J. exp. Biol. **158**, 583–589 (1991)
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SHORT COMMUNICATION

NONINVASIVE DETECTION OF ELECTRICAL EVENTS DURING THE STARTLE RESPONSE IN LARVAL MEDAKA

BY DAVE FEATHERSTONE*, CHARLES D. DREWES
Department of Zoology and Genetics, Iowa State University, Ames, IA 50011, USA
AND JOEL R. COATS
Department of Entomology, Iowa State University, Ames, IA 50011, USA

Accepted 1 February 1991

In teleosts, a startle response occurs when one of a pair of Mauthner cells (M-cells) originating in the fish hindbrain is excited by sensory afferents and fires a single action potential. This action potential propagates caudally along the M-axon, which crosses the midline and continues tailward within the spinal cord. In passage, the M-spike excites (*via* collaterals) primary motor neurons within the spinal cord which, in turn, activate nearby axial musculature. The most obvious behavioral result of this highly conserved (at least in teleosts) and stereotypic sequence is a fast body bend toward the side of the active M-axon such that the fish forms the shape of the letter C. This is stage 1 of the teleost 'C-start', also previously called the M-reflex (for reviews, see Nissanov and Eaton, 1989; Eaton and DiDomenico, 1986; Eaton and Nissanov, 1985; Eaton and Hackett, 1984; Eaton and Bombardieri, 1978; Diamond, 1971).

Owing to the M-axon's large caliber and the relatively low electrical resistance of the body wall of larval fish, some of the electrical events associated with the M-reflex can be measured transcutaneously by using noninvasive recording techniques. Such recordings were originally made by Prugh *et al.* (1983) using larval zebrafish and a recording electrode grid consisting of parallel copper wires etched onto a printed circuit board. Following stimulation by movement of a sharpened capillary tube positioned against the body surface, they recorded a short-latency (7.8±4 ms) spike-like potential from the body surface. Since the potential appeared to propagate from head to tail, was correlated with an M-cell spike, as measured with tungsten microelectrodes placed within the hindbrain, and invariably preceded a startle response, they concluded that this large extracellular potential was the M-spike.

However, as pointed out by Eaton and DiDomenico (1986), an alternative explanation of these results is that the large potential (recorded either inside or

* To whom all correspondence should be addressed.

Key words: medaka, Mauthner, startle reflex, noninvasive technique, *Oryzias latipes*.

outside the body) may actually be the muscle electrical response associated with the initiation of the C-start, and the M-spike, occurring earlier, may have been undetected. Here, noninvasive recordings from larval medaka (*Oryzias latipes*) were used in combination with intracellular recordings to demonstrate that the large potential does indeed originate in the axial musculature. In addition, high-sensitivity noninvasive recordings were employed to characterize two small spike-like potentials that precede the muscle potential. We propose that these two small potentials represent an M-axon unitary action potential and the corresponding motor neuron compound potential.

Recordings were made by transferring 6–10 mm long Japanese medaka (1 month old, post hatch), *via* an eyedropper, to an electrode grid consisting of parallel, tinned-copper lines (0.5 mm apart) etched onto a printed circuit board. Excess water surrounding the fish was then removed and immediately replaced with a 1 % SeaPrep agarose solution. Next, the position of the fish was carefully changed so that its dorsal surface was closest to the electrodes and its longitudinal axis was perpendicular to them. Finally, the recording grid, with agarose and fish, was cooled at 10 °C for 3–4 min to gel the agarose and immobilize the fish. Preparations in which the fish did not maintain this position relative to the electrodes were never used.

The agarose remained a gel throughout testing at various temperatures (8–23 °C). After testing (which required up to 2 h per fish), fish could be freed from the agarose; more than 95 % survived and showed no obvious after-effects.

For recordings, a tactile stimulus was delivered with a hand-held glass rod (tip diameter 15–30 μm), and the resulting electrical events were picked up differentially *via* the grid electrodes, preamplified in two stages (by a factor of 100 or 1000 at each stage), filtered, and led into a Tektronix 2221 digital storage oscilloscope with parallel connections to an audio monitor and plotter. In the absence of evoked activity, noise levels ranged from 3 to 5 μV.

As in zebrafish, a tactile stimulus evoked a sudden C-start-like movement accompanied by a large (about 1 mV), stereotyped, biphasic potential comparable to that observed by Prugh *et al.* (1983). Furthermore, as in zebrafish, the waveform of this potential varied systematically in relation to the recording site along the body and seemed to propagate from head to tail (Fig. 1A).

However, using the same recording apparatus, but with greater preamplification, we discovered that this large potential is invariably preceded by two much smaller electrical events (Fig. 1B). Measurements from 115 recordings in the mid-tail region of 10 different fish at 22 °C revealed the following characteristics. The first spike was smallest (6±2 μV, S.D.) and monophasic, always appearing as a smooth, unitary spike. Based on simultaneous multichannel measurements from two regions of the tail, the apparent conduction velocity of this spike (as well as the whole sequence of potentials) was 16.4±5.8 m s^{-1}. This conduction velocity varied greatly with age, increasing from about 7 m s^{-1} at hatching to more than 25 m s^{-1} in 2-month-old fry. The second spike was slightly larger (15±4 μV) and also monophasic, but its waveform was variable, sometimes with an obvious inflection

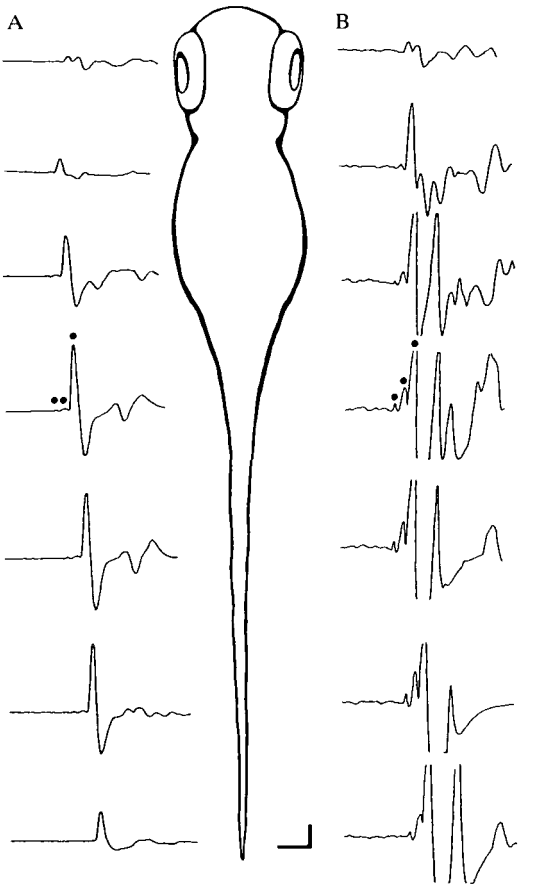

Fig. 1. Maps of low- (A) and high- (B) sensitivity noninvasive recordings from a 1-month-old medaka larva stimulated by touch. The waveforms, obtained as a series of separate responses, are arranged with baselines corresponding approximately to the positions of the recording electrodes along the fish (center) and with latencies that reflect the relative timing of potentials along the fish. The three dots in each of the center recordings indicate the three successive peaks discussed in the text. Time scale: 2 ms (A,B). Voltage scale: 0.2 mV (A); 0.02 mV (B).

The time lag from the peak of the first spike to the peak of the second potential was 0.55±0.05 ms. Finally, the peak of the second small potential was followed after 0.58±0.06 ms (measured from peak to peak) by a large biphasic potential (1.0±0.10 mV).

To test the possibility that this large potential might originate from the muscle, we obtained simultaneous grid and intracellular recordings from axial muscle cells in undissected fish (Fig. 2). For these recordings, larval medaka were immobilized on a recording grid as before but, in addition, an aluminum silicate glass microelectrode (resistance 15–30 MΩ) was driven through the body wall and into the musculature at the location of the active grid electrodes. Following cell

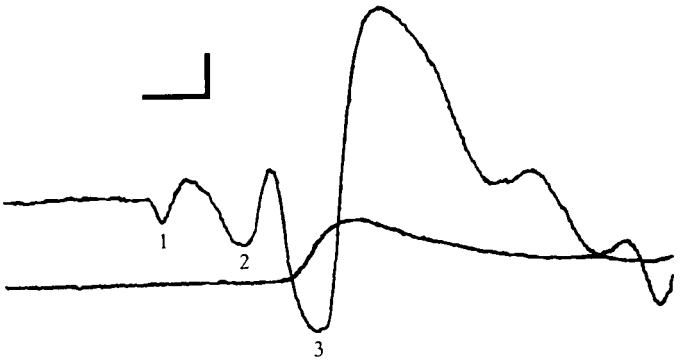

Fig. 2. Simultaneous noninvasive extracellular recording (upper trace) and intracellular recording (lower trace) from midtail axial musculature (12°C). Note that the muscle EPSP coincides with the largest wave of the extracellular recording. The three main peaks in the extracellular waveform are labelled 1–3. Time scale: 1 ms. Voltage scale: 10 μV (upper); 10 mV (lower).

penetration (as indicated by a sudden, negative shift in the baseline), a tactile stimulation was applied to the fish as before. In all cases (six successful penetrations in four fish), the onset of the intracellularly recorded muscle potential (EPSP) was synchronous with the onset of the large third potential. Furthermore, if intracellular recordings were lost due to movement, focal extracellular recordings were obtained consisting of small negative potentials synchronous with the large biphasic potential in the grid recording.

Since it is well established that an M-spike results in motor neuron activity that leads to large contractions of axial musculature, the first of the two smaller potentials preceding the muscle activity might represent the extracellular correlate of the M-spike as it propagates caudally within the spinal cord, while the second potential might represent, at least in part, the concomitant motor neuron spike potentials. Although correlated grid and intracellular recordings of either of these events have not been obtained, several lines of evidence indirectly support this hypothesis.

First, in medaka, the time interval from the putative M-cell spike to the peak of the muscle electrical response (both recorded at the same level of the spinal cord) was 1.1±0.08 ms (N=115). This compares favorably with that seen in other species. For example, Zottoli (1977) recorded, *via* chronically implanted electrodes, the electrical activity of the axial musculature and an M-cell in unrestrained goldfish during the C-start and found that the M-spike preceded the EMG by 1.1–2.1 ms. Similarly, Eaton *et al.* (1981) found that the M-spike occurred a little over 2 ms before the EMG. Note that these values include M-axon conduction time, which would be approximately 0.7 ms, assuming a conduction distance of 6 cm and velocity of 82 m s^{-1} (Funch and Faber, 1982).

Also, the second small potential preceded the muscle potential by a time consistent with that expected for motor neuron to muscle excitation (Katz and Miledi, 1965) and followed the putative M-spike by the expected synaptic dela

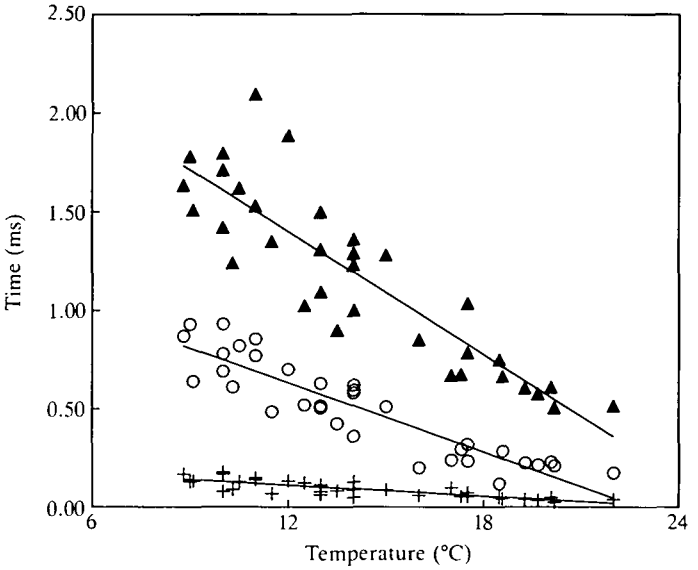

Fig. 3. Graph of temperature effects of M-spike to motor neuron transmission time (▲), motor neuron to EMG transmission time (○) and M-spike conduction time (+).

between M-axon and primary motor neurons (Diamond, 1971; Fetcho and Faber, 1988).

Finally, if the three peaks shown in Figs 1B and 2 represent independent, yet synaptically coupled, events, they would be expected to respond to an environmental variable such as cooling in different, but predictable, ways. When fish were gradually cooled by placing the underside of the recording grid in contact with a cold block of aluminum, a gradual temporal separation of the three peaks was observed, as well as a decrease in velocity of the putative M-spike. After plotting each of these times against temperature and determining the slopes, it was evident that each measurement was affected differently by temperature (Fig. 3). For example, M-spike conduction time (over 1 mm) decreased with a slope of $-0.009\,\text{ms degree}^{-1}$, whereas the transmission times from M-spike to motor neuron spike and motor neuron spike to EMG onset were affected much more, with slopes of $-0.104\,\text{ms degree}^{-1}$ and $-0.059\,\text{ms degree}^{-1}$, respectively. Such differences are consistent with temperature effects seen in other poikilothermic animals (Katz and Miledi, 1965; Bradfisch *et al.* 1982).

Although the hypothesis that the two small potentials derive from M-axon activity and primary motor neurons is consistent with known physiology, the possibility that other neural events may contribute to the observed potentials cannot be excluded. For example, in an extensive study of the spinal circuitry of the M-reflex in goldfish, Fetcho and Faber (1988) described two other groups of cells in the spinal cord besides primary motor neurons that were activated by the M-cell. One of these cell groups, the descending interneurons, was activated parallel to, and with about the same latency as, primary motor neurons.

Therefore, despite an expected large field potential resulting from synchronous excitation of large motor neurons, contributions to the second small potential from other neuronal populations cannot be excluded.

Fetcho and Faber (1988) also described a group of 'crossing interneurons' that were electrotonically excited by the M-axon and thus activated only 0.11 ± 0.02 ms after the M-spike. Although our extracellular recordings showed no potential with comparable timing characteristics, the possibility exists that field effects from this cell population also contributed to the observed field potentials. In addition, other reticulospinal neurons may be activated in parallel with the M-cell during the startle response, but the timing, speed and consistently unitary waveform of the first peak argue that the M-axon current is probably the sole source of this potential.

In conclusion, three sequential, but otherwise distinct, electrical events associated with a startle response can be detected in larval fish using noninvasive recording techniques. Evidence is provided that the third and largest potential in this sequence derives from the axial musculature, and it is hypothesized that the first and second smaller potentials preceding it derive from the M-axon and, at least in part, from primary motor neurons. The capability for measuring the timing of these events may be useful in future studies of the effects of environmental factors (such as pesticides and neurotoxins) on startle reflex function in intact, freely behaving fish.

We thank Gardner Cooley for expert fish care. This research was funded by the US Environmental Protection Agency, through a Cooperative Agreement with the US EPA Environmental Research Laboratory-Duluth, Duluth, MN (CR-816228). This article is Journal Paper no. J-14205 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa; Project no. 2306.

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