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Losses of immunoreactive parvalbumin amacrine and immunoreactive $_{\alpha}$protein kinase C bipolar cells caused by methylmercury chloride intoxication in the retina of the tropical fish *Hoplias malabaricus*

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Abstract

To quantify the effects of methylmercury (MeHg) on amacrine and on ON-bipolar cells in the retina, experiments were performed in MeHg-exposed groups of adult trahiras (*Hoplias malabaricus*) at two dose levels (2 and 6 µg/g, *ip*). The retinas of test and control groups were processed by mouse anti-parvalbumin and rabbit anti-$_{\alpha}$protein kinase C ($_{\alpha}$PKC) immunocytochemistry. Morphology and soma location in the inner nuclear layer were used to identify immunoreactive parvalbumin (PV-IR) and $_{\alpha}$PKC ($_{\alpha}$PKC-IR) in wholemount preparations. Cell density, topography and isodensity maps were estimated using confocal images. PV-IR was detected in amacrine cells in the inner nuclear layer and in displaced amacrine cells from the ganglion cell layer, and $_{\alpha}$PKC-IR was detected in ON-bipolar cells. The MeHg-treated group (6 µg/g) showed significant reduction of the ON-bipolar $_{\alpha}$PKC-IR cell density (mean density = 1306 ± 393 cells/mm^2) compared to control (1886 ± 892 cells/mm^2; $P < 0.001$). The mean densities found for amacrine PV-IR cells in MeHg-treated retinas were 1040 ± 56 cells/mm^2 (2 µg/g) and 845 ± 82 cells/mm^2 (6 µg/g), also lower than control (1312 ± 31 cells/mm^2; $P < 0.05$), differently from the data observed in displaced PV-IR amacrine cells. These results show that MeHg changed the PV-IR amacrine cell density in a dose-dependent way, and reduced the density of $_{\alpha}$PKC-IR bipolar cells at the dose of 6 µg/g. Further studies are needed to identify the physiological impact of these findings on visual function.

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- Displaced amacrine cells
- ON-bipolar cells

Introduction

Mercury intoxication

Mercury can have several chemical forms (Hg^o, Hg^+, Hg^{2+}, and organic combinations such as methylmercury) with different characteristics such as volatility, water solubility and facility to cross the lipid membrane barrier. The elemental (Hg^o) and organic (MeHg) forms are easily transported through the pulmonary alveoli and blood-brain barrier, and consequently cause nervous system injury due to their high reactivity with proteins (sulfhydryl groups) which could lead to enzyme inhibition and autoimmune damage (1,2). Organomercury compounds such as methylmercury are rapidly absorbed by the gastrointestinal tract where 90% of ingested mercury is absorbed by the organism (3,4).

In the Amazon, gold-mining activities have caused water basin contamination with mercury, with serious consequences for the health of riverine populations, whose potential intoxication has been recently demonstrated (5-8). Fish differ in the amount of methylmercury they accumulate according to their foraging characteristics. Methylmercury content in tissues may be as much as 30 times higher in carnivorous than in non-carnivorous species (9). Exceedingly high mercury concentrations (112.4 to 2250 mg/kg) have been found by dos Santos et al. (10) in predatory fish (dourada, pescada, surubim, and tucunaré) from regions of current intense gold-mining activities, in the Tapajós River Basin (State of Pará). These levels are much higher than the maximal values established by Brazilian legislation (predatory fish: >1.0 mg/kg; other fish: >0.5 mg/kg).

Fish from other regions may have much lower mercury concentrations. Brabo et al. (11) measured mercury concentration in different species of carnivorous fish (trahira, barbado, surubim, and tucunaré) from the Munduruku reserve in an old gold-mining area in the Tapajós River Basin (State of Pará), having found levels from 0.173 to 0.546 mg/kg. Nevertheless, the authors argue that the large amount of fish consumed daily (at last tree times a day) by this population is an important factor for the risk evaluation of mercury contamination.

Methylmercury and the visual system

The central nervous system is one of the most important targets for methylmercury (12). Several studies have reported that methylmercury affects the cerebellum and visual system (13-15). Studies in humans have been carried out to evaluate the potential visual damage associated with occupational mercury contamination such as color vision losses, spatial contrast sensitivity reduction and visual field constriction (7,16-19).

In the marmoset (a primate species) it has been shown that mercury vapor inhaled by the mother accumulates in several tissues of the neonate's retina - the optic nerve, the internal plexiform layer and the ganglion cells - and also concentrates in the pigment epithelium and in the blood vessel walls (20). There is also mercury accumulation in the photoreceptor cells, but the metal accumulates in the rods and not in the cones (21).

Some studies have revealed scotopic and photopic visual deficits after methylmercury exposure (22,23), but there is no evidence of the effects of methylmercury on specific retinal cell types using immunocytochemistry.

Bipolar cells receive their input from photoreceptors at the outer plexiform layer and send their output to amacrine and ganglion cells in the inner plexiform layer. They are classified as either cone-bipolar or rod-bipolar cells, depending on the type of photoreceptor from which they receive their input. In the fish retina, some bipolar cells receive their input exclusively from cones, but several types of bipolar cells receive a mixed input from rods and cones (24).

The present study was undertaken to determine by immunohistochemistry and con-

focal analysis the possible effects of methylmercury on amacrine and bipolar cells of the rod pathway of adult trahiras (*Hoplias malabaricus*), a voracious fish widely distributed in South American basins. Antibodies against parvalbumin (PV) and protein kinase C ($_{\alpha}$PKC) were used as markers for amacrine and ON-bipolar cells, respectively.

Experimental procedures

Methylmercury chloride exposure

The animals were purchased from a supplier in São João da Boa Vista, SP, Brazil, and cared for in accordance with the guidelines of the NIH guide for care and use of laboratory animals. Ten trahiras from stock containers were transferred to individual 40-L aquaria. Each fish was anesthetized with 0.02% MS-222 (Sigma, St. Louis, MO, USA), and received an intraperitoneal injection of 2 (N = 3) or 6 µg MeHgCl/g (N = 3) in groups weighing 342.09 ± 29.9 g (mean ± SD) and 353.34 ± 36.69 g, respectively. After 10 days, the animals were decapitated and the retinas dissected and analyzed. The fish from the control group (N = 4; 315.46 ± 15.28 g) were placed in aquaria and submitted to the same conditions. Only one retina per fish was used. This was done in order to have three independent retinal samples of each treatment dose, rather than having dependent samples, by using the two eyes from the same fish. All animals were dark-adapted for 2 h, their posterior eyecups containing the retina were dissected from the sclera and pigment epithelium, and fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 3 h. Sections were obtained from the retina of the control group to identify the cell types that were stained by antibodies against PV and $_{\alpha}$PKC. After several rinses in 0.1 M sodium phosphate buffer, pH 7.4, retinas were cryoprotected in 30% sucrose in 0.1 M sodium phosphate buffer, pH 7.4, frozen in optical critical temperature compound (Tissue-Tek, Sakura, Torrance, CA, USA) and serially sectioned at 20-µm intervals with a cryostat (Leica, JUNG CM 3000, Wetzlar, Germany). Retinal sections were collected on gelatinized slides and stored at -20ºC for further use. Alternatively, retinas were dissected from the epithelium and vitreous humor, wholemounted and stored in 0.1 M sodium phosphate buffer, pH 7.4, at 4ºC for topographic analysis.

Immunocytochemistry

The transverse retinal sections were processed for immunofluorescence with mouse monoclonal antibody against PV (1:1.000, Sigma) and rabbit anti $_{\alpha}$PKC (1:1.000, Sigma). Non-specific binding sites were blocked for 1 h with 0.1 M sodium phosphate buffer, pH 7.4, containing 10% normal goat serum and 0.3% Triton X-100. Sections were incubated overnight at room temperature with the primary antibodies against PV and $_{\alpha}$PKC diluted in blocking solution. Goat antisera against mouse or rabbit IgG tagged with fluorescein or Cy3 (1:200, Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA) were diluted in 0.1 M sodium phosphate buffer, pH 7.4, with 0.3% Triton X-100, and the sections were incubated for 2 h. After washing, the tissue was mounted using 0.1% paraphenylenediamine (Sigma), and analyzed under a light microscope. For the control experiments, primary antibodies were omitted and no specific cellular staining of wholemounts or frozen sections was observed. The wholemounts were treated similarly, except for the time of incubation time with the primary antibody (72 h).

Morphologic analysis

Quantitative determinations of the number of both anti-$_{\alpha}$PKC and anti-PV-immunopositive cells were made and the cells were

examined with an inverted Zeiss 410 confocal laser scanning microscope (LSM 410, Carl Zeiss, Oberkochen, Germany), using 40X oil immersion objectives. Digitized images were counted in square 102.93-mm^2 fields using the systematic random method (25). Counts were made from the optic disk across the whole retina using NIH Scion Image 2.0 and were converted to cells/mm^2. Original pictures were mounted with Adobe Photoshop 4.0 (San Jose, CA, USA) software to obtain optimal contrast within the same figure plate. To obtain isodensity, contours were drawn on a map of the wholemount retina using DeltaGraph 4.0 software (Delta Point, Monterey, CA, USA) and Canvas 5.0 software (Deneba Systems, Miami, FL, USA). Data obtained for MeHg-treated and control retinas were analyzed statistically using the Sigma Stat 2.0 program (Jandel Scientific Corporation, San Rafael, CA, USA).

Results

Parvalbumin and $_{\alpha}$PKC

The antibody against $_{\alpha}$PKC predominantly stained bipolar cells located in the middle third of the inner nuclear layer (Figure 1A). In turn, besides being distributed in a few bipolar cells, PV was largely expressed by amacrine cells in the inner nuclear layer and in some displaced amacrine cells of the ganglion cell layer (Figure 1B). Although expression of PV is species-dependent and several kinds of cells can be labeled in the same retina, the anti-PV antibody has been shown to be a good marker for amacrine cells (26-29).

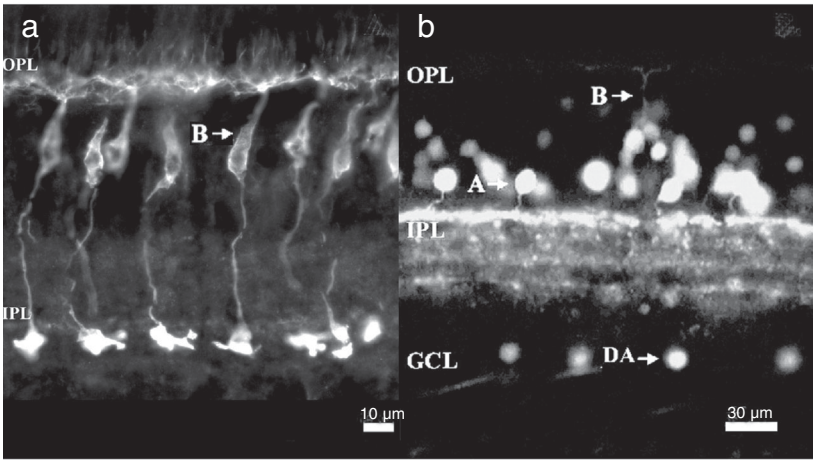

Figure 1. Distribution of PKC-IR and PV-IR in cross-sections of the fish retina. *a*, PKC-IR bipolar cells (B) sending their dendrites to the outer plexiform layer (OPL) and their axons to the inner plexiform layer (IPL) can be observed. *b*, Arrows indicate examples of PV-IR amacrine cells (A) between the OPL and IPL, displaced amacrine cells (DA) in the ganglion cell layer (GCL), and a bipolar cell (B). PKC-IR = immunoreactive protein kinase C; PV-IR = immunoreactive parvalbumin.

In wholemounted retinas, the mean density of immunoreactive PV (PV-IR) amacrine cells was 1312 ± 31 cells/mm^2 (N = 3) in the control retinas, and fell to 1040 ± 56 cells/mm^2 (N = 3) and 845 ± 82 cells/mm^2 (N = 3) after treatment with 2 and 6 µg/g of MeHgCl, respectively. Statistical comparison revealed that the lower dose group differed from control at the $P = 0.03$ level and the higher dose group differed from control with high significance at $P < 0.001$ (Mann-Whitney rank sum test). Thus, our data showed a dose-dependent reduction of immunoreactivity in amacrine cells with MeHg treatment.

A separate analysis of the dorsal and ventral regions showed no significant differences between the treated and control groups in the dorsal part of the retina. In the ventral retina, however, there was a highly significant difference between treated and control groups ($P < 0.001$, Kruskal-Wallis one-way analysis of variance on ranks).

PV-IR displaced amacrine cells in the ganglion cell layer, on the other hand, showed very similar densities in retinae from control (146 ± 11 cells/mm^2) and treated fish (135 ± 10 and 126 ± 45 cells/mm^2, 2 and 6 µg/g of MeHgCl, respectively). No significant effect was detected in immunoreactivity ($P < 0.153$, ANOVA). Similarly to the topic PV-IR amacrine cells in the inner nuclear layer, the cell density peak of displaced PV-IR amacrine cells was found in the temporo-ventral area.

Finally, the number of $_{\alpha}$PKC-positive

bipolar cells decreased only with the highest dose of MeHg ($P < 0.001$, one-way ANOVA and *post hoc* Tukey test). Cell densities were 1886 ± 892, 1476 ± 621, and 1306 ± 393 cells/mm^2 for control, and 2 and 6 µg/g MeHgCl-treated animals, respectively.

Discussion

Methylmercury chloride is one of the most toxic forms of mercury, causing irreversible damage to animal cells. According to Durrieu et al. (30), *H. aimara* showed a predominance of methylated form of mercury accumulation in skeletal muscle (94% of total mercury; 5.11 µg/g dry weight). Indeed this organic form is absorbed very efficiently through the digestive barrier (4) and is excreted very slowly (31). This level of mercury concentration exceeds the WHO safety limit for human consumers (2.5 µg/g dry weight) (12). Regarding human exposure, Kehrig et al. (32) estimated that a daily intake of 110 g fish (0.5-0.9 µg MeHg/g) corresponds approximately to 55-88 µg MeHg/day. These investigators detected accumulated hair MeHg levels of 13.1-23.5 µg/g in the Balbina Village population, Amazon Region. As observed above, the MeHg concentrations were higher in *H. aimara* than those reported by Kehrig and colleagues (32), so that the doses used in the present study were realistic even though they were injected intraperitoneally.

Histopathological studies have shown necrosis in the exocrine pancreas and capsular hepatic inflammation (33,34). Other effects on the visual system of fish have been described, such as alteration in the scotopic and photopic spectral sensitivity (23).

To our knowledge, there is no published information about the effects of MeHg on the fish retina. The present results showed a reduction of immunoreactivity in amacrine PV-IR and bipolar $_{\alpha}$PKC-IR cells in the retina of fish after exposure to MeHg. The reduction was dose-dependent for PV-IR amacrine cells and significant at the highest dose for $_{\alpha}$PKC-IR bipolar cells. This difference may suggest differential sensitivity of the two cell types.

PV is a calcium-binding protein that is present in certain neuronal populations (35). Although its role is not well understood, PV immunocytochemistry has been extensively used to identify several cell types in mammalian, avian and fish retinas (26-29). Depending on the species, PV-positive horizontal, bipolar and/or ganglion cells were also described, in addition to amacrine cells (26-29). In the ganglion cell layer of the fish retina, it has been recently shown that PV-positive cells were displaced amacrine cells (28). In addition, the anti-PV antibody has been shown to be a good marker for AII glycinergic amacrine cells (26-29). Thus, it is possible that the identified PV-IR amacrine cells may be inhibitory interneurons that modulate chromatic and achromatic processing in the visual system and that are affected by MeHg intoxication. The same occurred in bipolar $_{\alpha}$PKC-IR cells, which are part of the rod pathway. The damage to the rod and cone pathways agrees with the behavioral findings reported by Hawryshyn et al. (23) showing reduction of the scotopic and photopic spectral sensitivity.

The present findings suggest that the different cell types may have distinct degrees of susceptibility to mercury intoxication. Further experiments are necessary to determine whether the decrease in immunoreactivity is caused by a reduction of gene expression or by apoptosis. In this context, Kunimoto (36) evaluated the effects of MeHg *in vitro* and observed the presence of programmed cell death in brain neurons.

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