A Non-Invasive Neurotoxicity Assay Using Larval Medaka

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ABSTRACT: We present a method for non-invasive electrophysiological analysis of rapid escape responses in intact, freely behaving larval medaka (Oryzias latipes) before and after short-term exposure to environmental toxicants. Recordings are obtained as a larval medaka swims in a small chamber of water above pairs of recording electrodes etched onto a printed circuit board. When the chamber is briefly vibrated by a sinusoidal pulse from a speaker attached to the grid, a stereotyped Mauthner cell-initiated escape response is evoked. The following parameters were quantified from recordings: 1) Mauthner axon conduction velocity, 2) delay between Mauthner axon spike and motoneuron spike in the spinal cord, 3) latency from stimulus onset to Mauthner spike, and 4) percent of success (or failure) of Mauthner responses during a series of stimuli. Toxicant-induced changes in these parameters were described following exposure to phenol, which tended to increase central delay, onset latency, and percent of response failure. This contrasts with effects of strychnine, which tended to decrease central delay and onset latency.

KEYWORDS: neurotoxicity, Mauthner, medaka, escape reflex, fish

Many previous toxicity tests in fish have used death (i.e. LC50) as an endpoint (Maltby and Calow 1989). However, an organism's ability to function normally in the ecosystem may be impaired at sublethal toxicant concentrations. The nervous system is a logical place to test for physiologically important and ecologically relevant sublethal effects because it is a primary target for many toxicants, as well as a sensitive integrator of many physiological and behavioral processes (World Health Organization 1986).

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Japanese medaka (Oryzias latipes) possess an escape reflex which is well suited for non-invasive electrophysiological monitoring (Featherstone et al. 1991). Therefore, the medaka may be useful for studying sublethal neurophysiological effects of environmental toxicants. In medaka, as in most other teleost fish and some amphibians, escape is mediated by a pair of large interneurons (Mauthner cells), with cell bodies in the hindbrain and axons which extend down the spinal cord. Unilateral excitation of one of the Mauthner cells (i.e. left or right) occurs in the hindbrain by integration of cranial nerve sensory inputs to the Mauthner cells. Typically, these inputs are generated by sudden tactile, vibrational, auditory, or visual stimulation. The result is initiation of a single action potential in one of the Mauthner cells. This spike then propagates down the Mauthner axon and excites, via Mauthner axon collaterals in the spinal cord, primary motoneuron pools that in turn activate nearby fast (white) axial musculature. The result of these events is a large body bend away from the stimulus, followed by a series of quick tail flips as the fish swims away (Diamond 1971, Eaton and Bombardieri 1978, Eaton and Hackett 1984, Eaton and Nissanov 1985, Eaton and DiDomenico 1986, Nissanov and Eaton 1989).

In this paper we demonstrate that printed circuit board recording grids can be used to non-invasively detect all of the above events (i.e., Mauthner axon spike, compound spike from primary motoneurons, fast axial muscle potential, and subsequent bursts of electromyographic activity) in freely behaving fish in response to a controlled vibrational stimulus. Our objectives were (a) identification of several quantifiable, electrophysiological measures related to medaka escape reflex function in vivo, (b) description of the neurophysiological basis of each, (c) demonstration that each can be affected by short-term toxicant exposure, and (d) discussion of the applicability of these testing methods for rapid and reliable screening of sublethal neurotoxicant effects on escape reflex function in larval medaka. Using a non-traditional paper format, we attempt to integrate these salient points for each selected measure.

EXPERIMENTAL PROCEDURES

Waveform Acquisition and Timing Measurements

Non-invasive recordings were made as follows: Thirty-day-old (6-10 mm long) medaka were transferred by pipet to a small, rectangular Plexiglass recording chamber. The bottom of the chamber consisted of a printed circuit board on which parallel, tinned-copper lines were etched, 0.5 mm apart and perpendicular to the long axis of the chamber. The chamber was filled with distilled water (~ 0.04 ml capacity). A comparison of the effects of exposing the fish to this volume of distilled water with the effects of artificial pond water showed that: (a) signal strengths of the Mauthner neuron spikes were actually greater when using distilled water, apparently because lower electrolyte strengths reduced the possibility of undesirable shunting of signal currents through parallel pathways in the fluid surrounding the fish, and (b) there were no significant differences in the timing of escape
reflex electrical events when comparing fish exposed to pond water and distilled water for the normal recording period (<5 min).

Differential signals picked up from fish by two adjacent pairs of recording electrodes were then amplified in two stages (100X per stage), filtered, and led simultaneously into two channels of a Tektronix 2221 digital storage oscilloscope. To enhance signal:noise ratios, the two inputs were electronically added within the oscilloscope and displayed on the screen as a single trace. For conduction velocity measures, however, the two channels were separately displayed rather than added. Although timing measurements were usually made "on-line" using cursors, waveforms were also printed on a plotter, or acquired by computer for automated data analysis and storage (Fig. 1).

Stimulus Delivery

Previous studies have shown that one or more cycles of sinusoidal water displacement is effective in evoking escape behavior in fish (Blaxter and Hoss 1981, Blaxter et al. 1981). In the present study, stimuli were delivered by a 7-inch speaker removed from a Grass AM5 audio monitor. The recording chamber was rigidly attached to the drum of the speaker by a 1 inch cylindrical glass extension (weight of recording chamber and extension = 82 g).

A Grass SD9 square-wave stimulator provided a gating pulse of sufficient duration for a Tektronix FG501 function generator to produce one cycle of a 20-40 Hz sine wave output through the audio monitor amplifier to the speaker. This resulted in a 1.5-2.0 mm vertical

FIG. 1--Arrangement for electro-mechanical stimulation and non-invasive recording of Mauthner-mediated escape in larval medaka.
displacement of the recording chamber. During a series of 30-40 consecutive stimuli delivered through the speaker, with an inter-stimulus interval of only 5-10 seconds, stereotyped Mauthner responses were evoked in response to 70-80% of stimuli to untreated fish. Responsiveness appeared uniform throughout the series; that is, the first ten stimuli in such a series were no more or less likely to elicit Mauthner responses than the last ten. Furthermore, the percentage of test stimuli that evoked successful responses was not affected by increasing the interval between consecutive test stimuli to one minute. This reliability in evoking escape responses, when using short inter-stimulus intervals, allowed rapid acquisition of repeated measurements of several neural parameters from each fish, usually within a five minute test period.

**Toxicant Exposure**

Prior to treatment, neural recordings were obtained, as described above, and corresponding measurements were taken from each fish, as described below in sections entitled "Waveforms" and "Measurements." For chemical exposure (ten fish/concentration), animals were placed in cylindrical containers filled with 20 ml of a solution made from a known concentration of the test chemical dissolved in artificial pond water. After exposure (4 or 24 h at room temperature), neural recordings and measurements were again obtained, and post-test means were compared to pre-test means for each fish. Statistical significance was determined using a t-test for related measures.

All examples of toxicant effects in this paper are results of exposure to one of two chemicals which produce disparate effects: phenol, and strychnine. Phenol is a polar narcotic (Bradbury et al. 1989) and depresses nervous system function, probably through nonspecific mechanisms (Veith and Broderius 1990). Strychnine, a convulsant, blocks inhibitory pathways by acting as a glycine antagonist (Bradbury et al. 1991).

**WAVEFORMS**

Typical electrophysiological recordings (Fig. 2) during an escape response in control fish consisted of three stereotyped peaks, followed by one or more bursts of electrical activity. The first of these three peaks (indicated as peak "1" in inset box, Fig. 2) was a small monophasic spike. The mean amplitude of the spike, baseline to peak, was 6 μV (Featherstone et al. 1991). This was followed, about 0.5 ms later, by a slightly larger spike (indicated as peak "2" in inset box, Fig. 2). The mean amplitude of this second spike, baseline to peak, was 15 μV (Featherstone et al. 1991). These two potentials represent extracellular recordings of (1) the Mauthner axon action potential as it propagates caudally within the spinal cord, and (2) the compound action potential derived from the nearly synchronous excitation of primary motoneuron pools, also within the spinal cord. Approximately 0.3 ms after the motoneuron spike was a large (about 1 mV) biphasic potential (indicated as peak "3" in the inset of Fig. 2). This latter potential represents fast axial muscle activity from one side of the body as the
fish forms the large initial body bend typical of the Mauthner-initiated escape response. This large muscle potential is the first in a series of oscillatory electrical waves that comprise one to four relatively distinct bursts of electromyographic activity, each lasting 30-60 ms. By simultaneously comparing (a) unilateral intracellular microelectrode recordings from axial muscle of a partially restrained, fully intact larval medaka with (b) simultaneous whole-body grid recordings and videotape recordings of tail movements, we have confirmed that each burst derives from muscle activity in alternate sides of the body as the fish swims away from an applied stimulus (Featherstone, unpublished).

MEASUREMENTS

Because non-invasive recordings of escape episodes consist of a series of stereotyped electrical events, several parameters, each

![Diagram of non-invasive recording of electrical events during an escape episode. Stimulus delivery (stim) results in an escape sequence consisting of two bursts. The first burst always begins with three stereotyped peaks (inset box below). Labelled peaks represent: (1) Mauthner axon spike, (2) motoneuron compound action potential, and (3) axial muscle potential. Other labels: latency = time from stimulus to Mauthner spike; delay = time from Mauthner spike to motoneuron spike.](image)
obtained from the same fish before and after treatment, or during different developmental stages, can be measured. All measurements of time intervals were made to the nearest 0.01 ms using the digital readout of oscilloscope cursor positions that were referenced to either spike peaks or onset of stimulus delivery (Fig. 2), the latter corresponding to the triggering of the oscilloscope sweep.

Mauthner Axon Conduction Velocity

Medaka, like most fish, have a pair of bilateral Mauthner cells whose cell bodies lie within the hindbrain and whose relatively large axons decussate and project down the entire length of the spinal cord. When one of these Mauthner cells is sufficiently depolarized, via one or several of the sensory modalities that have inputs to the Mauthner cell, a single action potential is generated. This action potential propagates tailward along the axon by saltatory conduction (Funch and Faber 1982, Funch et al. 1984, Fetcho and Faber 1988). As it propagates, it generates electrical currents which are detectable outside the body, due to the Mauthner axon's large size and relatively low electrical resistance of the fish's body wall (Featherstone et al. 1991).

Dual recordings of Mauthner axon spikes were simultaneously obtained from two sites along the tail; conduction distance was either 1 or 2 mm, depending on body length. Measurements of the conduction time (peak-to-peak) were then used to calculate the conduction velocity of the Mauthner axon spike as it propagated within the tail. This conduction velocity varied with age, increasing from about 7 m/sec at hatching to more than 25 m/sec in 2-month-old fry (Fig. 3). Such increases in velocity apparently reflect increases in Mauthner axon diameter during development (Funch et al. 1981).

Although we have not yet used this developmental measure for toxicity studies, it might prove useful for studying long-term exposure effects on age-dependent changes in conduction velocity. It should be noted, however, that velocity measurements were more readily obtained by temporarily restraining fish in agarose. Despite the additional stress from this procedure, the majority of untreated fish survived and were readily re-tested.

Electromyographic Burst Number and Duration

As mentioned previously, an escape episode contains a series of one to four bursts of electromyographic activity. This "swim" phase of escape behavior is probably initiated in parallel to the Mauthner system, possibly by other reticulospinal neurons (see Nissanov and Eaton 1989).

Although the number (usually 1-4) and duration (approximately 30-60 ms) of the bursts may be too variable to be useful quantitative measures, they may reflect important qualitative effects related to toxicant action on central pattern generation and motor control. Phenol, for example, causes an apparent decrease in tail beat frequency as the fish swims, and strychnine sometimes causes convulsions.
FIG. 3.—Relationship of Mauthner axon conduction velocity to age of medaka (posthatch). Each point represents an average of 4-6 measurements from a different fish.

Delay Time from Mauthner Axon Spike to Motoneuron Spike

Throughout the length of the spinal cord, the Mauthner axons monosynaptically excite large primary motoneurons that innervate fast (white) muscle fibers involved in the initial, fast body bend of escape behavior (Fetcho and Faber 1988). Because primary motoneurons are relatively large, become activated nearly simultaneously, and have processes that extend into the periphery (Westerfield et al. 1986), their activity can be detected as a single compound spike following the Mauthner axon spike in non-invasive recordings (designated peak "2" in the inset box; Fig. 2).

Assuming that the Mauthner-to-motoneuron synapse is chemical (Fetcho and Faber 1988), it appears that the delay time (peak-to-peak) between the Mauthner axon spike and compound motoneuron spike (mean delay in untreated fish = 0.55 ms; Featherstone et al. 1991) is mainly consumed by the expected synaptic delay for chemical transmission.
Changes in Mauthner axon potential-to-motoneuron delay were readily induced by exposure to both phenol, which lengthened the delay, or strychnine, which slightly shortened it (Fig. 4). The former effect would be expected if phenol depressed the efficacy of Mauthner-to-motoneuron synaptic transmission. The latter effect could be explained if one assumes that normally there was a slight (and tonic) inhibitory bias on spinal motoneurons and that this inhibition was blocked by strychnine treatment, thus enhancing the efficacy of Mauthner-to-motoneuron synaptic transmission.

**Stimulus-Mauthner Spike Latency**

Mauthner-mediated escape can be initiated via several sensory modalities. In this study, speaker-driven water displacement probably
resulted in activation of the polysynaptic acoustico-lateralis sensory system. Consequently, after a relatively short latency (Fig. 2), a Mauthner cell was brought to threshold due to one or perhaps many simultaneous inputs from the VII, VIII, and X cranial nerves (Blaxter and Batty 1985).

Delivery of a standardized stimulus, as in this study, allowed measurement of an onset latency for the Mauthner axon spike. As shown in Fig. 5, the average time from onset of the gating pulse (which triggers a single sweep of the oscilloscope) to initiation of the Mauthner axon spike in untreated fish was $14.96 \pm 3.34$ ms S.E.M. ($N = 516$ measurements, 40 fish). However, to ascertain whether there was a delay from triggering to actual displacement of the recording chamber, a needle from a ceramic phonograph cartridge was used as a sensitive detector of initial movement. Output from the cartridge showed that, due to probable effects of electro-mechanical delay and inertia, the earliest detectable movement of the recording chamber (which was probably detected by the fish) occurred about 6.5 ms after the onset of the gating pulse. Thus, the corrected onset latency of the response was

![Frequency histogram showing distribution of latency measurements (time from stimulus prepulse to onset of Mauthner spike).](image)

**FIG. 5.**--Frequency histogram showing distribution of latency measurements (time from stimulus prepulse to onset of Mauthner spike).
about 8 ms, a value consistent with that measured in other studies (Prugh et al. 1982, Eaton et al. 1981). Because the exact time in the stimulus cycle that the fish detected movement is not known, we have chosen to express toxicant-induced changes in latency as deviation (in ms) from pre-treatment mean.

As expected by their presumed modes of action and previously mentioned effects on stimulus-to-Mauthner spike latency (Fig. 4), both phenol and strychnine changed reflex latency significantly, and in opposite ways. Phenol lengthened this delay, while strychnine decreased it (Fig. 6).

Response Success Rate

Measurement of the percent of tests in which Mauthner responses are successfully evoked provided an additional measure of escape reflex sensitivity. In untreated fish, 70-80% of stimuli successfully evoked electrophysiologically identifiable Mauthner responses; exact percentages varied depending on the individual fish. Individual response failures, in treated or control fish, may reflect mechanical

![Graph showing treatment-induced changes in response latency following four-hour exposure to phenol or strychnine. Each bar represents the mean difference between post-hatch and pre-hatch values from each of ten fish. (Error bars = ± S.E.M.; * p ≤ 0.05).]
Figure 7 shows two examples of toxicant-induced changes in the percent of response failures. Phenol increased the rate of failure, while strychnine (20 ppm) had little effect on it. These results are consistent with known mode(s) of phenol action. Phenol, as a polar narcotic (Bradbury et al. 1991), would be expected to non-specifically depress nervous system function (Veith and Broderius 1990). Additional studies are needed to determine if treatment with other toxicants produces reliable increases or decreases in reflex latency. If so, then this measure may ultimately serve as a means of quantifying commonly observed behavioral effects, such as "lethargy" or "hyperexcitability."
CONCLUSION

Predation appears to be the main cause of mortality in larval and juvenile fish (Batty 1989). Given that Mauthner-initiated escape is an important means of predator avoidance (Taylor and McPhail 1985, Eaton and DiDomenico 1986), any toxicant-induced changes in reflex latency (e.g., Fig. 6), or response success rate (e.g., Fig. 7), would likely have important implications for predator avoidance and predator-prey interactions in natural environments.

The electrophysiological parameters discussed in this paper offer a set of measures for assessing (in intact, freely behaving fish) the effects of toxicants on escape-related neural performance after environmentally realistic exposures to toxicants. By further testing we hope to show that different toxicants can be characterized and classified with respect to the specific combinations of sublethal neurophysiological effects which they produce.

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