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Decrease in occurrence of fast startle responses after selective Mauthner cell ablation in goldfish (*Carassius auratus*)

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Abstract A single action potential in one of a pair of reticulospinal neurons, the Mauthner cells, precedes a short-latency electromyographic response of the trunk and tail musculature on the opposite side of the body and a fast startle response in goldfish. It has been postulated that not only the Mauthner cell, but also an array of neurons can trigger or participate in fast startle responses (Eaton et al. 1991). We have selectively ablated the Mauthner cells in goldfish to study how neurons of the brainstem fast startle response network interact. The probability of eliciting a fast startle response was significantly less in fish with double Mauthner cell ablations, as compared to the responsiveness of control fish. The finding that there is a significant decrease in the occurrence of fast startle responses in animals with no Mauthner cells, implies that the Mauthner cell may play a role in triggering the involvement of the other network elements in fast startle responses. We hypothesize that Mauthner cell activation may be important in bringing those reticulospinal neurons that are “primed” by the behavioral context to threshold and provides the basis for studies focused on the interactive nature of the brainstem startle response network.

Key words Reticulospinal neurons · Teleost fish · Kinematics · C-starts · Neuroethology

Abbreviations *C-Start* fast startle response · *EMG* electromyographic · *M-cell* Mauthner cell

Introduction

Many fish display fast startle responses to an abrupt vibratory stimulus (Eaton et al. 1977; Webb 1978; Blaxter et al. 1981; Currie 1991; Domenici and Blake 1991; Meyers et al. 1998). Cyprinid fish have a characteristic fast startle response (*C-start*) in which the animal's body initially forms a C shape (Eaton et al. 1981), and the so-called *C-start* is considered to be important for escape from predation (Bartelmez 1915; Diamond 1971; Eaton et al. 1977; Webb 1978; Eaton and Hackett 1984) as well as other behaviors (Canfield and Rose 1993; Zottoli et al. 1995b).

The neuronal basis of *C-starts* is only partially understood. An action potential in one of a pair of reticulospinal neurons, the Mauthner cells (*M-cells*), is thought to initiate *C-starts* in goldfish (Nissanov and Eaton 1989). *M-cell* activity precedes a short-latency *EMG* response of the trunk and tail on the opposite side of the body (Zottoli 1977) and a *C-start* (Eaton et al. 1981, 1982, 1988) in goldfish. A *M-cell* action potential in a restrained animal results in an early electromyographic (*EMG*) response 1.5–2 ms later followed by activation of contralateral musculature (Hackett and Greenfield 1986). The latency from *M-spike* to *EMG* response in restrained animals is the same as that recorded after selective excitation of a *M-axon* in freely swimming fish (Nissanov et al. 1990). Although the activity of the *M-cell* is correlated with *C-start* behavior and the initial *EMG* response is time-locked to this cell's activity, other reticulospinal neurons are thought to play a role in the execution of the response. Selective stimulation of *M-axons* in freely swimming fish produces a movement which is significantly weaker and less variable than responses evoked by sensory stimuli (Nissanov et al. 1990). To account for the variability in *C-start* trajectories after sensory stimulation, Eaton and his colleagues have proposed a *parallel network model*. The *M-cell* is the first to fire, determining the initial direction of movement, and other neurons (non-*M-cells*) control

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the strength of and interval between muscle contractions on both sides of the fish which results in a remarkable variety of escape trajectories (Eaton et al. 1991; Foreman and Eaton 1993). The non-M-cells are capable of initiating C-starts in the absence of M-cells (Eaton et al. 1982, 1988), and these responses have a longer latency on average but are otherwise indistinguishable from Mauthner-initiated responses (Eaton et al. 1982, 1988, 1991).

Given that M-cell and non-M-cell circuits do exist and that each is capable of eliciting a C-start to a vibratory stimulus, what is the nature of interactions between them? To address this question, we have selectively ablated the M-cells of goldfish. Our result shows that in animals with no M-cells there is a significant decrease in the occurrence of C-starts when compared to control animals. Such results imply that the M-cell may play a role in triggering non-M-cells to participate in C-start responses to certain vibratory stimuli.

A preliminary report of this work was presented previously (Zottoli et al. 1995a).

Materials and methods

Thirty-two common goldfish (*Carassius auratus*; purchased from Hunting Creek Fisheries) of 11.2 ± 1.0 cm (mean \pm SD) body length were housed individually in 23 cm \times 17 cm \times 14 cm deep tanks. The fish were allowed to acclimate for a minimum of 2 weeks before use. Fish were kept in 4 l of conditioned (Novaqua; Kordon), aerated water held between 21 and 24 °C and presented with an alternating 12-h light-dark cycle. They were fed Hikari Staple food (Kamihata) three times a week followed by replacement of their water with fresh, conditioned tap water.

Behavioral observations

Our behavioral testing system is similar to that described by Wieland and Eaton (1983) and Eaton et al. (1988) except for the following modifications: 1) a circular arena was used instead of a square one to prevent fish from settling into corners, 2) the water depth was decreased by 5.1 cm to restrict vertical movement of the fish, and 3) the stimulus was more intense (i.e., 600 μ m vertical tank movement compared to 3–6 μ m used by Eaton et al. 1988) to insure the delivery of a suprathreshold stimulus. We describe some of the general features of the test tank, the stimulus, and imaging system below.

Fish were placed in a circular arena, 20.3 cm in diameter and 10 cm deep to restrict movement. The arena was centered in a tank with opaque sides and a clear bottom, 43.5 cm square and 23.5 cm in depth. The tank and arena were filled to a depth of 7.7 cm with conditioned tap water. The water in the test tank was equilibrated to the temperature at which the animals were held. The central arena was aerated and fish were allowed to acclimate for at least 10 min before testing.

Fish were allowed to orient randomly in a test tank prior to stimulation with an abrupt vibratory stimulus. The vibratory stimulus was created by lifting the test tank with a solenoid and was delivered when the fish was stationary with its body oriented radially in the circular arena. The solenoid was separated by 0.69 mm (a feeler gauge was used) and was triggered by computer with 1.5 waves of the 60-Hz line voltage. The fish tank was lifted approximately 600 μ m.

Two cameras were located below the test tank to record fish movement within the arena. Fish were videotaped with a conven-

tional video camera/VCR system. In addition, silhouettes were captured by a customized matrix camera, consisting of a 10 000-pixel array of photodiodes (EG & G Reticon Camera/Controller MC521/RS521; EG & G Reticon). The matrix camera and solenoid-activated vibratory stimulus were triggered at the same time, and silhouettes were stored on computer memory every 2 ms for a total of 104 ms (i.e., 52 images; Wieland and Eaton 1983; Eaton et al. 1988). A schematic of the test chamber, stimulation arrangement and imaging equipment is shown in Fig. 1.

Fish were screened during preoperative testing to meet the following three criteria: 1) the fish had to respond to the stimulus with C-starts in at least five of the six trials, 2) at least one C-start had to be to the left and one to the right and 3) the silhouette had to be compatible with the thinning algorithm (i.e., some fish had silhouettes that included too much of the tail, see below). Three of the 32 fish were excluded as a result of this screening procedure.

The C-start has been divided into two stages (Blaxter et al. 1981; Eaton et al. 1981). During stage 1 there is a major contraction of the musculature on one side of the body resulting in a characteristic C-like shape. Stage 2 is characterized by forward propulsion that may be associated with a turn. To analyze C-start responses, 52 matrix camera silhouettes (Fig. 2A) were each reduced to a midline one pixel thick, using a thinning algorithm (Fig. 2B). The rostral 40% of each midline was then converted to a regression line (Fig. 2C; Nissanov 1991). The computer used the regression lines, representing the head and rostral trunk, to calculate stage 1 and stage 2 kinematic parameters.

Stage 1 parameters are:

- 1) *Stage 1 latency*. This is the latency from the onset of the vibratory stimulus to the beginning of the response. Regression lines were used to determine whether significant movement had occurred based on the criteria discussed below. The latency from activation of the solenoid to sound pressure onset was 4.4 ms as determined with a hydrophone. In addition, the matrix camera started filming 0–2 ms after camera activation. The maximum latency, as determined by the computer, was therefore adjusted by subtracting 2.4 ms (i.e., computer determined latency -4.4 ms $+ 2$ ms).
- 2) *Stage 1 angle*. This is the angle formed between the regression line at the stage 1 latency and the regression line at the stage 2 latency.

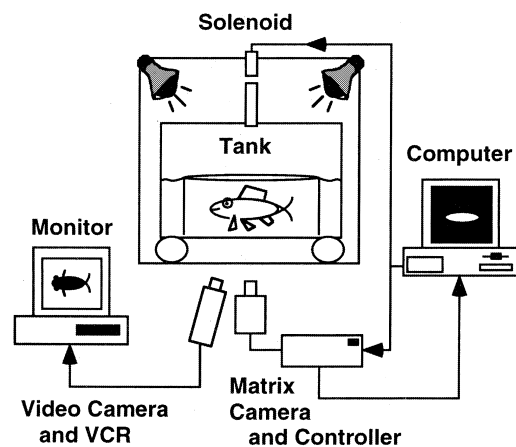


Fig. 1 Schematic diagram of the behavioral testing apparatus used to elicit and analyze goldfish fast startle responses (C-starts). Light is projected from above and fish images are captured from below the tank by a video camera and a matrix camera. Goldfish are stimulated with a vibratory stimulus created by lifting the whole tank with a solenoid. The computer simultaneously triggers the solenoid and the matrix camera which starts saving silhouettes at a rate of 500 s^{-1} (i.e., every 2 ms). These fish silhouettes are stored in the expanded memory of a controller and then loaded onto the computer hard drive for analysis

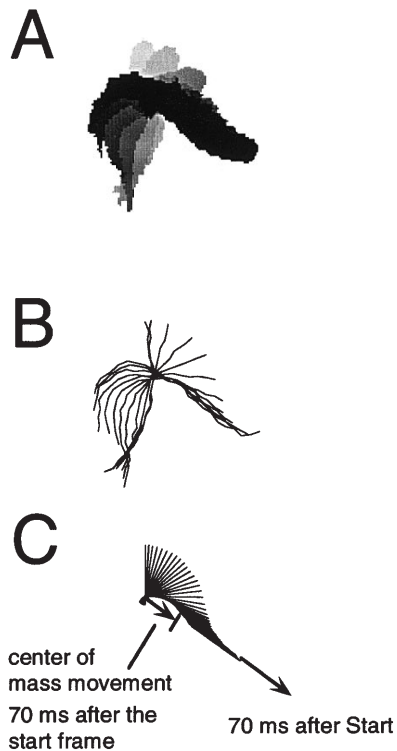


Fig. 2A–C Method of analysis of the goldfish fast startle responses. **A** Superimposition of silhouettes of the C-start of the goldfish. Silhouettes were taken every 2 ms but, for clarity, only those occurring at 14-ms intervals after the beginning of the response are shown. The lightest image is the silhouette just before the beginning of the response and it has been arbitrarily oriented with the upper body axis of the fish in the vertical plane and the nose upward. The darkest image is the fish silhouette at about 104 ms after the stimulus delivery. The initial orientation of silhouettes are the same for all figures that follow. **B** Superimposition of midlines determined from silhouette images. The silhouettes were reduced to a midline a single pixel thick using a thinning algorithm. For clarity every fourth midline is shown (i.e., every 8 ms). **C** The rostral portion of each midline, corresponding to the length of the head and rostral trunk which underwent minimum curvature during a startle response, was then converted to a regression line (Nissanov 1991). This length of minimum curvature corresponded to the rostral 40% of the midline for goldfish. Regression lines are shown in 2-ms increments and were used to determine latencies, angles and center of mass measurements (Nissanov 1991). The straight-line center of mass distance traveled during 70 ms after the start (i.e., the first regression line) is shown

Stage 2 parameters are:

- 1) *Stage 2 latency*. This latency is measured from the stage 1 latency to the time when the center of mass has become displaced 0.75 cm from its position at the start (Eaton et al. 1988).
- 2) *Escape trajectory angle*. The angle formed between the regression line at the stage 1 latency and the regression line 70 ms later (Fig. 2C; Eaton and Emberley 1991).
- 3) *Center of mass movement*. This movement is the straight-line distance that the center of mass travels during 70 ms after the stage 1 latency (Fig. 2C).
- 4) *Linear velocity of the center of mass movement* (Nissanov 1991).

Physiological localization and ablation of M-cell somata

We found that general anesthetic hindered the localization of the M-cells in goldfish. Therefore topical anesthetic and cooling of the

fish were used in these experiments. Fish were secured in the operation chamber with rods whose tips were coated with topical anesthetic (20% benzocaine in a water-soluble glycol base; ULTRA-CARE Ultradent Products), and chilled water was recirculated through the mouth and over the gills (the chilled water reduces the gill temperature of the fish from about 22 °C to 8 °C). The skin over the skull was then coated with topical anesthetic. After 10 min, a hole was drilled in the skull to expose the area from the rostral portion of the corpus cerebellum to the caudal edge of the vagal lobes. Overlying muscle, cartilage and fat were removed and care was taken not to damage the anterior and posterior semicircular canals. The choroid plexus just caudal to the cerebellum was removed and the cerebellum was gently pushed forward and held in place with a small piece of Kimwipe (Kimberly-Clark), thereby exposing the surface of the medulla oblongata. A reference electrode was placed in the muscle just behind the skull. Topical anesthetic was reapplied to the area around the skull hole about every 15–20 min.

Topical anesthetic was first applied to the scales and skin at the caudal edge of the dorsal fin just above the lateral line scales before placing stimulation electrodes on the vertebral column. After about 5 min scales were removed, a small hole was made in the skin with forceps, and bipolar stimulation electrodes were inserted through the hole and through muscle until the vertebral column prevented further movement. The voltage was increased in steps while adjusting the position of the stimulation electrodes on the vertebral column until head, trunk and tail movements were evoked reliably, using a stimulus rate of 1/1.3 s. Once the optimal location (i.e., the spot that evoked a head and body response at the lowest voltage) was found, the stimulus rate was increased to 3 s⁻¹. The higher rate of stimulation caused a reduction of movement in the trunk and tail and loss of movement in the head, presumably due to synaptic depression (Diamond 1971).

M-cell somata were located with a microelectrode by stimulating their axons in the spinal cord. The antidromic action potential generates a short-latency, extracellular field potential that can be up to 40 mV in amplitude within the axon cap (a specialized structure surrounding the initial-segment axon hillock region of the M-cells; Furshpan and Furukawa 1962). This electrophysiological “signature” provides a point of reference from which any part of the soma, the two major dendrites and axon can be located.

A microelectrode (filled with 3 mol · l⁻¹ KCl; 3–7 MΩ) initially penetrated the surface of the medulla oblongata about 400 μm to one side of the midline and at the rostrocaudal level where the corpus cerebellum joins the medulla (Fig. 3). Electrode tracks about 1.5 mm in depth were used to search for the antidromically evoked field potential in the M-cell’s axon cap (Furshpan and Furukawa 1962).

The criteria for localizing that region was set as extracellular field potential of around 15 mV or more (Fig. 4A; this potential was not maximized to prevent any damage to the initial segment; Furshpan and Furukawa 1962). Once this site was localized, the electrode was removed and reinserted into the brain 50 μm laterally. The M-cell soma was identified by its depth (1.5 mm), and its short-latency extracellular field potential that was smaller than that in the axon cap (Fig. 4B; less than 5 mV compared to 15 mV at the cap). Identification of the M-cell was confirmed after intracellular penetration by the occurrence of both postsynaptic potentials elicited by auditory stimulation (i.e., clapping) and the short-latency action potential evoked by antidromic stimulation (Fig. 4C1, C2). The diameter of the soma was determined by the excursion of the microelectrode without the loss of resting potential (70–100 μm), the microelectrode was then moved to the center of the soma, and the manipulator was then tapped so that the electrode mechanically disrupted the membrane of the cell. Once the resting potential was less than 30 mV with a concomitant decrease in action potential amplitude (Fig. 4D) and remained low for at least 4 min, the cell was considered ablated. During this interval, the electrode was occasionally lowered through the cell so that the soma was “skewered” and the manipulator was again tapped. Subsequently, the electrode was moved to the other side of the medulla and the other M-cell was located and ablated in the same way.

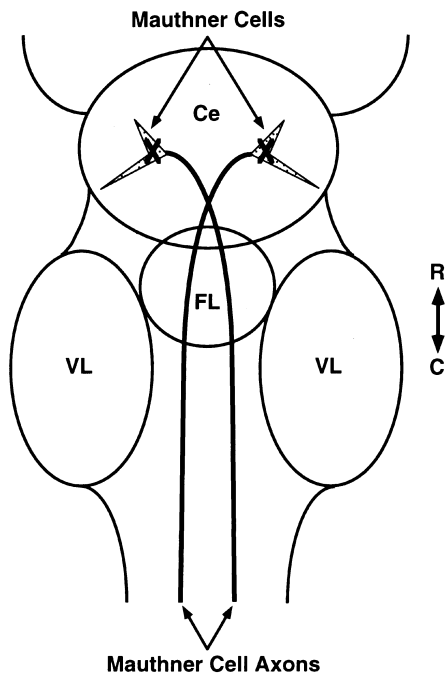


Fig. 3 Brain level at which selective ablations of Mauthner cells (M-cells) were attempted. The M-cells are superimposed on a dorsal view of the goldfish brain. The cell somata lie below the cerebellum within the medulla oblongata. Selective ablations of M-cell somata are designated by an X. *Ce* cerebellum; *FL* facial lobe; *OL* optic lobe; *VL* vagal lobe; *C* caudal; *R* rostral

Control fish had their brains exposed as described above. The axon caps of both M-cells were located with a microelectrode in the same way as experimental fish (Fig. 5) but the M-cell soma was not penetrated. One control fish had a M-cell that was chromatolyzed, which may have resulted from inadvertently damaging the M-axon initial segment. This fish was not used in this study. An attempt was made to make the same number of brain microelectrode penetrations in control and experimental fish in order to control for damage that might result. Microelectrode penetrations averaged 6.4 on the right and 4.6 on the left sides of the brain for experimental fish and 5.3 and 4.2 for the corresponding sides of control fish.

After both cells were damaged, the brain was protected from osmotic shock by covering it with a Vaseline-paraffin oil mixture to a level just below the skull. A piece of thin plastic the size of the hole was placed on the mixture. Thirty-gauge stainless steel wire was looped through three small accessory holes and twisted tightly together to act as an anchor for the vinyl polysiloxane impression material (Imprint, 3 M) used to "cap" the skull (Zottoli 1977). After the impression material had solidified, the fish was put back in its home tank. Fish were initially sluggish, and all animals regained equilibrium and "normal" activity levels in about 10 min. In the first half hour of recovery experimental fish displayed spontaneous "C-starts" which were not observed in control animals. All postoperative fish maintained an upright posture and actively ate throughout the study.

Postoperative behavioral testing

Three fish were used to test the reliability of the ablation technique and to provide preliminary short-term behavioral data. The group consisted of two fish with double M-cell ablations that were tested 3 h postoperatively with a set of six trials followed 30 min later by another set of six trials. The interval between individual trials was 2 min. The third fish was tested 10 days postoperatively with a set of six trials followed 24 h later by another set of six trials. The interval between trials was 5 min. A fourth fish had only one M-cell ablated and no postoperative trials were conducted.

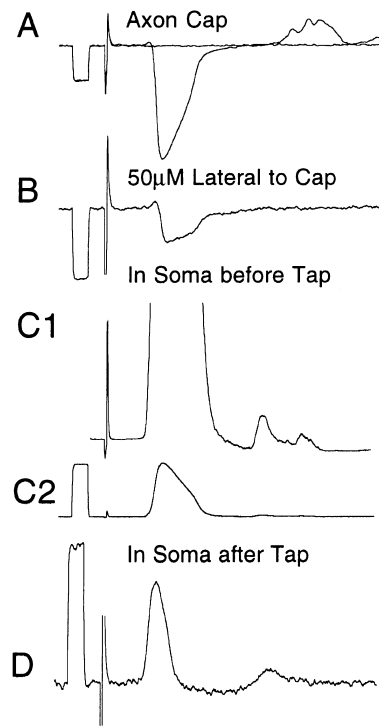


Fig. 4A–D Physiological identification of the M-cell axon cap and mechanical ablation of the soma. **A** The left M-cell axon cap was located in the medulla oblongata by the large negative field potential evoked by antidromic stimulation of the M-axon in the spinal cord. It took three microelectrode penetrations to locate this site. The M-cell was stimulated below and above threshold to highlight the all-or-none nature of this field potential. Calibration pulse = 5 mV, 1 ms. **B** After physiological localization of the cap, the microelectrode was removed from the brain and reinserted 50 μ m laterally. The M-cell soma was identified by its depth, and smaller extracellular field potential. Calibration = 5 mV, 1 ms. **C** The microelectrode was then inserted into the soma (resting potential = -75 mV) and stimulated by antidromic activation of the M-axons. High (C1) and low (C2) gain records are shown. **C1** Calibration the same as that in **D**; **C2** Calibration = 40 mV, 1 ms. **D** The manipulator was then tapped so that the electrode mechanically disrupted the membrane. When the resting potential was lowered below 30 mV and remained below this level for at least 4 min, the electrode was removed and the cell was considered ablated. Calibration = 2 mV; 1 ms

Ten fish were tested 30 days postoperatively to determine the effect of the loss of M-cells on C-start behavior. This group consisted of five fish with double M-cell ablations and five sham-operated controls. These fish were tested with a set of six trials followed 1 day later by another set of six trials. The interval between individual trials was 5 min.

Thirteen fish were tested at four postoperative intervals (3 h, 1 day, 30 days, 60 days) to determine the effect of the loss of M-cells on C-start behavior at short and long postoperative intervals. This group consisted of seven fish with double M-cell ablations and six sham-operated controls. These fish were tested with a set of six trials followed 30 min later by another set of six trials. The interval between individual trials was 2 min. One additional fish that died 13 days postoperatively was not used in determining the occurrence of C-start responses.

Histological processing

In order to determine the presence or absence of the M-cell soma after the attempted ablation, fish were anesthetized in 0.03% ethyl-

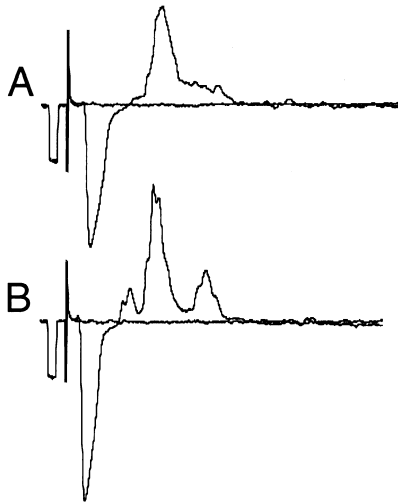


Fig. 5A, B Physiological identification of the M-cell axon caps in control fish. The left (**A**) and right (**B**) M-cell axon caps were located in the medulla oblongata by the large negative field potentials evoked by antidromic stimulation of the M-axons in the spinal cord. The M-cells were stimulated below and above threshold. The characteristic short-latency negative field potential was followed by a positive-going potential (EHP, extrinsic hyperpolarizing potential). It took one microelectrode penetration to locate the left M-cell while it took nine penetrations to locate the right M-cell. After localization of the cap, the microelectrode was removed from the brain. Calibration in **A** and **B** = 5 mV, 1 ms

m-aminobenzoate after the last set of behavioral trials. When respiration had ceased, they were perfused with 100 ml of freshwater tealeost Ringers with 0.015% anesthetic added followed by 100 ml of 10% formalin in phosphate buffer (Fisher). The brains were removed and placed in fresh fixative overnight. The brains were then dehydrated, cleared in methyl salicylate and embedded in paraffin. Transverse sections (15 μ m) were mounted on glass slides and the tissue was stained with cresyl violet acetate.

Statistical analyses

Comparisons of the probability of eliciting a C-start

Observation of videotapes provided a preliminary screening for the occurrence and direction of startle responses. Since fish were stimulated when their bodies were straight and after they had come to rest, the identification of a rapid response coupled to the stimulus was usually clear. Final determination of C-start occurrence was made by computer software (KNOWAL; Nissanov 1991).

Specifically, the regression lines determined from the head and upper body portion of fish silhouettes (see above) were used to determine whether significant movement had occurred based on the following criteria: 1) the angle of the linear regressions between the start silhouette (fish silhouette before the onset of significant movement; each silhouette represents 2 ms) and start + 1 silhouette must be greater than 3 degrees, 2) no directional reversal past the start position occurs within the four silhouettes subsequent to the start silhouette, and 3) if the angle between start and start + 2 silhouettes is less than 10 degrees, then start + 3 cannot be situated between start and start + 2 silhouettes. Computer analysis of some trials was not possible and occurrence of a C-start was determined from the videotapes.

The frequency of eliciting a C-start for control fish was compared to that of experimental fish at 3 h, 1 day, 30 days, and 60 days. Proportions were arc-sine transformed. The data was analyzed using a repeated measures analysis of variance. In addition

the individual control and experimental values at 60 days postoperatively were compared using a *t*-test.

To check for possible effects of habituation resulting from repeated trials, the probabilities of C-start responses were sorted according to condition (experimental versus control) and position of trial (i.e., 1–12) at each postoperative testing interval. There was no significant relationship between probability of response and position of the trial in either control or experimental fish at any postoperative interval. Therefore, habituation appears insignificant in terms of explaining the observed behavioral changes.

Comparisons of C-start kinematic parameters

To determine if parameter values differed over time (i.e., the four postoperative intervals) or by treatment (i.e., control versus experimental) and to determine if there was a possible interaction between the two, a MANOVA was run on mean parameter values determined for each fish at each postoperative interval. The treatment was the only significant factor. Since time had no significant effect on parameter values, we chose to use a reduced data set to minimize the effect of an unequal number of responses between fish. Specifically, for each parameter analyzed, we chose the first response at a given postoperative interval by a given fish and discarded the rest of the values for that fish at that interval. Thus, a maximum of four responses were chosen for each fish (in some cases less than four responses were available since the fish did not respond in one or more of the four postoperative intervals). Means were calculated for each parameter, and the control and experimental means were compared using a MANOVA. Since the MANOVA indicated an effect of treatment, one-factor ANOVAs were run to determine what control parameters differed from the corresponding experimental ones. The *P* values were adjusted with a sequential Bonferroni adjustment to correct for type 1 error. A significance level was set at 0.05.

Results

Morphology of ablated cells

To determine the extent of damage resulting from the selective ablation technique per se, M-cells were studied histologically 3 h and 10 days postoperatively. All seven attempted M-cell ablations (i.e., one single ablation and two double ablation fish at 3 h postoperatively; one double ablation fish at 10 days postoperatively) in these fish were successful based on light microscopic evaluation of cresyl violet-stained sections. For example, in the single ablation fish the area that was occupied by the M-cell contains reactive cells as early as 3 h postoperatively. All that remains to identify the location of this cell is its axon cap, a unique structure that surrounds the axon-hillock initial segment of the M-cell (Zottoli 1978; Fig. 6A). In contrast the left M-cell of the fish with a single ablation appears "normal" despite ten microelectrode penetrations in its vicinity (Fig. 6B).

All of the double ablation attempts, analyzed histologically at the end of the longer-term studies (i.e., 30 and 60 days) were successful. The M-cells of the sham-operated control fish (i.e., fish with M-cells that had been electrophysiologically identified but not intentionally damaged) looked normal (Fig. 7A) except in one case in which one M-cell was inadvertently damaged as judged by its chromatolytic reaction (Zottoli et al. 1984).

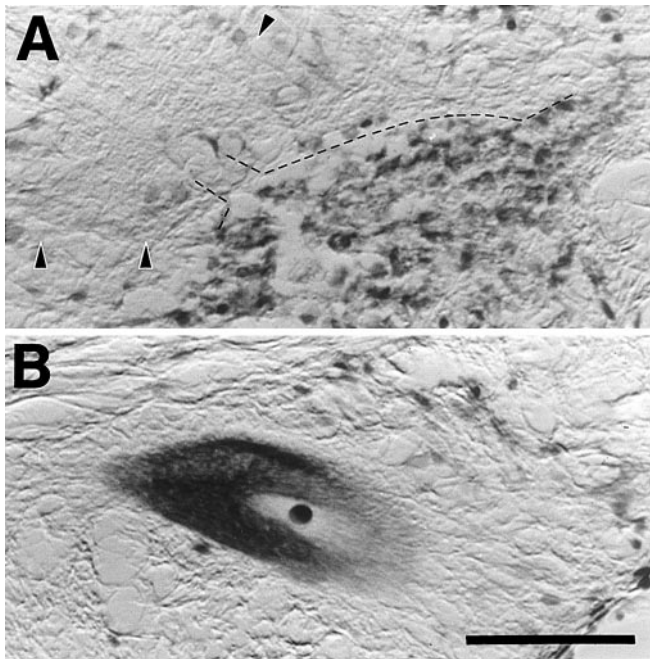


Fig. 6A,B Morphological status of the M-cell soma 3 h after selective ablation. M-cell axon caps were located electrophysiologically in the medulla oblongata by the large negative field potentials evoked by antidromic stimulation of the M-axons in the spinal cord. The M-cells of experimental fish were subsequently ablated with the microelectrode. **A** Three hours after ablation all that remains to identify the former location of the right M-cell is the axon cap (*within arrowheads*) and reactive cells (to the right of the axon cap and *below the dashed line*). The *dashed line* designates what was the dorsal edge of the cell and the initial axonal segment). Dorsal is up and the midline is to the left. **B** The left M-cell appears “normal” even though a microelectrode was brought in close proximity to the axon cap and soma during seven separate penetrations. Dorsal is up and the midline is to the right. Calibration = 50 μ m

This fish was not used in this study. In experimental fish all that remained to identify the previous location of the M-cells was the axon cap as shown in Fig. 7B

Probability of eliciting a C-start response to a vibratory stimulus

Initial results suggested that ablation decreased the probability of eliciting C-starts. Two of the three fish that were used to test the reliability of the ablation technique responded with C-starts to vibratory stimuli in 100% and 83% of the preoperative trials, respectively, but did not display any C-starts when tested 3 h postoperatively. The third fish displayed C-starts in 100% of the preoperative trials but in only 17% 10 days postoperatively. However, these results do not provide proper controls for microelectrode penetrations and the surgical procedures themselves.

The probabilities of eliciting a C-start are shown in Fig. 8 for ten fish (five experimental and five control) tested at 30 days postoperatively. C-start probability was not different between preoperative trials and the

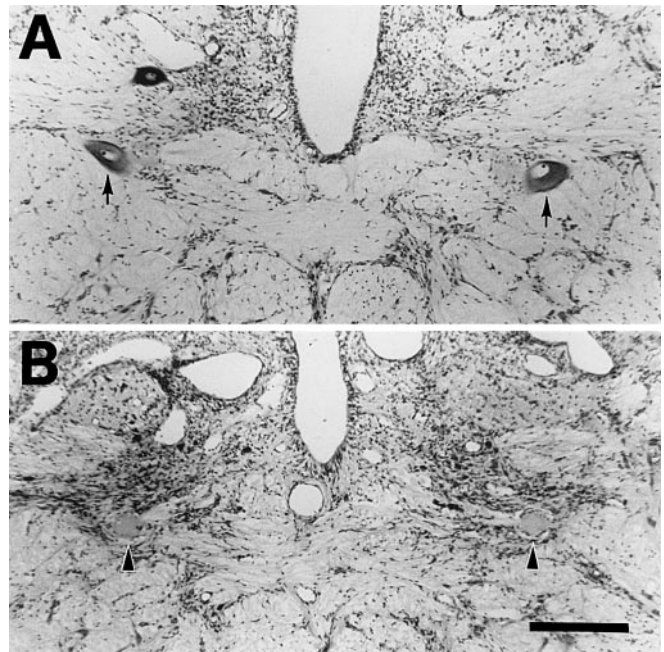


Fig. 7A, B Morphological determination of ablation success. M-cell axon caps were located in the medulla oblongata by the large negative field potentials evoked by antidromic stimulation of the M-axons in the spinal cord. The M-cells of experimental fish were subsequently ablated with the microelectrode. **A** The M-cells of a control goldfish. After the axon cap was localized, the microelectrode was removed, the cranial cavity was sealed and the fish was allowed to recover from anesthesia. Thirty days postoperatively both M-cells (*arrows*) are intact. **B** Thirty days after double ablation all that remains to identify the former location of the M-cells are the axon caps (*arrowheads*). The midline is the center of each photograph and dorsal is up. Calibration = 200 μ m

postoperative control trials (0.9 ± 0.15 versus 0.87 ± 0.09 ; *t*-test), indicating that the electrophysiological and surgical procedures did not influence responsiveness. In contrast, the postoperative experimental fish displayed a significantly lower responsiveness (0.12 ± 0.26) than the postoperative controls (0.87 ± 0.09 ; $P < 0.001$; *t*-test). In fact, three of the five experimental fish showed no C-start responses postoperatively.

The probabilities of eliciting a C-start in 13 fish (7 experimental and 6 control) tested at 3 h, 1 day, 30 days and 60 days postoperatively are presented in Fig. 9. A repeated measures analysis of variance indicated that there was a significant effect of time ($F_{3,33} = 18.2$; $P < 0.001$), treatment (experimental versus control; $F_{1,11} = 74.2$; $P < 0.001$) and of the interaction between the two ($F_{3,33} = 7.0$; $P < 0.001$). There was a significant difference between control and experimental responsiveness 60 days postoperatively (*t*-test, $P < 0.05$).

Comparison of C-start kinematic parameters

There was a great deal of variation in the kinematic parameters (i.e., stage 1 angle, escape trajectory angle,

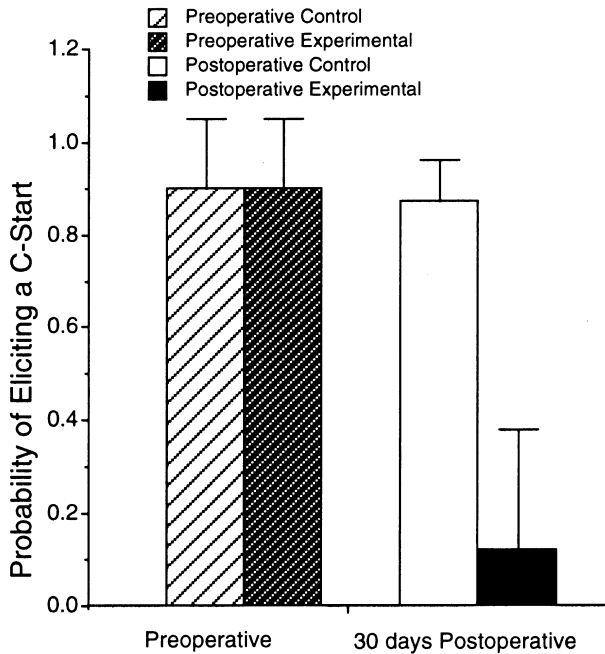


Fig. 8 Probability of eliciting C-starts for double M-cell ablated experimental fish and their sham-operated controls 30 days postoperatively. The mean C-start probabilities with their standard deviations are plotted for double ablated experimental fish ($n = 5$) and sham-operated control fish ($n = 5$) preoperatively and 30 days postoperatively. Experimental and control values are significantly different postoperatively ($P < 0.001$; t -test)

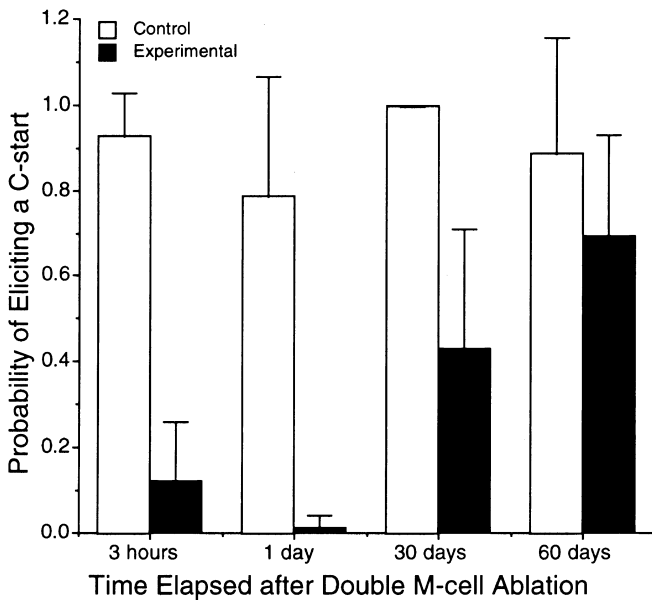


Fig. 9 Probability of eliciting C-starts for double M-cell ablated fish and their sham-operated controls 3 h, 1 day, 30 days and 60 days postoperatively. The mean C-start probabilities with their standard deviations are plotted for experimental fish ($n = 7$) and control fish ($n = 6$). There was a significant effect of time, treatment and of the interaction between the two

center of mass movement and the linear velocity of the center of mass movement) measured between trials of different fish and between trials of each fish. For

example, regression lines of six preoperative trials for two different fish are presented in Fig. 10. The responses of the fish in Fig. 10A show a great deal of variability while those of the fish in Fig. 10B are much more homogeneous. A comparison of their kinematic parameters, highlighting this difference, is presented in Table 1.

A MANOVA indicated a significant effect of treatment (i.e., control versus experimental; Wilks' Lambda, 0.018; $P < 0.0001$). Stage 1 latency is apparently determined by activity of the M-cell in control fish. We therefore considered whether this and other kinematic

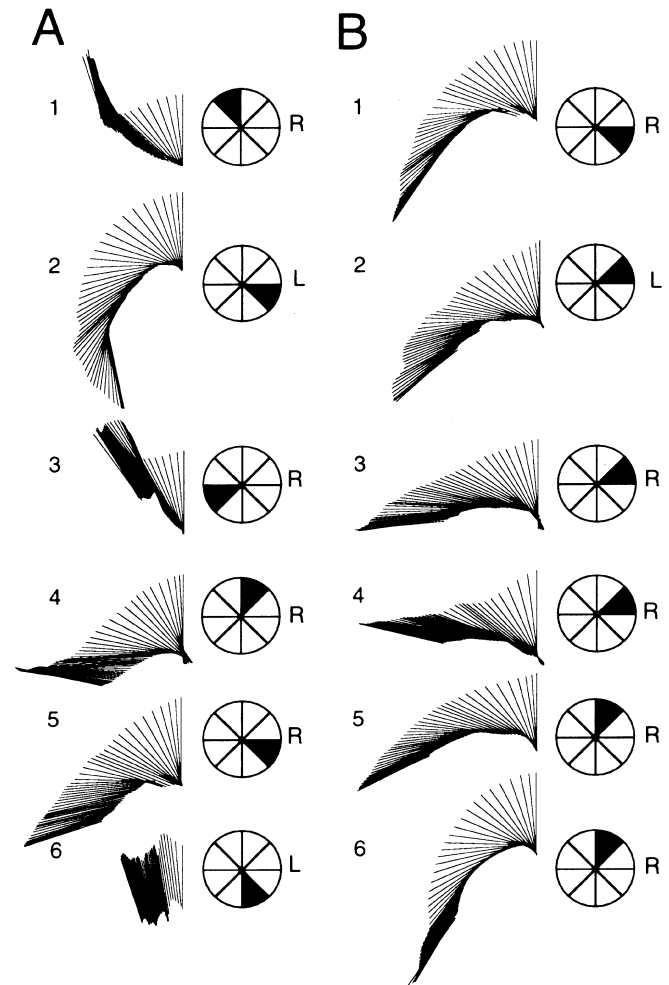


Fig. 10A, B C-start variability between trials of two different fish and between trials of individual fish. Regression lines are shown for each of six consecutive preoperative C-starts for two fish. Each regression line represents 2 ms as described in Fig. 2. The regression lines are displayed as if all responses are directed to the left for convenience of presentation. Actual direction is designated as either right (R) or left (L). The position of the fish on stimulation is shown by shading one of eight segments of the circular arena within the test tank. **A** The C-starts of this fish are highly variable in relation to their final position. One measure of this variability as compared to the trials in **B** is the straight-line center of mass distance which is 3.8 ± 1.5 cm (mean \pm SD; range = 1.3–5.2 cm). **B** The C-starts of this fish display a great deal of consistency in relation to their final position. In this fish the straight-line center of mass distance is 5.2 ± 0.2 cm (range = 4.9–5.4 cm)

Table 1 Comparison of kinematic parameters between sets of six preoperative trials of two fish (*CM* center of mass, *ET* escape trajectory angle)

| | Stage 1 latency (ms) | Stage 1 angle (°) | Stage 2 latency (ms) | ET angle (°) | CM distance (cm) | Velocity of CM movement (cm · s ⁻¹) |
|---|----------------------|-------------------|----------------------|--------------|------------------|---|
| A | 18.9 ± 4.7 | 61.7 ± 19.1 | 44.6 ± 10.5 | 81.8 ± 55.5 | 3.8 ± 1.5 | 72.2 ± 29.7 |
| B | 18.3 ± 2.1 | 72.5 ± 11.9 | 40.9 ± 3.7 | 121.9 ± 24.7 | 5.2 ± 0.2 | 92.2 ± 11.0 |

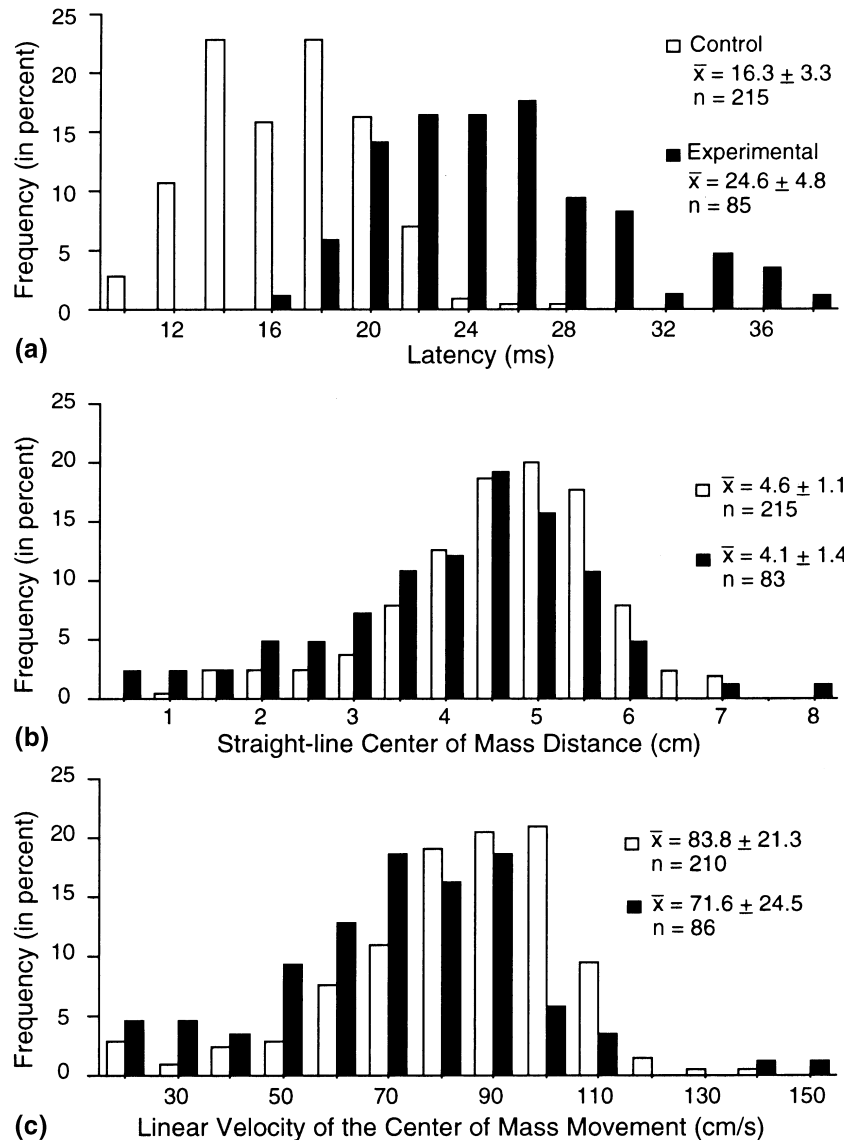
* ET = escape trajectory angle
 + CM = center of mass

parameters change after M-cell ablation. The stage 1 and 2 latencies for experimental fish were significantly longer than the corresponding latencies of control fish ($P < 0.01$). All other stage 1 and stage 2 response parameters measured were not significantly different between control and experimental animals. The pooled data (data from all responses at all postoperative intervals) for stage 1 latency, center of mass movement and the linear velocity of that movement are shown in Fig. 11.

Discussion

The probability of eliciting a C-start was significantly less in fish with double M-cell ablations when compared to the responsiveness of control fish. As discussed below, these results suggest that the M-cell may not only initiate C-starts but may play an important role in triggering other non-M-cells involved in this response.

Fig. 11A–C The distribution of C-start parameters in control and experimental fish. Data from all postoperative intervals is combined on the graph. Stage 1 latencies (A), the straight-line distance that the center of mass travels during 70 ms after the stage 1 latency (B), and the velocity of the straight-line center of mass movement (C) are presented. Statistical analyses indicate that stage 1 and 2 latencies between control and experimental fish are significantly different while no other C-start parameter differs significantly



C-starts occur in the absence of M-cells

C-starts can be elicited in goldfish after selective ablation of both M-cell somata, albeit with a lower probability of occurrence and a longer latency than when the M-cell is intact. Thus, other neurons (non-M-cells) are capable of initiating C-starts. Although the stage 1 latencies from stimulus to response of experimental fish are significantly longer on average (8.3 ms) in experimental fish than in controls, there is some overlap between the two distributions (see Fig. 11). The shortest response latencies of non-M-cell responses were 8 ms longer than the shortest M-cell responses. The shorter latency of M-cell-initiated C-starts may result from a number of factors including a lower number of synaptic relays in the neural network involving the M-cell. Other differences that may be involved include number and size of active afferents, cell membrane properties and feedforward inhibitory networks. The finding that kinematic parameters other than stage 1 and 2 latencies are not significantly different between M-cell- and non-M-cell-initiated responses confirms previous studies in which the M-cells were damaged by electrolytic lesioning in goldfish (Eaton et al. 1982; DiDomenico et al. 1988) and by radiation-induced developmental deletions in zebrafish (Eaton and Kimmel 1980; Kimmel et al. 1980).

Decreased probability of eliciting a C-start after selective M-cell ablation

The selective double ablation procedure has a profound effect on C-start occurrence in the goldfish. Some fish show no C-start response to vibratory stimuli, and overall there is a significant decrease in the probability of eliciting a C-start in experimental fish relative to that found for control fish. Separation of the effects of M-cell somata damage (primary) on the occurrence of C-start responses from morphological and physiological events that occur secondarily as a result of that damage is difficult. Such secondary effects include changes in the physiological properties of neurons that lose their M-cell target (Faber 1984; Titmus and Faber 1990). Feedforward inhibitory neurons (PHP neurons; Faber et al. 1991) which modulate the excitability of the M-cell (and possibly non-M-cells) may change their spontaneous activity patterns as a result of target removal. If spontaneous activity of the inhibitory neurons increased at short behavioral test intervals, then non-M-cells that are involved in C-starts may be inhibited such that they are less likely to reach threshold and initiate a C-start. To control for such a possibility, both M-axons were selectively axotomized in experimental fish and the probability of C-start occurrence was compared to control fish 3 h postoperatively. Such a procedure left the M-cell intact but removed its spinal output. Double selective axotomy resulted in a significant reduction in the occurrence of C-starts (Zottoli et al. 1995a). This result supports our interpretation that the decrease in the

probability of eliciting C-starts is due to the loss of the M-cells.

The M-axon separated from its soma survives, and, if activated, it can elicit EMG responses (Zottoli et al. 1987; Blundon et al. 1990). Such survival should have no effect on our results because there are no reports of direct or indirect afferent connections to the goldfish M-axon in the brain which makes it unlikely that the M-axon could subserve M-cell function at any of the postoperative test intervals. Indeed, if that were the case, and the isolated M-axon was activated, one might expect little if any change in responsiveness.

Calcium-imaging studies of the activation pattern of reticulospinal neurons in zebrafish have shown that head stimulation activates the M-cells and their "homologs" (see below, MiD2 cm and MiD3 cm; Lee and Eaton 1991; O'Malley et al. 1996) while tail stimulation activates only the M-cell and not the homologs. Thus, a decrease in the probability of eliciting C-starts after double M-cell ablation could result if after ablation the fish perceives a caudal stimulus that normally would activate M-cells exclusively. This seems unlikely in goldfish for two reasons. M-cell activity almost always precedes responses to various vibratory stimuli (two cycles of 150 Hz, Zottoli 1977; ball drop, Eaton et al. 1981; tank displacement, Eaton et al. 1988). Secondly, goldfish in this study did not remain in the same tank position from trial to trial. As a result, it is highly unlikely that they consistently perceived the vibratory stimulus as caudally directed.

In summary, the significant reduction in the probability of C-starts in experimental fish appears to be due to the loss of the M-cells.

Are kinematic parameters of M-cell- and non-M-cell-initiated C-starts the same?

The kinematic parameters (i.e., stage 1 angle, escape trajectory angle, center of mass movement, linear velocity of center of mass movement) of M-cell-initiated C-starts are not significantly different from those initiated by non-M-cells (see also Eaton et al. 1982). The lack of significant differences in those parameters may be misleading, especially in the light of the non-uniformity of sound intensity within the test arena (percentage differences are similar to those measured by Eaton et al. 1988). Since fish are not positioned or restricted to a discrete arena location, a particular fish will perceive the source of the stimulus as emanating from a different direction from one trial to the next (see Fig. 11). However, goldfish are known to respond directionally to a vibratory stimulus (Eaton and Emberley 1991), and such a variety of possible source locations could result in the fish displaying the maximum range of trajectories (see Fig. 2 of Foreman and Eaton 1993). Such diversity in response trajectories will result in a maximum range of kinematic parameter values, decreasing the ability to distinguish differences between experimental and control

groups. Despite the large variability, kinematic parameters of experimental fish measured in this study were consistently lower than those measured from control fish. We hypothesize that, if fish are presented with a uniform stimulus, the responses will be more stereotyped and the kinematic parameters of M-cell-initiated C-starts will differ from those initiated by non-M-cells. This hypothesis could be tested by restricting the fish to a particular portion of the test arena in an attempt to elicit uniform response trajectories or by designing testing chambers with constant directional information (Canfield and Rose 1996).

Changes in responsivity over time

The mean probability of eliciting C-starts in experimental fish increases markedly with postoperative time, from around 10% at 1 day to 70% at 60 days. This trend is statistically significant and implies a plasticity in non-M-cell circuits such that threshold is attained more often with increased postoperative intervals. To understand the changes underlying the increase in responsiveness, non-M-cells involved in C-starts must be physiologically characterized to determine how they interact with other reticulospinal neurons including the M-cell. Behavioral studies have provided some evidence for common features of M-cell and non-M-cell circuits. They include 1) input from the ear and/or lateral line (this paper; DiDomenico et al. 1988), 2) similar thresholds (DiDomenico et al. 1988), and 3) networks that involve reciprocal inhibition between M-cells and non-M-cells (DiDomenico et al. 1988; Eaton et al. 1991). Three pairs of segmentally "homologous" neurons have been termed the "Mauthner series" (M-cells, MiD2 cm and MiD3 cm; Fig. 8 of Lee et al. 1993). Each of these neurons has a lateral dendrite and an axon that crosses the midline to descend in the contralateral fasciculus longitudinalis medialis. All three cells in the Mauthner series are unilaterally active during C-starts elicited by head stimulation of zebrafish (O'Malley et al. 1996). Therefore, MiD2 cm and MiD3 cm are candidates for involvement in non-M-cell-initiated C-starts. The ability to locate M-cells physiologically (Furshpan and Furukawa 1962) combined with the morphological location of the homologs (Lee et al. 1993) should allow the physiological identification of the serial homologs and the characterization of their role in C-start responses.

Possible physiological mechanism for a decrease in the probability of eliciting a C-start after double M-cell ablation

Response probability and threshold of non-M-cell C-starts were the same as M-initiated responses after electrolytic lesioning of both M-cells (Wieland and Eaton 1984). These results would seem to contradict the findings of this study. However, the differences in the

techniques used to kill the M-cells provide a possible explanation for and insight into the physiological mechanisms underlying the decrease in the occurrence of C-starts after M-cell ablation.

Eaton and his colleagues have used metal microelectrodes to locate the axon cap and then to electrolytically lesion an area of about 100 μm in diameter (Eaton et al. 1982; DiDomenico et al. 1988). The M-cell, the axon cap and some surrounding tissue is damaged by this technique. The axon cap is a specialized structure around the initial-segment axon-hillock region of the M-cell that is the site of termination of excitatory interneurons (i.e., spiral fiber neurons, Bartelmez 1915; Bodian 1937; Nakajima 1974; Scott et al. 1994) and inhibitory interneurons, or PHP cells, named for a passive hyperpolarizing potential (PHP) that occurs as a result of the M-cell's action current flowing inwardly across the membranes of these cells (Faber and Korn 1973; Korn and Faber 1975). One class of PHP cells (commissural) forms bilateral feedforward inhibitory networks that regulate M-cell threshold to sensory inputs (Faber and Korn 1978; Korn and Faber 1983; Faber et al. 1991). Another class of PHP neurons (collateral) is activated by relay neurons and forms feedback networks with each M-cell and reciprocal networks between M-cells. This arrangement helps ensure that only one M-cell fires and that it does so only once (Furukawa and Furshpan 1963; Faber and Korn 1978; Hackett and Faber 1983; Korn and Faber 1983; Faber et al. 1989). The endings of excitatory (spiral fiber cells) and inhibitory (PHP cells) interneurons are undoubtedly damaged by electrolytic lesions. The bilateral, commissural PHP cells would have the most extensive damage due to the relationship of their neurites to the area damaged by electrolytic lesioning. Specifically, the main neurite of an ipsilateral soma, after sending projections into the ipsilateral M-cell axon cap as well as to other reticulospinal neurons, crosses the midline in a commissure just above the M-cell. Branches project to the contralateral M-cell axon cap as well as to other reticulospinal neurons (Zottoli and Faber 1980; Triller and Korn 1981). An electrolytic lesion in the region of both M-cells would undoubtedly sever connections of commissural PHP cells to M-cells and other neurons. The hypothesis that commissural PHP cells form a common feedforward inhibitory control (either directly or indirectly) of M-cells and non-M-cells alike can be used to explain a difference in behavioral results between this selective ablation study and previous M-cell lesion studies. Specifically, electrolytic lesioning of M-cells and associated damage of PHP cells would "release" the non-M-cells from feedforward inhibition.

The selective ablation technique used in this study results in minimal damage to surrounding tissue other than that caused by the multiple microelectrode penetrations themselves. We hypothesize that the selective technique results in minimal alteration of inhibitory inputs common to both M-cells and non-M-cells involved in C-start behavior. Thus, we have removed the M-cells

and their excitatory (or the inhibition of inhibitory input) influence on non-M-cells. Thus, feedforward inhibition predominates and the non-M-cells are often unable to initiate a C-start with a resultant decreased probability of response.

A further comparison of the behavioral output of fish with electrolytic versus selective M-cell lesions, possibly in combination with population lesions of PHP cells, would be interesting. In addition, physiological studies on candidate non-M-cell neurons (Lee et al. 1993) during stimulation of PHP neurons will help to determine the role of feedforward inhibition in the brainstem C-start network.

The M-cells and the neuronal basis of C-start responses

The activity of an ipsilateral M-cell in goldfish precedes trunk EMG responses by 1.5–2 ms and a C-start to the contralateral side (Zottoli 1977; Eaton et al. 1981, 1988; DiDomenico et al. 1988). For each C-start only one M-cell fires a single action potential, and this M-cell is generally on the side of the perceived stimulus (Eaton and Emberly 1991; Eaton et al. 1991). The active M-cell initiates contraction of contralateral musculature. The M-cell determines the initial direction of movement and other reticulospinal neurons are thought to determine the ultimate trajectory of the fish (Eaton et al. 1991). Eaton and his colleagues have proposed a *parallel network model* that involves the corollary discharge of many interacting reticulospinal neurons (non-M-cells and M-cells) acting in parallel to produce C-starts (Eaton et al. 1988; Nissanov and Eaton 1989; Foreman and Eaton 1993). The variety of C-start trajectories that can be elicited (Foreman and Eaton 1993) emphasizes the complexity of these interactions. Since fish respond by moving away from the direction of the sound source (Eaton and Emberly 1991), the “behavioral context” of the animal before delivery of the stimulus will determine which reticulospinal neurons are more likely to be activated (Eaton and Hackett 1984; Foreman and Eaton 1993; Zottoli et al. 1995b; Canfield and Rose 1996). Our finding that there is a decreased occurrence of C-starts when the M-cells are selectively ablated indicates that M-cell activity may have a greater role than just determining the initial direction of movement. We hypothesize that M-cell activation may be important in bringing those reticulospinal neurons that are “primed” by the behavioral context to threshold. This hypothesis, along with studies that demonstrate the ability of M-cell activation to override competing motor commands (e.g., swimming, Eaton et al. 1995; Svoboda and Fetcho 1996), provides the basis for studies focused on the interactive nature of the brainstem startle-response network. Imaging of multiunit arrays of neurons will provide us with the best insight of how these interactions might occur to control C-start responses (O’Malley et al. 1996).

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