids (Stell *et al*. 1994), the H2 CHCs of *A. pulcher* show a marked preference for M and S cones. By using the position within the mosaic as a clue for identifying the cone types, we found an average of 7.05 SCs, 9.72 smaller DC partners and 1.42 larger DC partners invaginated by an H2 CHC ($n = 10$; Braun *et al*. 1997). These cells may therefore be expected to yield biphasic, chromaticity responses, whereas H1 CHCs would generate monophasic luminosity signals.

Rearing fish in different light regimes affected the number of contacts of CHCs with the S SCs. Blue light increased the number of S cones contacted by H1 cells

(from 1.52 to 1.92), whereas in the bright-white light group, the number of S cones contacted by H2 cells was significantly reduced (from 7.05 to 2.81, $p < 0.01$, ANOVA; Braun 1997; Braun *et al*. 1997). Therefore, the connectivity of CHCs is determined by both the intensity and the spectral composition of the rearing lights. Interestingly, the luminosity-type H1 cells are more sensitive to the spectral component, whereas the chromaticity-type H2 cells respond to intensity cues. Our finding about the involvement of the S cones in this process does, however, not rule out changes in the contacts between CHCs and the L/M DCs. These could affect the size of the synaptic contacts, and would be accessible only at the electron microscopic level.

(ii) *Spinules*

Spinules are finger-like extensions from the dendrites of CHCs that invaginate the cone cytoplasm opposite the synaptic ribbons. Their presence during the day and absence during the night has been correlated with the adaptation-dependent dynamic of the biphasic response of the H2 CHCs, and therefore implicated with chromatic feedback processes within the M cone pedicles (Weiler & Wagner 1984). Altering the balance between the spectral cone channels was therefore expected to affect the number and/or dynamics of spinules.

In the light-adapted state, the green-light group was no different from the baseline values of the white-light group; by contrast, spinules of the red-light group were more numerous and those in the blue-light group significantly less numerous (Kröger & Wagner 1996). The situation in SCs was similar to that, and statistically more significant than, in DCs. Dark-adaptive reduction of spinule numbers was equal to controls in the red and blue rearing groups, whereas, in the blue-light group, there were as many spinules as during daytime, indicating a complete absence of spinule dynamics. Spectral deprivation in the medium- and long-wavelength domains thus has a severe impact on the formation and degradation of spinules, indicating changes in the control mechanisms. It remains to be seen in electrophysiological recordings whether this is accompanied by imbalances in the chromatic feedback activity as reflected by the biphasic response pattern of the H2 HCHs. As long as these data are not available, it is difficult to interpret the functional significance of the changes in connectivity and synaptology between cones and CHCs.

4. CONCLUSIONS

Our observations demonstrate that the spectral composition, as well as the intensity of environmental lighting, strongly influences the morphology of cones and CHCs. Such an approach mimics to a certain degree natural conditions, where some fish species may grow up and live in long-wave-dominated water bodies ('black waters'), and others in the long-wave-deprived mesopelagic habitats of the oceans. Among the experimental conditions blue light had the most profound effects. This may be due to the fact that, in the stimulation paradigm, the S pathway was most clearly separated from the L and M channels, but could also indicate a special role of this particular pathway. The reactions of cone photoreceptors are easily interpreted in terms of compensatory mechanisms. In the case of cone–HC connectivity and synaptology, however, ultrastructural studies of the synaptic complexes and electrophysiological recordings of light-evoked responses are necessary for a functional understanding of the induced changes.

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