Effects of mercury intoxication on the response of horizontal cells of the retina of thraira fish (*Hoplias malabaricus*)

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Abstract

Methyl mercury (MeHg) is highly neurotoxic, affecting visual function in addition to other central nervous system functions. The effect of mercury intoxication on the amplitude of horizontal cell responses to light was studied in the retina of the fish *Hoplias malabaricus*. Intracellular responses were recorded from horizontal cells of fish previously intoxicated with MeHg by intraperitoneal injection (IP group) or by trophic exposure (T group). Only one retina per fish was used. The doses of MeHg chloride administered to the IP group were 0.01, 0.05, 0.1, 1.0, 2.0, and 6.0 mg/kg. The amplitudes of the horizontal cell responses were lower than control in individuals exposed to 0.01 (N = 4 retinas), 0.05 (N = 2 retinas) and 0.1 mg/kg (N = 1 retina), whereas no responses were recorded in the 1.0, 2.0, and 6.0 mg/kg groups. T group individuals were fed young specimens of *Astyanax* sp previously injected with MeHg corresponding to 0.75 (N = 1 retina), 0.075 (N = 8 retinas) or 0.0075 (N = 4 retinas) mg/kg fish body weight. After 14 doses, one every 5 days, the amplitude of the horizontal cell responses was higher than control in individuals exposed to 0.075 and 0.0075 mg/kg, and lower in individuals exposed to 0.75 mg/kg. We conclude that intoxication with MeHg affects the electrophysiological response of the horizontal cells in the retina, either reducing or increasing its amplitude compared to control, and that these effects are related to the dose and/or to the mode of administration.

Key words

- Methyl mercury
- Horizontal cells
- Electrophysiology
- *Hoplias malabaricus*
- Retina

Introduction

Mercury intoxication in humans and animals is caused in part by atmospheric pollution and in part by natural causes such as corrosion of the earth’s crust, volcanic activity, or evaporation of large masses of water (2700-6000 ton/year), as well as by direct human activities (3000 ton/year) (1). Many aquatic environments in the Amazon region have been exposed by gold mining activities (2-5).

Due to their position in the feeding chain, aquatic animals such as fish become intox-
cated with methyl mercury (MeHg) mainly through the gills and the trophic pathway. Trophic intoxication occurs at higher levels in carnivorous than in herbivorous or omnivorous species (4). Pinheiro et al. (6) found high concentrations of MeHg in carnivorous species of fish, and other investigators have reported elevated concentrations of MeHg in Brazilian fish (2,4,5).

MeHg affects synaptic transmission both in the peripheral and the central nervous systems, and this action is mediated by several mechanisms (7). The visual system is an important target of mercury intoxication (8-12). Investigation of the neuro-ophthalmological aspects of the mercury intoxication that caused Minamata disease started with the measurement of the visual field performed by Iwata and Abe (8).

The effects of mercury intoxication on the retinas of vertebrates are still little known. In studies on the squirrel monkey, mercury vapor inhaled by the mother accumulated in several tissues of the neonate’s retina - the optic nerve, the inner plexiform layer and the ganglion cells, - and was also concentrated in the pigment epithelium and in blood vessel walls (13,14). It has also been observed that mercury accumulates in the rod photoreceptor cells of the monkey retina (15,16). Cats intoxicated with mercury exhibited an increase in the amplitude of the b-wave of the electroretinogram, and also a decrease in the response latency compared to untreated cats (17). In a pioneering study describing changes due to mercury exposure in the functioning of the retina, Fox and Sillman (18) showed that rods, but not cones, were affected in the electroretinogram of the frog. Their study, however, used mass action potentials in the two-flash technique to extract the rod responses by subtracting the mixed rod and cone response obtained with a flash from the pure cone response obtained with a second flash.

In fish there is little evidence of the effects of mercury exposure on vision. Hawryshyn et al. (19) demonstrated a reduction of the behaviorally determined visual spectral sensitivity function in the rainbow trout injected intraperitoneally (ip) with MeHg. The reduction of sensitivity involved both photopic and scotopic mechanisms, in contrast to the previous results obtained by Fox and Sillman (18).

The present study investigated the activity of single retinal neurons in mercury-exposed Hoplias malabaricus fish. We studied the responses of monophasic horizontal cells of the fish retina, which correspond to morphological type H1 (20), with the purpose of evaluating cone impairment due to the effects of MeHg intoxication. Monophasic horizontal cells reflect the activity of cones, mainly of the L-type cones, and are also called luminosity horizontal cells, as opposed to chromaticity horizontal cells, since they are thought to process luminance information (21,22). Due to the relative ease of recording from H1 horizontal cells compared to other retinal cells in the fish retina, these cells offer a good model for investigating impairment of the functions of the outer retina.

**Material and Methods**

**Experimental animals**

Specimens of thraira (Hoplias malabaricus), obtained from Toledo Station (a lake in the northwest of Paraná State, Brazil), approximately 30 cm in length, were maintained in glass aquaria (40 L) under constant aeration. An untreated control group (N = 6 retinas) and nine mercury-exposed groups were used. The untreated control group (N = 6 retinas) and nine mercury-exposed groups were used. The IP groups were acclimated to the experimental conditions for 10 days and then received an intraperitoneal (ip) injection of MeHg (IP group) at one of the following doses: 0.01 (N = 4), 0.05 (N = 2), 0.1 (N = 1), 1.0 (N = 2), 2.0 (N = 4), 6.0 (N = 4) mg/kg MeHg, under anesthesia with 0.02% 3-aminobenzoic acid ethyl ester. The
retinas were analyzed 15 days after ip injection. Fish from the other treated group, exposed to mercury through feeding (trophic or T group), were acclimated to the experimental conditions for 30 days (1 fish per 30-L aquarium containing dechlorinated tap water, at a temperature of 21 ± 2ºC and under a 12:12-h photoperiod). The fish were fed young live specimens of Astyanax sp previously injected ip with 0.1 mL of an aqueous solution of MeHg. The mg/kg doses were calculated for each thraira based on its weight. Since each thraira was kept in one aquarium, it was possible to determine with precision the number of Astyanax specimens ingested by each fish. The final dose was based on the total amount of MeHg ingested by the fish. This total amount was the sum of the mercury content that had been injected into 14 Astyanax fish eaten by the thraira. Three dose groups of 7 individuals each were exposed to MeHg (H3C-Hg+) (14). Doses of 0.75, 0.075, and 0.0075 mg H3C-Hg+/kg wet thraira weight, corresponding to each of the three groups, were administered every 5 days (corresponding approximately to a daily dose exposure of 0.015 mg MeHg/kg). After 14 doses of MeHg, retinas exposed to 0.75 (N = 1), 0.075 (N = 8), and 0.0075 mg/kg (N = 4), were used for electrophysiology.

Recording equipment

The electrophysiological responses of the retinal cells were recorded through glass microelectrodes (Boron-silicate, WPI, New Haven, CT, USA) filled with a conductive solution of 3 M KCl, with high tip resistances (200 to 400 MOhms). The microelectrodes were prepared immediately before the experiments with a Sutter model P-2000 laser puller. A David-Kopf (Tujunga, CA, USA) hydraulic micropositioner was used to advance the electrode in constant steps (2.5 µm) to penetrate cells.

Recordings were made with a WPI model 767 intracellular amplifier monitored on a Tektronix D13 oscilloscope (Alta Loma, CA, USA), and recorded on magnetic tape initially with an HP model 3968A analogue recorder and later with a Cygnus CDAT4 (Delaware Water Gap, PA, USA) digital recorder. Recorded responses were transferred off-line to paper using a Gould model 3000 (Cleveland, OH, USA) chart recorder, and response parameters were measured using an in-house computer program for data acquisition (Data Acquisition Devices, model: PCI-MIO-16E-1), with a National Instruments card (Austin, TX, USA).

Optical stimulation

The system consisted of a 75-W xenon light source, a monochromator and an all-quartz optical system that projected a disk of light onto the preparation (for details, see Ref. 23). The intensities, wavelengths and diameters of the light stimulus were controlled electromechanically by a computer. The optical stimulation was programmed to emit appropriate sequences of stimuli for the identification and measurement of the characteristics of the cells penetrated.

The xenon lamp coupled to the monochromator generated the light stimulus. The light was transmitted through a fiber optic bundle, an electromagnetic shutter, a neutral
density filter to reduce the luminous intensity (Starna, Atascadero, CA, USA, model 522, 0-4 log), a diaphragm of variable diameter that projects a circular spot of light from 20 to 1700 µm onto the retina and an optical system composed of two lenses that focus the image of the diaphragm onto the retina. Calibration of the luminous source is made periodically, with an International Light radiometer model IL 1700 and a SED 033 photodetector (both from Peabody, MA, USA). Calibration was performed for each of the 205 positions of the neutral filter at each of the 101 wavelengths of the monochromator, in 4-nm steps from 300-700 nm.

**Stimulation procedure**

The preparation was kept in a light-shielded cage in which background light was very low. To find cells, we used a continuously flashing light of medium intensity, thus adapting the stimulated region of the retina (a spot of about 1.5 mm in diameter, centered on the electrode tip).

After penetrating a cell, we immediately turned off the probing light and presented a series of stimuli of increasing diameters, at a fixed wavelength, for the determination of the type of cell penetrated. Next, a stimulation program generated four 400-ms pulses at 370, 450, 540, and 640 nm, with intervals of 500 ms between each pulse. This series of four pulses was presented at three different intensities (-1, -2, and -3 log) relative to the maximum intensity (3 x 10^13 q s^-1 (cm^2)^-1). The maximum diameter of the light spot (1500 µm) was used. If the cell’s responses and baseline were stable, a spectral sequence was presented at a larger number of wavelengths, extending up to 700 nm. With this more detailed series, the peak frequency could be determined more precisely.

**Statistical analysis**

One-way ANOVA using the Kruskal-Wallis and Duncan post hoc tests from the Statistica 6.0 (Stat Soft, Inc., Tulsa, OK, USA) statistical analysis program was used for data analysis. In this analysis the averages of the amplitudes and latencies obtained for each retina of the treated and control groups were compared, with the level of significance set at P < 0.05.

**Results**

No electrophysiological responses were observed in the retinas of fish injected with 1.0, 2.0, or 6.0 mg MeHg/kg. Examples of intracellular recordings of monophasic horizontal cell responses from the groups in-

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Figure 1. Intracellular recordings obtained in monophasic horizontal cells in response to 640-nm flashes of 3 x 10^{13} q s^{-1} (cm^2)^{-1}. For the group receiving intraperitoneal injections of MeHg there was a decrease of the response amplitude with increasing doses. Different results were obtained for the fish that received mercury through feeding. They showed an increase in the response amplitude with the increase of the dose at low doses and a decrease of the responses amplitude with the highest doses.
Effect of mercury intoxication on the retina of *H. malabaricus*

JECTED with lower doses of MeHg, for which it was possible to record activity, are shown in Figure 1. The left column presents the responses of the IP group and the right column those of the T group. The corresponding dose is indicated next to the response trace. A horizontal cell response from a retina from the control group is also shown at the top of each column; the same trace is displayed for both the IP and T groups. Notice that the concentration range was 10-fold for the IP group and 100-fold for the T group.

In the control group 19 horizontal cells were recorded from 6 retinas. The amplitudes of the responses to flashes of $3 \times 10^{13}$ quanta $(\text{cm}^2)\text{s}^{-1}$ had an average value of $-18.4 \pm 7.7 \text{ mV}$ at 640 nm. The response amplitude to this stimulus ($3 \times 10^{13}$ quanta $(\text{cm}^2)\text{s}^{-1}$ at 640 nm) will be compared below for all experimental conditions. Figure 2 presents the averages and standard deviations of the response amplitudes obtained at all wavelengths and intensities under the different experimental conditions.

In retinas from fish exposed to 0.1 mg MeHg/kg, two cells recorded from the same retina responded with a smaller amplitude to 640-nm flashes at -1 log compared to the control group (average response amplitude 640 nm, -1 log = $-6.48 \pm 1.7 \text{ mV}$, $P = 0.495$). In addition to almost complete absence of electrophysiological responses, extensive hemorrhaging was observed in the choroids of all the retinas from the IP group exposed to 0.1, 1.0, 2.0, and 6.0 mg MeHg/kg. Fish from these groups were less responsive to handling compared to control. It was only at the two lowest *ip* MeHg doses used that a higher number of intracellular responses were recorded. Three cells from 2 retinas were...
recorded in the 0.05-mg MeHg/kg IP group (average response amplitude: 640 nm, -1 log = -10.66 ± 0.78 mV, P = 0.598). In the 0.01-mg MeHg/kg IP group, recordings were obtained for 8 cells from 4 retinas (average response amplitude: 640 nm, -1 log = -10.33 ± 3.66 mV, P = 0.585).

In the T group exposed to 0.75 mg MeHg/kg, 6 cells were recorded from one retina (average response amplitude: 640 nm, -1 log = -9.7 ± 4.6 mV, P = 0.658) and in the 0.075 mg MeHg/kg T group, 27 cells were recorded from 8 retinas (average response amplitude: 640 nm, -1 log = -40.31 ± 17.49 mV, P = 0.009). These responses had the same characteristics as those recorded from the control group, but their amplitudes were higher than control. Eighteen cells from 4 retinas were recorded in the 0.0075-mg MeHg/kg T group (average response amplitude: 640 nm, -1 log = -15.0 ± 12.09 mV, P = 0.950).

The mean amplitudes recorded at all doses for the IP and T groups, as well as those for the control group, are presented in Figure 2. Response amplitudes increased with intensity at all wavelengths. The response amplitude peaked at 640 nm, which is characteristic of monophasic horizontal cells. The amplitude differences as a function of intensity were more apparent at the highest intensity and even more for the intermediate dose of the T group.

Discussion

We have recorded electrophysiological responses of horizontal cells in the species *H. malabaricus*. The horizontal cells that we recorded from were basically characterized by their large receptive fields and slow graded potentials, similar to those recorded in several other species such as the goldfish (*Carassius auratus*) (22,24), carp (*Cyprinus carpio*) (21), or the Amazonian species *Prochilodus lineatus* (25). The characteristics of the spatial response and the response to different light intensities were similar to those observed in many other species of fish.

In both the IP and T groups the monophasic horizontal cell responses were affected by intoxication with MeHg in a dose-dependent way. This finding extends results that contradict the report that the neurotoxic action of mercury on the retina is restricted to the rod system. We observed here that the cone system is affected, and furthermore that it is affected differently by different administration procedures. Intraperitoneal injection is an acute procedure, while the T group is submitted to a slow, chronic intoxication procedure, much closer to what could happen in the natural habitat. In the group intoxicated with *ip* injection, no responses were recorded at the three highest doses, possibly due to widespread cellular damage caused by intoxication. The damage caused by the intoxication was also evident in the extensive hemorrhage of the choroids and in the alteration of the behavior of the intoxicated fish. Some responses were recorded in the groups intoxicated with injections of 0.1, 0.05, and 0.01 mg/kg MeHg, but they were much more difficult to obtain, and had much smaller amplitudes compared to control. We conclude that intoxication with MeHg is definitely harmful to retinal horizontal cells, and that larger doses eliminate all intracellular responses.

The *ip* design used here was applied according to Hawryshyn et al. (19,26) who determined scotopic sensitivity behaviorally in the rainbow trout (*Salmo gairdneri Richardson*), a species that inhabits colder waters. These investigators injected the fish with 4.6 and 6.2 mg MeHg/kg and obtained reliable behavioral responses. The same doses applied to *thrairas* resulted in behavioral unresponsiveness to handling and in complete absence of electrophysiological activity in the retina, suggesting a higher sensitivity of tropical fish species to mercury exposure, as also reported by de Oliveira Ribeiro et al. (27), as compared to the
species used in the study by Hawryshyn et al. (19,26). According to the same authors, this could be due to the higher metabolic rate of tropical species, resulting in damage to the retina and in the impossibility of intracellular recordings.

The trophic exposure group was designed according to the procedure used by Rabitto et al. (28), in which the doses used simulated the conditions found in nature. As reported by Kehrig et al. (29), it is estimated that carnivorous fish from the Amazon region, used daily in the human diet, have about 0.5-0.9 mg MeHg/kg, a higher concentration than used in the present study.

The retina recordings in the trophic preparations had larger amplitudes than those of the control group. This result suggests that for very low doses there is an excitability effect, followed by a decrease in response amplitude at intermediate concentrations and finally the cessation of activity with high doses. Increases in electrophysiological responses from the retina have also been found in the electroretinograms of cats intoxicated with mercury (17) and of humans intoxicated with lead (30).

At the same time as the present study, a morphological study was carried out on the effects of MeHg intoxication on the retina (31,32). For each retina used in an electrophysiological experiment the other retina from the same fish was used for the morphological study. Morphological analysis did not suggest a complete absence of activity in the exposed cells, as found in the electrophysiological experiments. In fish injected with the two highest ip doses (2 and 6 mg MeHg/kg) a dose-dependent reduction of the number of cells with immunoreactivity to parvalbumin was detected, probably amacrine and displaced amacrine cells (31,32) and bipolar cells of the ON type, with immunoreactivity to protein kinase C. The quantification of these data should provide a better understanding of the electrophysiological data.

An explanation of the opposite effects obtained in the IP and T groups is the U-shaped dose-response curve of synaptic transmission of the MeHg effect. As reported by some investigators, one of the primary effects of MeHg on synaptic transmission is the increase, followed by a decrease, of the spontaneous liberation of neurotransmitters, as well as a decrease in the liberation of neurotransmitters activated by the nerve impulses (33-35). The reduction of the provoked neurotransmitter release has been associated with a decrease in the amount of neurotransmitter available for liberation (36), with the blocking of the Ca²⁺-dependent voltage channels that control the exocytosis of synaptic vesicles, and with the decrease in the excitability of the neuronal membrane (37-40).

The present findings constitute the first intracellular demonstration of the retinal effects of MeHg. They demonstrate that the cone system is profoundly affected by the intoxication, thus confirming previous suggestions from the literature regarding fish (19,25) and humans (9,12). The effects ranged, in a dose-dependent manner, from complete elimination of the response to response reduction in the acute intoxication procedure, while in the chronic procedure, they ranged from reduction of the response to a hypersensitive response at low doses. These effects are compatible with the findings that cone-mediated functions such as color vision and contrast sensitivity are affected by mercury intoxication in humans (9,12).
References


