Recovery of C-starts, equilibrium and targeted feeding after whole spinal cord crush in the adult goldfish *Carassius auratus*

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Summary

Central nervous system neurons of many adult teleost fish are capable of regrowth across spinal cord lesions, which may result in behavioral recovery of swimming. Since there have been few, if any, studies that examine the return of behaviors other than swimming, we provide a quantitative analysis of the recovery of C-starts that occur in adult goldfish after spinal cord injury. In addition, we include a qualitative analysis of the return of targeted feeding and equilibrium. Whole spinal cord crushes near the junction of the brain and spinal cord [spinomedullary level (SML)] were made in 45 experimental fish. Eight sham-operated goldfish served as controls for the effects of the surgery procedures alone. After spinal cord crush and recovery from the anesthetic, experimental fish lay on their sides with no movement caudal to the wound. The fish were monitored for the return of behaviors for up to 190 days postoperatively. Twenty-five fish survived the course of this study. Of these fish, 12 regained equilibrium and C-starts, two regained equilibrium but not C-starts, and 11 did not regain equilibrium (one of these did display a C-start). Twenty-two of the 25 experimental fish that survived the 190 days were able to target food from the water surface. Quantitative analysis of recovered C-starts in this study revealed that the probability of eliciting the response is reduced, that latencies from stimulus to response are longer and that movement parameters (i.e. angles, distance and velocity) are reduced compared with those of sham-operated control animals for up to 190 days postoperatively. The recovery of C-starts, equilibrium and targeted feeding was due to re-growth across the wound site, since re-crushing the spinal cord at the SML resulted in the loss of these behaviors. Mauthner cells are known to initiate C-starts in goldfish. Since the majority of M-axons that regrow across a crush wound associate with an inappropriate pathway (i.e. the first ventral root), it is unlikely that these cells play a major role in the return of C-starts. We propose that regeneration of Mauthner cell homologues across the wound site is responsible for the recovery of most C-starts. The identifiability of the M-cell and its homologues provides a unique opportunity to analyze the mechanisms underlying behavioral recovery at the cellular level.

Key words: C-start, startle response, functional recovery, behavioral recovery, Mauthner cell, regeneration, spinal cord crush, goldfish, *Carassius auratus*.

Introduction

Central nervous system neurons of many adult teleost fish are capable of re-growth across spinal cord lesions (Koppányi and Weiss, 1922; Tuge and Hanzawa, 1937; Kirsche, 1950; Bernstein and Bernstein, 1969; Phelps, 1969; Bernstein and Gelderd, 1970; Coggeshall et al., 1982; Coggeshall and Youngblood, 1983; Bunt and Fill-Moebs, 1984; Al-Goshae and Bunt, 1992; Sharma et al., 1993; Yamada et al., 1995; Becker et al., 1997, 1998; Hanna et al., 1998; Van Raamsdonk et al., 1998; Becker and Becker, 2001; Doyle et al., 2001). This morphological regeneration has been shown to result in recovery of swimming (Koppányi and Weiss, 1922; Tuge and Hanzawa, 1937; Kirsche, 1950; Pearcy and Koppányi, 1924; Bernstein, 1964; Coggeshall and Youngblood, 1983; Van Raamsdonk et al., 1998; Doyle et al., 2001). Since there have been few, if any, studies that examine the return of behaviors other than swimming, we provide a quantitative analysis of the recovery of C-starts that occur in adult goldfish after spinal cord injury.

Goldfish display a rapid response to vibratory stimulation in which the animal's body typically forms a C shape (Eaton et al., 1977, 1981). This C-start is ideally suited for quantitative studies of behavioral recovery after spinal cord injury because (1) C-starts have been extensively characterized in normal animals (e.g. Eaton et al., 1988; Foreman and Eaton, 1993; see review by Eaton et al., 2001), (2) much of the neuronal network that controls C-starts has been described (Faber and Korn, 1978; Fetcho and Faber, 1988; Faber et al., 1989, 1991; Fetcho, 1991) and (3) the neurons that initiate C-starts are identifiable and accessible (Bartelmez, 1915; Furshpan and Furukawa, 1962; Zottoli, 1978).

Preliminary reports indicate that recovered C-starts differ from those of sham-operated control fish for up to 12 months

after spinal cord injury (Zottoli and Freemer, 1991; Zottoli et al., 1994; Zottoli and Faber, 2000). The present study provides a quantitative approach to analyze these C-start differences and also provides qualitative descriptions of the recovery of equilibrium and targeted feeding.

There is a vast literature on behavioral regeneration after spinal cord injury of many vertebrates (see Larner et al., 1995), including the larval and adult lamprey (Cohen et al., 1988, 1989; McClellan, 1994), amphibians (e.g. Davis et al., 1990), fish (Bernhardt, 1999; Doyle et al., 2001) and mammals (e.g. Schwab and Bartholdi, 1996). However, there are few preparations that provide the ability to determine the underlying neuronal basis for that recovery. The identification of neurons that are responsible for the return of C-starts will provide the unique opportunity to analyze the mechanisms underlying behavioral recovery at the cellular level.

Materials and methods
Fish care

Fifty-five common goldfish (*Carassius auratus* L.; purchased from Hunting Creek Fisheries Inc., Thurmont, MD, USA) 11.2±1.0 cm (mean ± S.D.) in body length were housed individually in 23 cm×17 cm×14 cm tanks. The fish were purchased in the autumn and were allowed to acclimate for a minimum of three weeks before use. Individual fish were kept in 4 liters of conditioned (Novaqua; Kordon, Inc., Hayward, CA, USA), aerated water at 22.4±1.1°C (mean ± S.D.; range, 19.1–24.3°C) and presented with an alternating 12 h:12 h light:dark cycle. They were fed Hikari Staple food (mini pellet, floating type, Kyorin Food Ind. Ltd, Himeji, Japan) three times a week, followed 2 h later by cleaning of the tank and replacement of the tank water with fresh, conditioned tap water.

The fish were between 6 months and 1.5 years in age (Hunting Creek Fisheries, Inc., personal communication).

Choice of wound type and site: whole spinal cord crush at the spinomedullary level

Spinal cord crush was chosen because it is most similar to the type of injury that might occur naturally. One distinct advantage of crush wounds is minimal bleeding compared with cut wounds.

The spinomedullary level (SML) was chosen as a site to crush the whole spinal cord in order to maximize behavioral deficits. After spinal cord crush and recovery from the anesthetic, experimental fish lay on their sides with no movement caudal to the wound; i.e. fin, trunk and tail movements caudal to the wound were abolished by this wound while those movements that control vital functions rostral to the wound (i.e. respiration and feeding from the bottom of the tank) were spared. The progressive return of behavior could be unambiguously documented by visual observation after SML crushes.

The SML level was also chosen to allow visibility of the spinal cord during and after the crush operation. As described below, the spinal cord was exposed in the brain case. This approach did not require damage to either muscle or vertebrae and allowed the cord to be fully exposed and, as a result, visible with the aid of a dissecting microscope.

Spinomedullary crush technique

Forty-five goldfish had a spinal cord crush at the SML (i.e. the junction area between the spinal cord and medulla). Eight sham-operated goldfish served as controls for the effects of the surgical procedures alone. All spinal cord crushes were performed by one experimenter (i.e. S.J.Z.). A holding temperature of 22.4°C was chosen because preliminary results had indicated that goldfish may not regain C-starts at 16°C (Zottoli and Faber, 1977). Fish were initially anesthetized in 0.03% ethyl-m-aminobenzoate (Sigma-Aldrich Co., St Louis, MI, USA) until breathing ceased and were transferred to an operating chamber where chilled water containing 0.012% of anesthetic was re-circulated through the mouth and over the gills (the chilled water reduced the gill temperature of the fish from approximately 22°C to 10°C). A hole was drilled in the skull to expose the area from the caudal portion of the corpus cerebellum to the spinal cord. Overlying muscle, cartilage and fat were removed to expose the SML, and care was taken not to damage the posterior semicircular canals. The spinal cord was crushed at the SML. The tips of the forceps (No. 5 Dumont forceps) were lowered on either side of the spinal cord until they touched the floor of the brain case. The forceps were moved rostrally until they were at the junction of the vagal lobes and medulla oblongata (i.e. the SML). The tips were then closed tightly and held together for approximately 2 s. This crush procedure was then repeated. When the first crush was made, the anesthetized fish moved slightly, giving a preliminary indication that the brain tissue had been damaged. Although the crush did not result in disconnection of the spinal cord from the medulla oblongata, a distinct line was evident where the crush had been made. Very little bleeding resulted from this operation. 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 two small accessory holes drilled on either side and rostral to the operation hole. The wire was twisted together caudally where a loop was made on one of the ends. The caudal loop was anchored to musculature just behind the skull with silk suture thread. The twisted wire and string acted as a secure framework for the vinyl polysiloxane impression material (Imprint, 3M) used to 'cap' the skull. After the operation, the re-circulating anesthetic solution was replaced with conditioned tap water and the fish recovered, initiating breathing in approximately 10–15 min. Fish were returned to their home tanks and monitored closely for 30–60 min.

Behavioral observations
General observations

Experimental and sham-operated control fish were observed to determine their general health, whether there were any

noticeable movements caudal to the wound site at rest, and their position in the tank relative to the vertical plane. The fish were observed daily for the first 10 postoperative days to carefully monitor the effect of the operation. Following the 10th postoperative day, observations occurred three times per week until 128 postoperative days. Observations were then made weekly until 168 postoperative days and a final observation was made 190 days postoperatively. Testing for C-starts occurred at random times during the day, and, in general, a set of six trials took approximately 1 h to complete.

Targeted feeding

During the preoperative, three-week acclimatization period, fish were fed 10–12 mini-pellets of food three times a week. All fish easily located and targeted pellets floating on the surface at each feeding session during the 3-week period. The pellets were consistently consumed within a 2-h period, and the tanks were then cleaned.

During this preoperative period, fish were tested once for their ability to target the floating pellets on the water surface during a 4-min test period. The test was divided into two parts: (1) five food pellets were placed on the water surface within a plastic floating ring, 5.1 cm in diameter, and fish were given 2 min to target food within the ring, and (2) if a fish failed to target the pellets in the ring within 2 min, the ring was removed, five additional pellets were added and the fish's ability to target free-floating pellets for another 2 min was observed. A fish met the targeting criterion if it touched and/or ate one of the floating pellets within the 4-min test period.

Postoperatively, this test was done weekly for both experimental and sham-operated animals at one of the normal feeding sessions until the 146–158th postoperative day.

If six or more pellets had not been consumed within approximately 2 h, the remaining pellets were removed and the fish was provided with TetraMin Tropical Flakes (Tetra GMBH, Melle, Germany), which sank to the bottom of the tank. Fish were observed to ensure they were eating some of this food from the tank bottom. Two hours after the addition of flake food the tanks were cleaned to reduce the accumulation of organic matter in the tank.

Equilibrium

The position of experimental and sham-operated fish relative to the vertical plane was noted during the 190-day postoperative interval. Each fish was categorized as being on its side (i.e. the sagittal plane of the fish was perpendicular to the vertical plane of the tank), tilted (partial equilibrium) or upright (full equilibrium; the sagittal plane of the fish was parallel to the vertical plane of the tank). A transition from one of these categories to another required the change to be observed in two consecutive observation periods. When this condition was met, the first observation date was used as the transition or recovery date.

Fourteen fish that regained full equilibrium and the eight sham-operated control fish were tested in water circulated at two different speeds to determine their ability to maintain equilibrium while swimming between 167 days and 169 days postoperatively. A circular, Plexiglas tank, 10 cm high and 23 cm in diameter, was filled with conditioned tap water at 22°C to a depth of 8 cm. The tank was placed on a stir plate, and a magnetic stir bar (9.5 mm in diameter; 58 mm in length) was placed in the center. A fish was placed in the tank and, after a 2 min acclimation period, the stir bar was rotated at a slow speed (estimated water speed, 6.1 cm s^{-1}) for 2 min and then a faster speed (estimated water speed, 36 cm s^{-1}) for 2 min. Fish tended to swim near the edge of the tank to avoid the stir bar.

C-starts

Fish were tested preoperatively for their ability to respond to a vibratory stimulus with a C-start. One set of six trials with an inter-trial interval of at least 2 min was given preoperatively 4–11 days prior to the spinal cord crush. Fish were screened during preoperative testing to meet the following three criteria: (1) each fish had to respond to the stimulus with C-starts in at least three of six trials, (2) at least one C-start had to be to the left and one to the right and (3) the fish silhouette had to be compatible with the software thinning algorithm (e.g. some fish had silhouettes that made it difficult for the software analysis).

Postoperatively, a block of six trials with an inter-trial interval of at least 2 min was given every 14 days for up to 138–158 days postoperatively. Fish were tested with a block of six trials one final time between 182 and 195 postoperative days. Fish were fed and their tanks were cleaned after testing.

Our behavioral testing system for delivering the vibratory stimulus is similar to that described by Eaton and colleagues (Wieland and Eaton, 1983; 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 reduced by 5.1 cm to a final depth of 7.7 cm to restrict vertical movement of the fish and (3) the stimulus was more intense (i.e. 600 μm vertical tank movement compared with 3–6 μm used by Eaton et al., 1988) to ensure the delivery of a supra-threshold 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 the 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
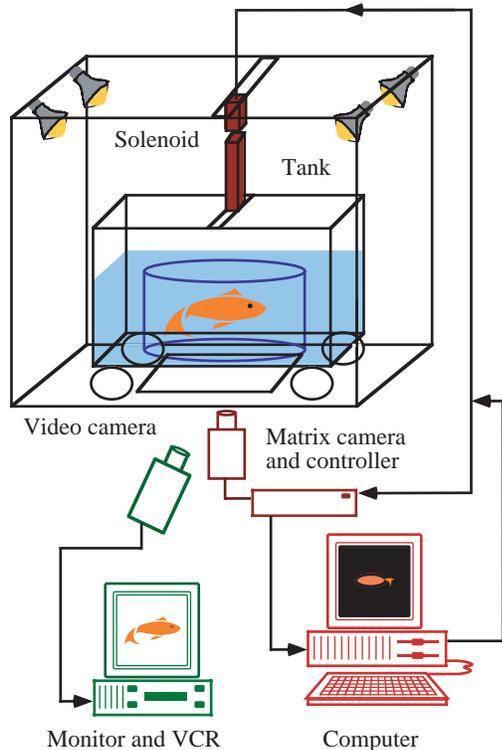
Fig. 1. Schematic diagram of the behavioral testing apparatus used to elicit and analyze goldfish C-starts. Light is projected from above and fish images are captured from below the tank by both a video camera and a matrix camera. Goldfish are stimulated with a vibratory stimulus created by lifting the whole tank with a solenoid. The computer simultaneously triggers the solenoid and the matrix camera, which starts saving silhouettes at a rate of 500 silhouettes 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.

triggered by computer with 1.5 waves of the 60 Hz line voltage. The fish tank was lifted approximately 600 µm.

Two cameras were located below the test tank to record fish movement within the arena (Fig. 1). Fish were videotaped with a conventional 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, Gaithersburg, MD, USA). 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).

Determination of the probability of eliciting a C-start

Observation of videotapes provided a preliminary screening for the occurrence and direction of C-starts. Since fish were stimulated when their bodies were straight and after they had come to rest, the identification of a response coupled to the stimulus was usually clear. The responses were categorized as one of the following. (1) Full body response, involving the whole body; the fish typically formed a C shape and, during these responses, were displaced from their original location. (2) Partial body response; movement of head structures (i.e. operculi, eyes, mouth), fins and upper trunk. The fish formed a shallow C shape that resulted in minimal displacement of the fish from its original location. (3) Head and fin response; movement of the head and fins occurred with no apparent movement of the trunk and tail. (4) No response.

Two different experimenters independently categorized these responses based on single frame analysis of the videotapes. In cases of disagreement, one of the investigators (i.e. S.J.Z) re-observed the trial and made a final judgement regarding the category.

Computer software (i.e. KNOWAL; Nissanov, 1991) was used to analyze all category 1 and 2 responses to determine whether they met the criteria for a C-start. The 52 matrix camera silhouettes (Fig. 2A) were each reduced to a midline 1 pixel thick (Fig. 2B) using a thinning algorithm. The rostral 40% of each midline was then converted to a regression line (Fig. 2C; Nissanov, 1991). These regression lines were used to determine whether significant axial 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 is greater than 3°, (2) no directional reversal past the start position occurs within the four silhouettes subsequent to the start silhouette and (3) if the angle between the start and start + 2 silhouettes is less than 10° then start + 3 cannot be situated between start and start + 2 silhouettes. Computer analysis of some trials was not possible, and occurrence of a startle response was determined from the videotapes.

Determination of C-start kinematic parameters

The C-start has been divided into two stages (Blaxter et al., 1981; Eaton et al., 1991). During stage 1, there is a major contraction of the body musculature on one side resulting in a characteristic C-like shape. Stage 2 is characterized by forward propulsion that may be associated with a turn. These two stages are the focus of this paper even though they may be followed by other movements, including swimming. Stage 1 and stage 2 kinematic parameters were automatically calculated by the computer from regression lines of the midlines, representing the head and rostral trunk. Fig. 2C provides a graphic representation of most of the following kinematic parameters.

The stage 1 parameter measured is stage 1 latency or start 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 above. 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).

Fig. 2. Method of analysis of the goldfish C-starts. (A) Superimposition of silhouettes of a goldfish during a C-start. Silhouettes were taken every 2 ms but, for clarity, only those occurring at 14-ms intervals starting at the beginning of the response are shown. The lightest image is the silhouette at the beginning of the response and it has been arbitrarily oriented with the nose (black circle) upwards. The darkest image is the fish silhouette at approximately 104 ms after the stimulus delivery. (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). The first midline is at the start of the response. (C) The rostral portion of each midline, corresponding to the rostral 40% of the midline (Nissanov, 1991). Regression lines are shown in 2 ms increments. The regression line that begins stage 2 and the line 70 ms after the start are labeled. The angle at the beginning of stage 2 is formed between the regression line at start and the regression line at the stage 2 latency. The escape trajectory angle (ETA) is formed between the regression line at start and the regression line 70 ms later. The straight-line distance that the center of mass travels during 70 ms after the start is delineated as well.
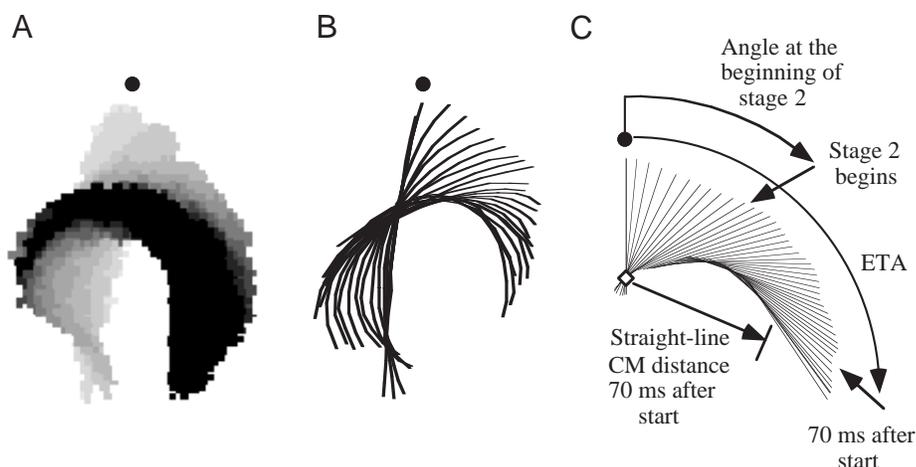
Stage 2 parameters are as follows:

(1) Stage 2 latency. This is the latency from the onset of the vibratory stimulus to the time when the center of mass was displaced 0.75 cm from the initial position (DiDomenico et al., 1988; Eaton et al., 1988). The maximum latency, as determined by the computer, was adjusted by subtracting 2.4 ms as described for stage 1 latency above. Stage 2 latency was always lower than stage 1 latency plus 70 ms (control fish, 44.8+6.7 ms lower; experimental fish, 35.9+11.8 ms lower).

(2) Angle at the beginning of stage 2. This is the angle formed between the regression line at the stage 1 latency and the regression line at the stage 2 latency.

(3) Escape trajectory angle (ETA). The angle formed between the regression line at stage 1 latency and the regression line 70 ms later (Eaton and Emberley, 1991). This latency was chosen to fall within predator closing times (see Foreman and Eaton, 1993).

(4) Stretched-body 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. In this interval, fish generally move approximately 4 cm (DiDomenico et al., 1988).

(5) Linear velocity of the center of mass movement. See Nissanov (1991).

The goldfish C-start not only has axial body movements but also includes closure of the jaw, adduction of the operculi and retraction of the eyes (Diamond, 1971; Hackett and Faber, 1983). These cranial components of the C-start were not analyzed in this study.

Statistical analyses

All data are reported as means ± S.D. For statistical analyses, data were organized into six, 25-day intervals (T1–T6) encompassing a total of 150 postoperative days. C-starts from eight sham-operated control animals and 11 experimental fish that recovered equilibrium and C-starts were analyzed. One fish that had not recovered equilibrium but had recovered C-starts and one other fish that had recovered a C-start on the 190th postoperative day were not included.

Comparisons of the probability of eliciting a C-start

The probabilities of eliciting a C-start were calculated for each fish at each of the last three time intervals (T4–T6). The proportions were arcsine transformed and analyzed using a repeated-measures analysis of variance (RM-ANOVA). In addition, the individual control and experimental probabilities in the longest postoperative interval (i.e. 126–150 days) were arcsine transformed and compared using an unpaired *t*-test.

Comparisons of C-start kinematic parameters

C-start kinematic parameter values analyzed in this study include stage 1 and stage 2 latency, angle at the beginning of stage 2, escape trajectory angle, center of mass movement and linear velocity of the center of mass movement. C-starts that did not have a second stage were not used in this analysis. To determine whether kinematic parameters differed over the last three time intervals (T4, 76–100 days; T5, 101–125 days; T6, 126–150 days) or by treatment (i.e. control *vs* experimental) and to determine whether there was a possible interaction between the two, a multivariate analysis of variance (MANOVA) was run on mean parameter values determined for each fish at each 25-day time 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 25-day time interval by a

given fish and discarded the rest of the values for that fish at that interval. Thus, a maximum of three responses was chosen for each fish (i.e. one from each of the last three time intervals; in some cases, less than three responses were available since the fish may not have responded in one or more of the time intervals). Means were calculated for control and experimental fish at each interval and were compared using a MANOVA. Since the MANOVA indicated an effect of treatment, one-factor ANOVAs were run to determine what control parameters (all T4–T6 values) differed from the corresponding experimental parameters (all T4–T6 values). The *P* values were adjusted with a standard Bonferroni adjustment to correct for type I error. A significance level was set at *P*=0.05.

Re-crush of spinal cords in fish that had recovered C-starts

In order to determine whether regeneration across the crush wound was responsible for behavioral recovery, the spinal cord was re-crushed 198–200 days after the original operation in five fish that had recovered C-starts, equilibrium and targeted feeding. The crush was at the same location (i.e. SML) and the procedure was identical to the original operation. General behavioral observations were made up to 10–12 days following the second operation. Fish were tested for their ability to respond to a vibratory stimulus with a C-start. One set of six trials was given 2–5 days after the re-crush with the standard stimulus strength. A second set of six trials was given 10–12 days after the re-crush with a greater stimulus strength.

Histological procedures: completeness of the wound

After spinal cord crush there was no evidence of movement caudal to the wound in any of the fish. To determine whether the crush wound actually damaged all nerve fibers, SML crushes were performed on three goldfish as described above. After recovery from the anesthetic, the fish were placed in their home tank. One of these fish displayed movement caudal to the wound site and was not used for histological observation.

The brains of the remaining two fish were re-exposed under anesthesia eight days postoperatively, and a whole spinal cord cut was made 1–2 mm caudal to the original crush. Biocytin (Sigma Chemical Co.), re-crystallized on the tip of 45-gauge stainless steel wire, was introduced into the cut wound. After the biocytin dissolved, the wire was removed, the skull was re-sealed and, after recovery from the anesthetic, the animals were placed in their home tank for 2 days. The fish were then anesthetized and perfused with fixative consisting of 4% paraformaldehyde, 1% glutaraldehyde in $0.1 \, \text{mol} \, \text{l}^{-1}$ phosphate buffer (pH 7.4). Brains were removed and placed in fresh fixative for 1 h, after which they were rinsed three times in phosphate buffer. Brains were stored in phosphate buffer for one week and then placed in 30% sucrose in $0.1 \, \text{mol} \, \text{l}^{-1}$ phosphate buffer 24 h before frozen sectioning. Brains were cut frozen in the transverse plane at $60 \, \mu\text{m}$. The sections were stored in $0.1 \, \text{mol} \, \text{l}^{-1}$ phosphate buffer and then processed with a procedure modified from Bass et al. (1994). Specifically, sections were: (1) incubated for 10 min in a hydrogen peroxide–methanol–phosphate buffer mixture to reduce endogenous peroxidase, (2) rinsed three times in buffer, (3) incubated for 30 min in 0.4% Triton X in buffer, (4) incubated for 3 h in an avidin–biotinylated horseradish peroxidase complex (Elite Kit, Vector Laboratories, Burlingame, CA, USA), (5) rinsed twice in phosphate buffer, (6) incubated for 3–5 min in 0.05% diaminobenzidine, 0.01% hydrogen peroxide in phosphate buffer, (7) rinsed twice in phosphate buffer and (8) stored in phosphate buffer until mounting on chrom–alum-coated slides. After the sections dried on the slide, they were dehydrated, placed in xylene and then coverslipped with Eukitt (O. Kindler, Freiburg, Germany).

The serial sections were studied to determine whether all nerve processes were separated at the wound site. Specifically, if a wound was complete, one would expect no biocytin-labeled fibers to be present rostral to the wound.

Results
Completeness of the crush wound

To determine whether the crush wounds were complete, spinal cords of two fish were crushed at the SML level (Fig. 3A) and biocytin was applied caudal to the wound to determine whether any fibers were spared the initial crush injury. Brain sections caudal, within and rostral to the crush site were observed to determine if any biocytin-filled profiles existed. Sections within the wound site had some biocytin-filled fibers (Fig. 3C). In one fish, no biocytin-labeled fibers could be found rostral to the crush site (Fig. 3B). The second animal had one thin biocytin-filled fiber that extended $540 \, \mu\text{m}$ rostral to the crush site. Since no cell body was identified rostral to the wound in this case, it is assumed that this fiber originated caudal to the crush site.

General behavioral status of fish up to 190 postoperative days

All eight sham-operated control fish survived the 190-day duration of this study. These fish remained healthy, maintained equilibrium at rest and, while swimming, were able to target food pellets from the water surface and displayed C-starts in response to the vibratory stimulus.

All 45 experimental fish were on their sides on the bottom of the tank immediately following spinal cord crush and recovery from anesthesia. Fish displayed movements of the eyes, operculi and jaws rostral to the crush wound. However, there was no evidence of movement caudal to the wound in any of the fish. However, movement could be evoked caudal to the wound by gently lifting the animals out of their tanks; this movement is thought to be due to spinal reflexes.

Twenty-five of the 45 experimental fish survived the course of this study. Of these surviving fish, 12 regained equilibrium and C-starts, two regained equilibrium but not C-starts, 11 did not regain equilibrium. Of the 11 that did not regain equilibrium six had no apparent body abnormalities, while five had body abnormalities that are believed to hinder behavioral recovery (Fig. 4A). Specifically, four had the

Fig. 3. Completeness of the spinal cord crush wound. (A) Schematic diagram of the dorsal view of a goldfish brain to show the location of the crush site at the spinomedullary level (SML). Biocytin was applied to a cut wound (1–2 mm caudal to the SML; not shown) 8 days after a crush wound at the SML to determine if any fibers were spared damage (see text for details). For reference, the Mauthner cells (M-cells) and their axons (M-axons) are superimposed on the diagram. Ce, cerebellum; FL, facial lobe; VL, vagal lobe; C, caudal; R, rostral. (B,C) Photomicrographs of 60-μm transverse tissue sections. The brain midline is centered on and the ventral edge of the brain is located at the bottom edge of each photomicrograph. (B) Section 1.5 mm rostral to the crush wound. The ventral area shown here has no biocytin-filled fibers and is representative of the entire medulla oblongata at this level. In fact, this fish had no biocytin-filled fibers rostral to the wound site, indicating that all fibers were damaged. (C) Section near the site of the SML crush. A few biocytin-filled fibers can be seen at this level. Scale bar, 100 μm for B and C.
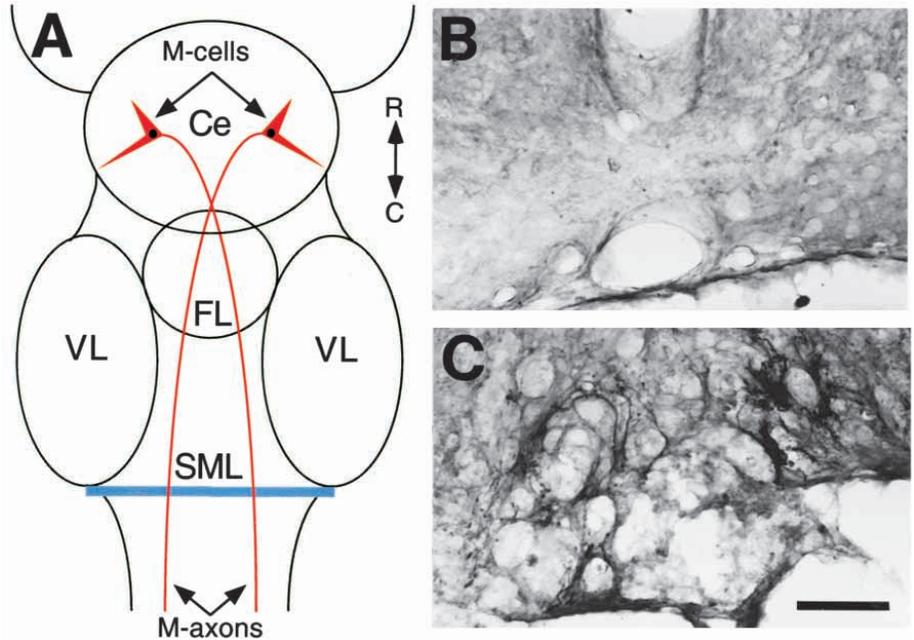
caudal half of their trunk and tail bent upwards and one had a bloated air bladder and was floating. One of these fish did recover a C-start while on its side. Twenty fish died during this study. The distribution of the number of fish that died over the 190-day postoperative interval (Fig. 4B) reveals two clusters, one between 3 and 52 days and another between 91 and 162 postoperative days.

The postoperative interval at which C-starts, equilibrium and targeted feeding returned varied between fish. Nonetheless, fish regained behaviors in a sequential manner with pectoral and/or pelvic fin movements appearing first, followed by targeted feeding, partial equilibrium, full equilibrium and finally C-starts (Fig. 5).

Recovery of targeted feeding

Preoperatively, all fish demonstrated the ability to target food pellets within a 4-min test period (i.e. either in the floating ring within 2 min or free floating in the subsequent 2 min of the test). Postoperatively, seven of eight sham-operated control fish met the targeting criterion on the first test day (3 days postoperative) while the 8th fish targeted food on the 2nd test day (10 days postoperative).

Twenty-two of the 25 experimental fish that survived the 190-day postoperative interval met the targeting criterion. The earliest recovery of the targeting criterion occurred on postoperative day 15 while the longest occurred at day 109 (Fig. 5). The three fish that did not meet the criterion were fish that never regained equilibrium; however, eight other fish that did not regain equilibrium were capable of targeting food pellets.

All eight sham-operated control fish and 19 of 22 experimental fish targeted pellets in the ring during the first 2 min of the test at least once during the 190-day postoperative interval.

Recovery of equilibrium

Sham-operated control animals were upright and swimming immediately following the operation and recovery from anesthetic (i.e. about 10–20 min after being returned to their home tanks). Thus, the operation itself did not result in damage to the spinal cord or semicircular canals. In addition, the 'cap' used to cover the skull wound did not hinder the ability of the fish to maintain full equilibrium.

Experimental fish were upright preoperatively. Immediately after recovery from crush wounds, all experimental fish were lying on their sides on the bottom of their tanks. Fourteen of 25 experimental fish gradually recovered full equilibrium (Fig. 5) during the 190-day postoperative interval. Three other fish regained partial equilibrium (i.e. they remained tilted) during this interval.

The 14 fish that regained full equilibrium and the eight sham-operated control fish were tested in water circulated by a stir bar at two different speeds to determine their ability to maintain equilibrium while swimming. Seven sham-operated control fish swam into the water current for the majority of the test while one swam with the current at both stir bar speeds. While swimming into the water current at both test speeds, the seven control fish maintained their position in the water column (i.e. did not drift backwards) and occasionally moved forward against the current.

At the slower stir bar speed, 10 of the 14 experimental fish that had regained full equilibrium and swam into the water current were able to hold their position while the other four fish were unable to maintain their position and drifted backwards.

Two of the 14 experimental fish lost the ability to maintain full equilibrium at the slower stir bar speed while 13 of the 14 fish were unable to maintain equilibrium at the faster speed.

Recovery of C-starts

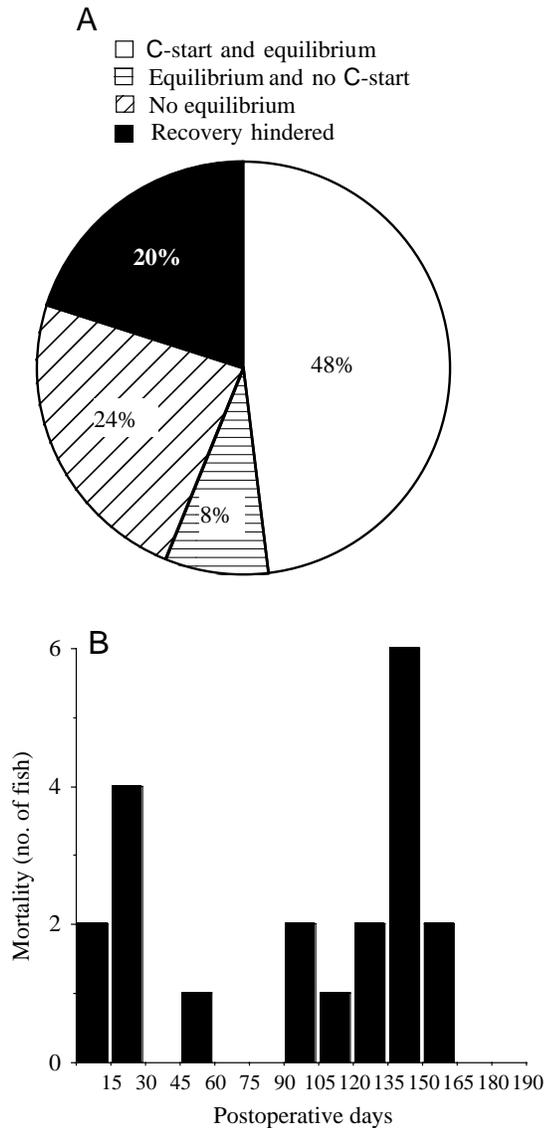All C-starts that were classified as a full body response (category 1) and could be analyzed met software criteria for a C-start (see Materials and methods). Three of 23 trials that were classified as partial body responses (category 2) met software criteria for a C-start (i.e. in 20 trials fish did not move enough for the computer to calculate a start frame).

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

Sham-operated control fish responded with a C-start to a vibratory stimulus in at least 75% of the trials on average throughout the six 25-day time intervals used for analysis (T1–T6; Fig. 6). By contrast, a C-start could not be elicited to a vibratory stimulus in experimental fish for the first 50 postoperative days. Two of the 12 fish that eventually regained C-starts displayed this response by 75 postoperative days, and 11 of the 12 fish regained C-starts by 150 postoperative days (Fig. 6). The 12th fish regained a C-start on the 190th day.

The probability of eliciting a C-start was compared over T4–T6 (T4, 76–100 days; T5, 101–125 days; T6, 126–150 days). A RM-ANOVA revealed a significant effect of treatment (i.e. control *vs* experimental) but not time. Comparison between experimental and control fish proportions at the 126–150-day interval indicated that the probability of eliciting a response was significantly greater in control animals ($P<0.0001$, $N=8$ for control; $N=11$ for experimental; t-test).

Comparison of C-start kinematic parameters

C-start kinematic parameters were compared over T4–T6 for 11 of the 12 fish that recovered C-starts. A MANOVA

Fig. 4. Fish survival and behavioral classification of goldfish after whole spinal cord crush. (A) Behavioral status of goldfish 190 days after spinal cord crush. Of the 25 surviving fish, 12 regained equilibrium and C-starts (48%), two regained equilibrium but not C-starts (8%), six did not gain equilibrium and had no apparent body abnormalities (24%; a C-start could be elicited in one of these fish), while five had body abnormalities (20%) that are believed to have hindered behavioral recovery. (B) Distribution of the number of experimental fish that died over the 190-day postoperative interval. Twenty of the 45 experimental fish died during the course of this study. The distribution of the number of fish that died reveals two clusters: one at 3–52 days and another at 91–162 days postoperatively.
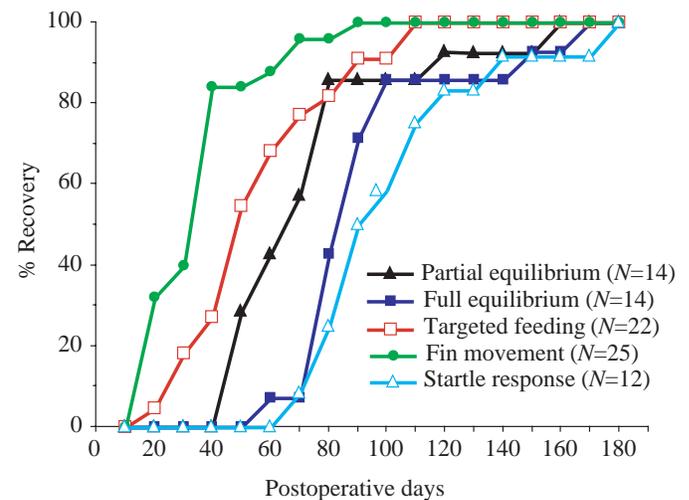

Fig. 5. Time course of behavioral recovery after whole spinal cord crush at the spinomedullary level (SML). The percentage of fish that recovered a particular behavior (# fish that recovered a behavior/total number that ultimately recovered the behavior) was calculated for each 10-day interval up to 190 postoperative days. Although there was a great deal of variability between fish as to when a particular behavior returned, return of pectoral and/or pelvic fin movement was generally first and was followed by targeted feeding, partial equilibrium, full equilibrium and C-starts. Eight sham-operated control fish targeted food, maintained full equilibrium and displayed C-starts throughout the postoperative interval (not shown).
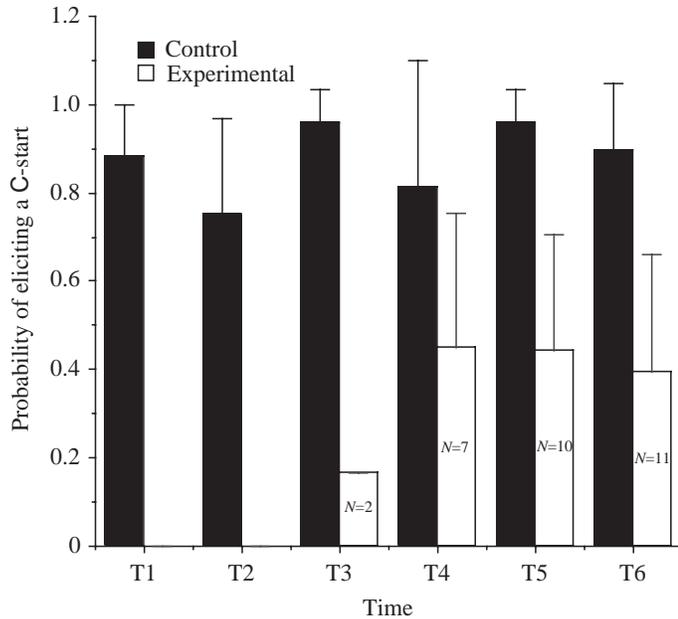
Fig. 6. Probability of eliciting C-starts from sham-operated control and experimental goldfish to a vibratory stimulus over six 25-day postoperative intervals (T1–T6). The mean probability and s.d. are shown for experimental fish that recovered C-starts and for eight sham-operated control fish. The first experimental responses occurred in two fish between 51 and 75 days postoperatively. C-starts could be elicited from 11 of the 12 fish by 150 days postoperatively. One other fish regained C-starts on the 190th postoperative day (not shown).
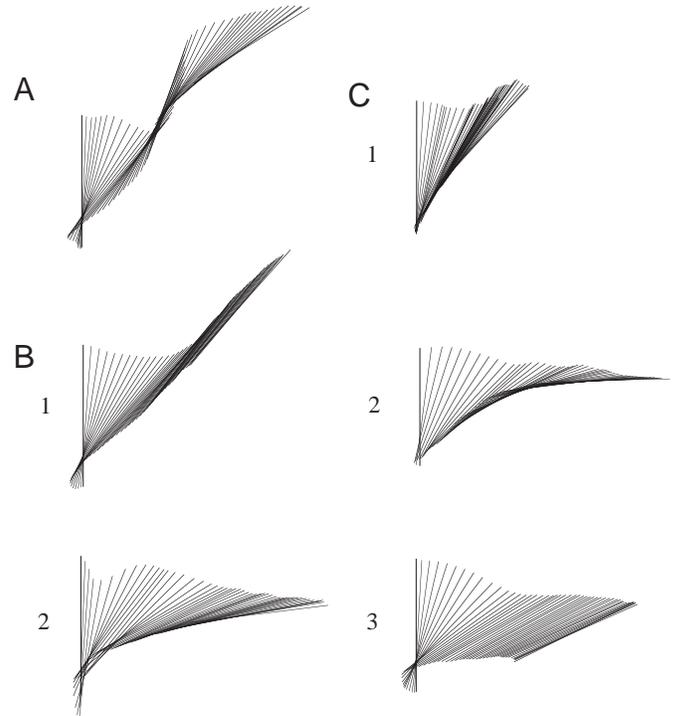

Fig. 7. Diverse trajectories of recovered C-starts from three experimental fish. Regression lines are shown for C-starts of three fish. Each regression line represents the position of a fish every 2 ms for a total of 104 ms, as described in Fig. 2. (A) A double C-start from fish 135, 120 days postoperatively (1st recovered response was elicited on day 64). (B) Two C-starts from fish 138, 120 days and 134 days postoperatively (1st recovered response was elicited on day 92). (C) Three C-starts from fish 112. Two were elicited 142 days postoperatively (C1,C2) and the third on 198 days postoperatively (1st recovered response was elicited on day 72).

indicated a significant effect of treatment (i.e. control *vs* experimental) but not time. Although there was a diversity in the trajectories of recovered C-starts, as shown for three fish in Fig. 7, statistical analyses indicate that recovered C-starts were slower and less robust than those elicited preoperatively or those of sham-operated control fish. A comparison between preoperative C-starts and those elicited 95–109 days postoperatively in Fig. 8 highlights some of the most dramatic differences in C-starts that we encountered. Stage 1 and 2 latencies for experimental fish were significantly longer than the corresponding latencies of control fish ($P<0.0001$, $N=8$ for control; $N=11$ for experimental; $P<0.008$ with Bonferroni adjustment). In addition, all other stage 1 and stage 2 response parameters, including the angle at the beginning of stage 2, escape trajectory angle (ETA), center of mass movement and linear velocity of the center of mass movement, were significantly smaller in experimental animals when compared with the corresponding control parameters ($P<0.0001$, $N=8$ for control; $N=11$ for experimental; $P<0.008$ with Bonferroni adjustment). For example, ETAs of ≥100° occurred in all eight sham-operated control fish and in 65.7% of the analyzed C-starts (69/105) but only occurred in two of the 11 experimental fish and in 2.5% of recovered C-starts (2/79). The largest control ETA was 199°, compared with 105° for an experimental fish. The pooled control and experimental

kinematic data (data from all responses between 75 and 150 postoperative days) for stage 1 latency, center of mass movement and the linear velocity of the center of mass movement are shown in Fig. 9. In addition, Table 1 contains mean control and experimental values for all kinematic parameters. These means were calculated by first averaging values from all trials of an individual fish for a particular parameter and then calculating a mean value for control and experimental groups. Therefore, the means in Fig. 9 (all trials) and those in Table 1 (mean of means) differ. Finally, the means in Table 1 were not those used to test for significant difference as described in the Materials and methods.

Both sham-operated control fish and experimental fish had a limited number of C-starts with no second stage (i.e. the center of mass did not become displaced 0.75 cm from its position at the start) between 75 and 150 postoperative days. Two of eight sham-operated control fish each had one C-start with no second stage. Six of 11 fish that regained C-starts and equilibrium had trials with no second stage. Examples of control and experimental C-starts with no second stage are shown in Fig. 10.

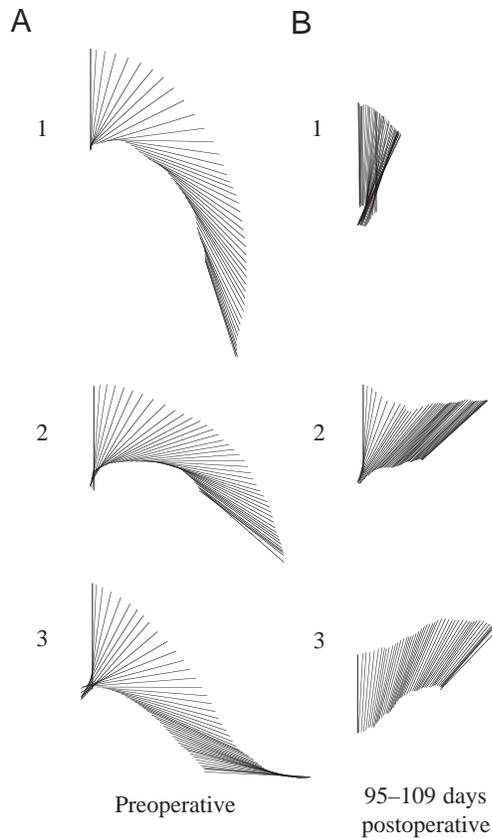A   B

Preoperative

95–109 days
postoperative

Fig. 8. In general, experimental fish displayed C-starts that were more restricted in their extent than those elicited preoperatively or by sham-operated control animals. Each regression line represents 2 ms as described in Fig. 2. Three consecutive startle responses preoperatively (A1–3) of fish 152 are compared with three responses (B1–3) elicited between 95 and 109 days postoperatively. The most obvious difference is the angle achieved 70 ms after the delivery of the stimulus. The mean angle of the trials in A is 134.1° while that in B is 53.8°.

Loss of recovered behaviors after spinal cord re-crush

Five fish were chosen from the 11 that had regained C-starts, equilibrium and targeted feeding by the 150th postoperative day. A re-crush of the original wound site resulted in the loss of these behaviors. Photographs in Fig. 11 provide a comparison of equilibrium before and after re-crush for one

fish. Three of the five fish had limited pectoral fin or caudal fin movement 10–12 days after the re-crush.

Discussion

Goldfish are capable of behavioral recovery of C-starts, equilibrium and targeted feeding. Although there is a great deal of variability of when a particular behavior returns, fish tend to first recover targeted feeding followed by partial equilibrium, full equilibrium and then C-starts. Recovered behaviors differ from those in sham-operated control fish. The focus of the present investigation was to quantitatively compare recovered C-starts with those of sham-operated control fish. Recovered C-starts were not as frequent, fast or robust as those of control animals. We speculate that the differences between experimental and control C-starts results from differences in the underlying neuronal circuitry.

Effectiveness of the stimulus in eliciting a C-start after spinal cord crush

The type and/or amplitude of the stimulus was critical for the successful elicitation of recovered C-starts. After an SML crush, C-starts could not be elicited for six months postoperatively with a sound pulse consisting of two cycles of a 200 Hz sinusoidal signal delivered by an underwater loudspeaker (Zottoli et al., 1989; Universal model UW-30; see Zottoli, 1977 for details). By contrast, the vibratory stimulus used in this study was effective in eliciting C-starts as early as 64 postoperative days. The ability to elicit C-starts at short postoperative intervals most likely resulted from the stimulus amplitude (600 µm displacement of the test tank), which is well above threshold levels determined for control fish (Eaton et al., 1988; 3–6 µm displacement).

Completeness of a crush wound

One disadvantage of a crush wound as compared with a cut wound is that there is no way to determine whether the wound is complete at the time of injury. After a cut wound, it is possible to use a probe to confirm that the proximal and distal pieces of spinal cord are completely separated (Pearcy and Koppányi, 1924); such an approach is not possible after a crush wound.

Only fish that showed no spontaneous movement below the level of the SML crush site within the first 10 postoperative

Table 1. *Kinematic parameters of control and experimental C-starts*

	Stage 1 or start latency (ms)	Stage 2 latency (ms)	Angle at the beginning of stage 2 (deg.)	Escape trajectory angle (deg.)	CM straight-line distance (cm)	CM linear velocity (m s^{-1})
Experimental ($N=11$)	44.7±8.9	75.8±9.0	38.7±6.8	51.9±7.2	2.4±0.4	39.0±10.8
Control ($N=8$)	19.2±1.9	44.4±4	63.5±12.9	92.6±42.1	3.6±0.5	56.8±23.3

All measurements are means ± S.D.; these means were calculated by first averaging values from all trials of an individual fish and then calculating a mean value for control and experimental groups. Only trials in time intervals T4–T6 were included in calculation of mean values. Trials with no second stage were not included in calculation of mean values.

The means ± S.D. in Fig. 9 represent means of all trials and therefore differ from the ones presented in this table.

CM, center of mass.

days were used in this study. It is generally accepted that paralysis caudal to a wound site is a good indicator of the completeness of the wound (Keil, 1940; Tuge and Hanzawa, 1937). For example, when the spinal cord was not completely cut at the high cervical level (level D; equivalent to the SML), Japanese rice minnows (*Oryzias latipes*) remained upright (Tuge and Hanzawa, 1935). However, behavioral evidence alone is not sufficient to determine the extent of a wound.

Our histological results indicate that spinal cord crush damages and ultimately results in separation of all descending axons at the wound site. However, an occasional afferent process may be spared. The effectiveness of an SML crush on damaging descending axons is supported by studies in which the Mauthner axon was filled with Lucifer yellow either rostral or caudal to the wound site 30–62 days postoperatively. In all cases, the axon had separated and retracted from the crush site and no longer extended across the wound (Zottoli et al., 1987, 1988).

Although it is difficult to compare behavioral studies that differ in wound level and type of wound, fish species and temperature, the similarity in the time course of recovery between cut and crush wounds lends support to the complete nature of our wound. The recovery of swimming in goldfish after whole cord transection at the high thoracic level, in which separation of the cord was confirmed, is maximized between 2 and 2.5 months (Koppányi and Weiss, 1922; Pearcy and Koppányi, 1924; Bernstein, 1964), an interval at which the majority of fish in this study were able to target food from the water surface. In Japanese rice minnows, movement caudal to a spinal cord transection at the high cervical level (equivalent to the SML) appeared between 15 and 30 postoperative days (Tuge and Hanzawa, 1935), which corresponds to our observations of movement caudal to the wound site occurring as early as 15 postoperative days. In addition, we found that full equilibrium returned as early as 60 postoperative days, which would explain why Tuge and Hanzawa (1935) did not observe upright posture of fish for up to 40 days postoperatively.

Recovery of behavior was due to morphological regeneration across the wound site

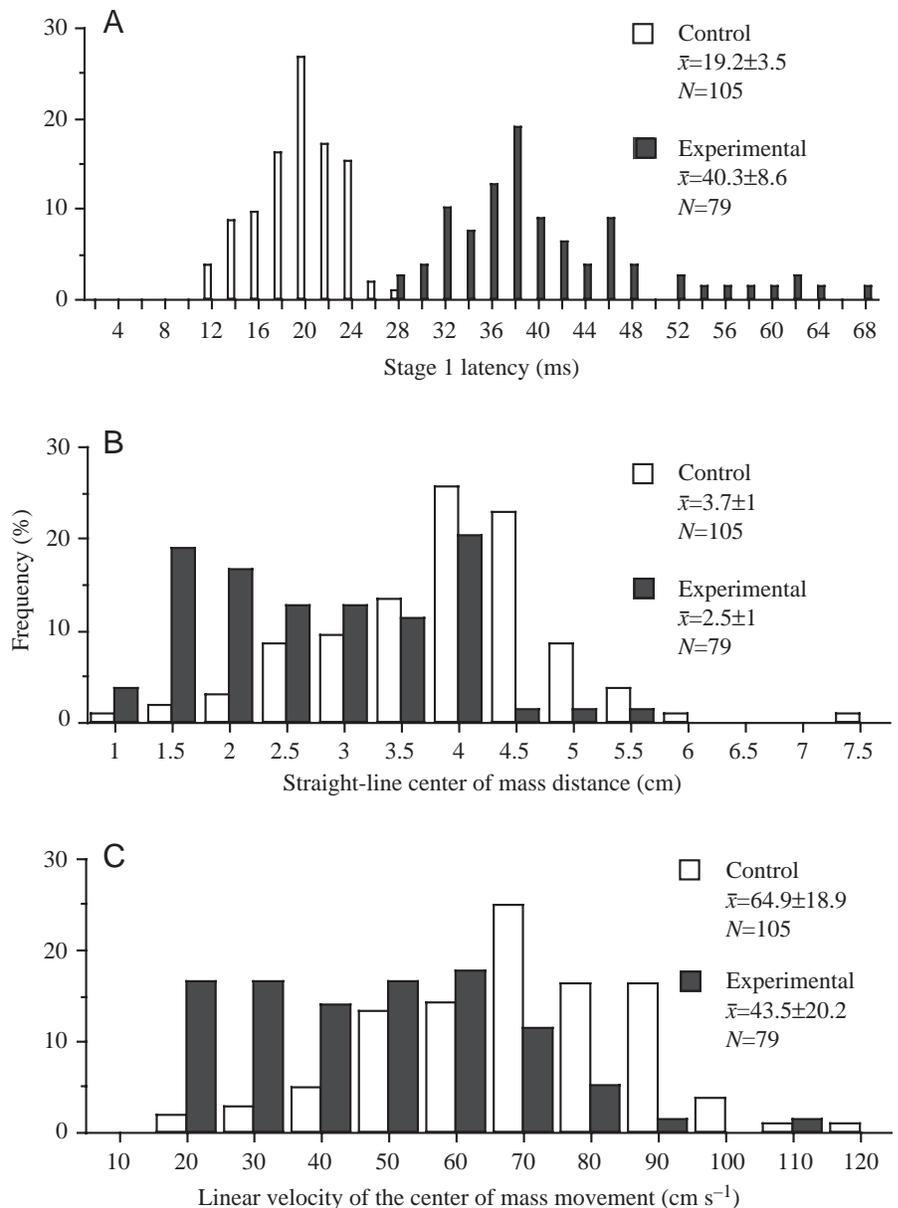Recovered C-start, equilibrium and targeted feeding

Fig. 9. The distribution of C-start kinematic parameters in control and experimental fish. All data up to 150 days postoperatively (T1–T6) are combined on the graph. Latency from the stimulus to the response (A), the straight-line distance that the center of mass traveled during 70 ms after the start of the response (B) and the velocity of the straight-line center of mass movement (C) are presented. Although there is no overlap between experimental and control latency values, there is substantial overlap with other parameters.

behaviors were lost when the spinal cord was re-crushed. This result indicates that morphological regeneration of nervous tissue across the original wound site was responsible for the return of these behaviors. It is interesting that a few fish had limited pectoral fin or caudal fin movement after the re-crush. These fin movements could have resulted from an incomplete wound or from extraspinal pathways. A spinal cord cut rather than a crush should be performed on fish that have recovered behaviors in the future to distinguish between these two alternative explanations.
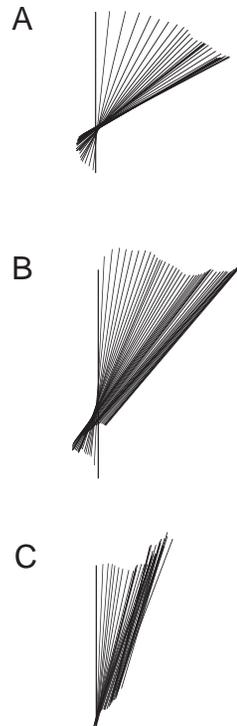
Fig. 10. The occurrence of C-starts with no second stage was greater in experimental fish than in sham-operated control fish. Each regression line represents 2 ms as described in Fig. 2. (A) A C-start with no second stage from a control fish. (B) A C-start from fish 112, 114 days postoperatively. (C) A C-start from fish 135, 106 days postoperatively.
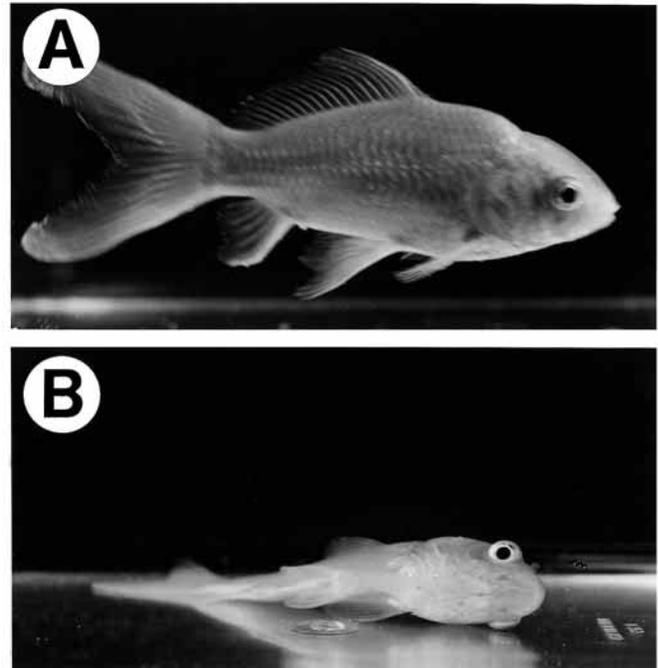

Fig. 11. Whole spinal cord re-crush at the spinomedullary level (SML) of fish that had recovered C-starts, full equilibrium and targeted feeding resulted in the loss of these behaviors. Photographs were taken from the side of the aquarium. (A) Goldfish 107, 200 days postoperatively. This fish was upright, was able to feed from the surface and displayed C-starts in response to the vibratory stimulus. (B) After spinal cord re-crush at the SML, this fish was on its side and was unable to target food from the water surface. In addition, vibratory stimuli did not elicit C-starts. This fish was 11 cm in body length.

All fish did not recover C-starts, full equilibrium or targeted feeding after spinal cord injury at the SML level

Mortality of experimental fish resulted from whole spinal cord crush since no sham-operated control fish died during the 190-day postoperative interval. Seven fish died 3–52 days postoperatively (mean, 22 days). Since bleeding was minimal after the spinal cord crush and no control animals died as a result of the operation, this short-term death was most likely due to infection related to the crush.

An additional 13 fish died 91–162 days postoperatively (mean, 133 days). None of these fish recovered equilibrium and, as a result, were provided with food that sank to the bottom of the tank. Although all of these fish were observed to ingest the food, seven of nine fish that were still alive after 121 postoperative days were noticeably emaciated. We suggest that these fish died due to lack of sufficient nutrition even though five of the nine fish were capable of targeting food pellets on the water surface. Gavage feeding may be an effective way to decrease mortality in this group of fish.

The mortality occurring over a 5–6-month postoperative interval in two separate studies, using identical protocols to those used in this study, was similar to that reported here (i.e. 44.4%). Specifically, the mortality was 32.2% in one study (N=31; 5 months postoperatively; Zottoli et al., 1994) and 36.7% in the other (N=49; 6 months postoperatively; S. J. Zottoli and J. E. Nierman, unpublished observations). Tuge and Hanzawa (1935) reported a somewhat higher mortality (65.6%) of those fish that had spinal cord transections at the high cervical level and survived for approximately 2 months (Tuge and Hanzawa, 1937).

All fish did not regain targeted feeding or full equilibrium. Approximately half (six of 11) of those fish that did not recover equilibrium had either a bent trunk (N=5) or an over-inflated swim bladder (N=1). Even though regeneration of nervous tissue could potentially support the return of equilibrium in these cases, it would be impossible for the fish to maintain equilibrium due to body abnormalities. The other five fish that did not regain equilibrium had no noticeable morphological restrictions that could explain the lack of behavioral recovery.

Two to three months after spinal cord transections at the high thoracic to cervical levels, many adult fish appeared 'normal', while others had partial or no behavioral recovery (Tuge and Hanzawa, 1937; Pearcy and Koppányi, 1924). There are many factors that might account for the lack of recovery of behavior after spinal cord injury. The age of the fish, subtle differences in the crush wound or the wound level may limit the return of behavior. In addition, regenerating central nervous system (CNS) neurons are known to make inappropriate pathway choices into the peripheral nervous system just caudal to an SML crush (Bentley and Zottoli, 1993; Zottoli et al., 1994). This inappropriate pathway choice may limit, delay or prevent the return of behavior caudal to the wound.

Recovered equilibrium differed from that in sham-operated control fish

Those goldfish that recovered equilibrium appeared 'normal' when left undisturbed in their home tanks. When they were challenged to swim in a water current, all but one experimental fish was unable to maintain full equilibrium. By contrast, sham-operated control fish were able to maintain full equilibrium under the same conditions. Differences in the amount of muscle (see below), in muscle fatigue or in the regenerated neuronal circuitry of experimental compared with control fish may contribute to the inability of experimental fish to maintain equilibrium.

Recovered C-starts differed from those in sham-operated control fish

Significant differences exist between C-starts of experimental fish and sham-operated controls. The differences include lower probability of response, longer stage 1 and 2 latencies, smaller turning angles and shorter distances traveled and velocities attained by the center of mass. We hypothesize that these differences can be explained by changes in neuronal circuitry that occur as a result of regeneration.

The Mauthner cell (M-cell) initiates C-starts in goldfish when exposed to a vibratory stimulus similar to that used in this study (Eaton et al., 1981). Morphological and physiological studies indicate that it is unlikely that M-cells are involved in the return of C-starts during the 190 postoperative days of this study.

After spinal cord cut or crush at the SML, the goldfish M-cell undergoes an axon reaction both morphologically (Zottoli et al., 1984) and physiologically (Faber, 1984; Faber and Zottoli, 1981; Titmus and Faber, 1990; Titmus et al., 1986). The proximal M-axon retracts about 1.5 mm from a crush site (Agostini and Zottoli, 1986). A few M-cells die after long postoperative intervals following SML crush (15.6°C holding temperature; Zottoli et al., 1984), and others display abortive regeneration (Zottoli et al., 1988). However, the majority of M-axons sprout extensively both rostrally and caudally for distances up to 5 mm within the CNS at 22°C (Zottoli et al., 1988). Few of the caudally projecting axons cross the wound site and those that do tend to be within or directed towards the first ventral root (Bentley and Zottoli, 1993; Zottoli et al., 1994).

Intracellular stimulation of M-axons in goldfish that have recovered C-starts did not elicit electromyogram (EMG) responses caudal to the wound in many cases, which supports the morphological findings. In general, the trunk EMG responses that were evoked in some cases were significantly smaller than those of controls and are thought to have a minimal contribution to the recovered response (Zottoli et al., 1989). Therefore, the M-cell may contribute to the recovery of C-starts in some fish but non-M-cells must underlie the recovery in most cases.

The regeneration of non-M-cells such as the M-cell homologues (Lee et al., 1993) may underlie the recovery of C-

starts. These neurons are active during C-starts in zebrafish (*Danio rerio* L.) larvae (O'Malley et al., 1996; Liu and Fetcho, 1999) and are thought to elicit non-M-cell C-starts in adult goldfish when M-cells are ablated (Eaton et al., 1982; DiDomenico et al., 1988; Zottoli et al., 1999). In addition, non-M-cell C-starts (Zottoli et al., 1999) and those C-starts that return after spinal cord crush are both characterized by a significantly lower probability of response and a longer latency from stimulus to response when compared with M-cell initiated C-starts of control fish. Retrograde labeling of axons that have regenerated across an SML crush would help determine whether the M-cell homologues or other non-M-cells are potential candidates for the recovery of C-starts.

The long-term fate of regenerating M-cells in the recovery of C-starts is not clear at this time. A decrease in C-start response latency in one goldfish between 2.5 and 12 months postoperatively indicates the possible plasticity in regenerated neuronal connections that may involve the contribution of additional cells such as M-cells (Zottoli et al., 1994).

Although there is no overlap between stage 1 latencies of experimental and sham-operated control fish, there is substantial overlap between other kinematic parameters (see Fig. 9B,C). Thus, many recovered C-starts have kinematic values that are comparable with those of controls. However, on average there were smaller turning angles and shorter distances traveled and velocities attained by the center of mass of recovered C-starts compared with control ones. Such a difference may result from changes in muscle mass after injury. Prior to recovery of movement, the trunk and tail musculature are not used except for occasional reflex responses evoked by the experimenter during routine handling and during tank cleaning. In a separate study, using identical protocols to those used in this study, fish weight normalized to the original weight prior to the crush decreased from 1.0 to 0.85±0.06 (mean ± S.D., $N=31$; J. E. Nierman, unpublished observations) one month postoperatively. Those fish that recovered equilibrium weighed 0.91±0.06 ($N=7$) of their original weight and returned to their original weights on average by six months postoperatively (1.0±0.07; $N=7$). Those fish that did not recover equilibrium did not gain back their lost weight during the same six month interval (0.84±0.12; $N=16$). Since recovered C-starts in this study could be elicited as early as 2 months postoperatively, the reduced muscle mass may influence some of the kinematic parameters measured. However, there was no effect of time on these parameters and, therefore, it is unlikely that muscle mass had a major effect on C-start kinematics.

Can the recovery of C-starts be explained by compensatory mechanisms?

Axonal regeneration across a crush wound could result in innervation of targets that would provide an alternative compensatory movement to that normally occurring in control animals. Axial motoneurons are responsible for the major components of C-starts (Fetcho, 1991, 1992). If regenerating axons predominantly innervated fin motoneurons rather than

axial motoneurons, their activation would result in a propulsive movement of the fish. This propulsive movement might be interpreted as a C-start that is slower and less robust than control C-starts. However, EMG responses of trunk musculature occur during recovered C-starts in free-swimming fish (S. J. Zottoli, unpublished observations). Therefore, if compensatory mechanisms exist, they do not appear to play a major role in the return of C-starts.

Conclusions

Numerous studies have shown that adult teleost fish can undergo behavioral recovery after spinal cord injury (see Koppányi, 1955; Zottoli et al., 1994). However, few studies on teleost fish (however, see Doyle et al., 2001) have provided the quantitative rigor in the analysis of behavioral recovery that has been the hallmark of swimming studies on the larval (e.g. Davis et al., 1993; McClellan, 1994) and adult (e.g. Cohen et al., 1989) lamprey. Our results provide the first quantitative description of the recovery of C-starts in adult teleost fish after spinal cord injury. M-cells in adult goldfish are known to initiate C-starts and after spinal cord injury can readily regenerate. However, morphological and physiological evidence indicates that M-cells would not contribute significantly to most recovered responses during the 190-day postoperative interval of this study. Therefore, recovery of C-starts does not involve restitution of the original patterns of neuronal connections. The identification of neurons that underlie the return of C-starts will provide the unique opportunity to analyze the mechanisms underlying behavioral recovery at the cellular level.

References

Agostini, M. A. and Zottoli, S. J. (1986). Retrograde degeneration of the goldfish Mauthner axon. *Soc. Neurosci. Abstr.* **12**, 509.

Al-Goshae, H. and Bunt, S. M. (1992). Selection of pathways by regenerating axons in the transected spinal cord of the goldfish. *J. Physiol.* **446**, 43P.

Bartelmez, G. W. (1915). Mauthner's cell and the nucleus motorius tegmenti. *J. Comp. Neurol.* **25**, 87-128.

Bass, A. H., Marchaterre, M. A. and Baker, R. (1994). Vocal-acoustic pathways in a teleost fish. *J. Neurosci.* **14**, 4025-4039.

Becker, T. and Becker, C. G. (2001). Regenerating descending axons preferentially reroute to the gray matter in the presence of a general macrophage/microglial reaction caudal to a spinal transection in adult zebrafish. *J. Comp. Neurol.* **433**, 131-147.

Becker, T., Wulliman, M. F., Becker, C. G., Bernhardt, R. R. and Schachner, M. (1997). Axonal regrowth after spinal cord transection in adult zebrafish. *J. Comp. Neurol.* **377**, 577-595.

Becker, T., Bernhardt, R. R., Reinhard, E., Wullimann, M. F., Tongiorgi, E. and Schachner, M. (1998). Readiness of zebrafish brain neurons to regenerate a spinal axon correlates with differential expression of specific cell recognition molecules. *J. Neurosci.* **18**, 5789-5803.

Bentley, A. P. and Zottoli, S. J. (1993). Central nervous system lesion triggers inappropriate pathway choice in adult vertebrate system. *Brain Res.* **630**, 333-336.

Bernhardt, R. R. (1999). Cellular and molecular bases of axonal regeneration in the fish central nervous system. *Exp. Neurol.* **157**, 223-240.

Bernstein, J. J. (1964). Relation of spinal cord regeneration to age in adult goldfish. *Exp. Neurol.* **9**, 161-174.

Bernstein, J. J. and Bernstein, M. E. (1969). Ultrastructure of normal regeneration and loss of regenerative capacity following teflon blockage in goldfish spinal cord. *Exp. Neurol.* **24**, 538-557.

Bernstein, J. J. and Gelderd, J. B. (1970). Regeneration of the long spinal tracts in the goldfish. *Brain Res.* **20**, 33-38.

Blaxter, J. H. S., Gray, J. A. B. and Denton, E. J. (1981). Sound and startle responses in herring shoals. *J. Mar. Biol. Assoc. UK* **61**, 851-869.

Bunt, S. M. and Fill-Moebs, P. (1984). Selection of pathways by regenerating spinal cord fiber tracts. *Dev. Brain Res.* **16**, 307-311.

Coggeshall, R. E. and Youngblood, C. S. (1983). Recovery from spinal transection in fish: regrowth of axons past the transection. *Neurosci. Lett.* **38**, 227-231.

Coggeshall, R. E., Birse, S. G. and Youngblood, C. S. (1982). Recovery from spinal transection in fish. *Neurosci. Lett.* **32**, 259-264.

Cohen, A. H., Mackler, S. A. and Selzer, M. E. (1988). Behavioral recovery following spinal transection: behavioral regeneration in the lamprey CNS. *Trends Neurosci.* **11**, 227-231.

Cohen, A. H., Baker, M. T. and Dobrov, T. A. (1989). Evidence for functional regeneration in the adult lamprey spinal cord following transection. *Brain Res.* **496**, 368-372.

Davis, B. M., Ayers, J. L., Koran, L., Carlson, J., Anderson, M. C. and Simpson, S. B., Jr (1990). Time course of salamander spinal cord regeneration and recovery of swimming: HRP retrograde pathway tracing and kinematic analysis. *Exp. Neurol.* **108**, 198-213.

Davis, G. R., Jr, Troxel, M. T., Kohler, V. J., Grossmann, E. M. and McClellan, A. D. (1993). Time course of locomotor recovery and functional regeneration in spinal-transected lamprey: kinematics and electromyography. *Exp. Brain Res.* **97**, 83-95.

Diamond, J. (1971). The Mauthner Cell. In *Fish Physiology*, vol. 5 (ed. W. S. Hoar and D. J. Randall), pp. 265-346. New York: Academic Press.

DiDomenico, R., Nissanov, J. and Eaton, R. C. (1988). Lateralization and adaptation of a continuously variable behavior following lesions of a reticulospinal neuron. *Brain Res.* **473**, 15-28.

Doyle, L. M. F., Stafford, P. P. and Roberts, B. L. (2001). Recovery of locomotion correlated with axonal regeneration after a complete spinal transection in the eel. *Neuroscience* **107**, 169-179.

Eaton, R. C. and Emberley, D. S. (1991). How stimulus direction determines the trajectory of the Mauthner-initiated escape response in teleost fish. *J. Exp. Biol.* **161**, 469-487.

Eaton, R. C., Bombardieri, R. A. and Meyer, D. L. (1977). The Mauthner-initiated startle response in teleost fish. *J. Exp. Biol.* **66**, 65-81.

Eaton, R. C., Lavender, W. A. and Wieland, C. M. (1981). Identification of Mauthner initiated response patterns in goldfish: evidence from simultaneous cinematography and electrophysiology. *J. Comp. Physiol. A* **144**, 521-531.

Eaton, R. C., Lavender, W. A. and Wieland, C. M. (1982). Alternative neural pathways initiate fast-start responses following lesions of the Mauthner neuron in goldfish. *J. Comp. Physiol.* **145**, 521-531.

Eaton, R. C., DiDomenico, R. and Nissanov, J. (1988). Flexible body dynamics of the goldfish C-start: implications for reticulospinal command mechanisms. *J. Neurosci.* **8**, 2758-2768.

Eaton, R. C., DiDomenico, R. and Nissanov, J. (1991). Role of the Mauthner cell in sensorimotor integration by the brain stem escape network. *Brain Behav. Evol.* **37**, 272-285.

Eaton, R. C., Lee, K. K. K. and Foreman, M. B. (2001). The Mauthner cell and other identified neurons of the brainstem escape network of fish. *Prog. Neurobiol.* **63**, 467-485.

Faber, D. S. (1984). Reorganization of neuronal membrane properties following axotomy. *Exp. Brain Res.* **Suppl. 9**, 225-239.

Faber, D. S. and Korn, H. (1978). *Neurobiology of the Mauthner Cell.* New York: Raven Press.

Faber, D. S. and Zottoli, S. J. (1981). Axotomy-induced changes in cell structure and membrane excitability are sustained in a vertebrate central neuron. *Brain Res.* **223**, 436-443.

Faber, D. S., Fetcho, J. R. and Korn, H. (1989). Neuronal networks underlying the escape response in goldfish: general implications for motor control. *Ann. N.Y. Acad. Sci.* **563**, 11-33.

Faber, D. S., Korn, H. and Lin, J.-W. (1991). Role of medullary networks and postsynaptic membrane properties in regulating Mauthner cell responsiveness to sensory excitation. *Brain Behav. Evol.* **37**, 286-297.

Fetcho, J. R. (1991). Spinal network of the Mauthner cell. *Brain Behav. Evol.* **37**, 298-316.

Fetcho, J. R. (1992). The spinal motor system in early vertebrates and some of its evolutionary changes. *Brain Behav. Evol.* **40**, 82-97.

Fetcho, J. R. and Faber, D. S. (1988). Identification of motoneurons and interneurons in the spinal network for escapes initiated by the Mauthner cell in goldfish. *J. Neurosci.* **8**, 4192-4213.

Foreman, M. B. and Eaton, R. C. (1993). The direction change concept for reticulospinal control of goldfish escape. *J. Neurosci.* **13**, 4101-4113.

Furshpan, E. J. and Furukawa, T. (1962). Intracellular and extracellular responses of the several regions of the Mauthner cell of the goldfish. *J. Neurophysiol.* **25**, 732-771.

Hackett, J. T. and Faber, D. S. (1983). Mauthner axon networks mediating supraspinal components of the startle response in the goldfish. *Neuroscience* **8**, 317-331.

Hanna, G. F., Nawar, N. N. and Sharma, S. C. (1998). Regeneration of ascending spinal axons in goldfish. *Brain Res.* **791**, 235-245.

Keil, J. H. (1940). Functional spinal cord regeneration in adult rainbow-fish. *Proc. Soc. Exp. Biol. Med.* **43**, 175-176.

Kirsche, W. (1950). Die regenerativen Vorgänge am Rückenmark erwachsener Teleostier nach operativer Kontinuitätstrennung. *Zschr. mikr. Anat. Forsch.* **56**, 190-265.

Koppányi, T. (1955). Regeneration in the central nervous system of fishes. In *Regeneration in the Central Nervous System* (ed. W. F. Windle), pp. 3-19. Springfield: Charles C. Thomas.

Koppányi, T. and Weiss, P. (1922). Functionelle Regeneration des Rückenmarks bei Anamniern. *Anz. Akad. Wiss. Wien Math. Nature Kl.* **59**, 206.

Larner, A. J., Johnson, A. R. and Keynes, R. J. (1995). Regeneration in the vertebrate central nervous system: phylogeny, ontogeny, and mechanisms. *Biol. Rev.* **70**, 597-619.

Lee, R. K. K., Eaton, R. C. and Zottoli, S. J. (1993). Segmental arrangement of reticulospinal neurons in the goldfish hindbrain. *J. Comp. Neurol.* **329**, 539-556.

Liu, K. S. and Fetcho, J. R. (1999). Laser ablations reveal functional relationships of segmental hindbrain neurons in zebrafish. *Neuron* **23**, 325-335.

McClellan, A. D. (1994). Time course of locomotor recovery and functional regeneration in spinal cord-transected lamprey: *in vitro* preparations. *J. Neurophysiol.* **72**, 847-860.

Nissanov, J. (1991). *Knowal: User's Guide and Programmer's Manual.* Philadelphia: Current Press.

O'Malley, D. M., Kao, Y.-H. and Fetcho, J. R. (1996). Imaging the functional organization of zebrafish hindbrain segments during escape behaviors. *Neuron* **17**, 1145-1155.

Pearcy, J. F. and Koppányi, T. (1924). A further note on regeneration of the cut spinal cord in fish. *Proc. Soc. Exp. Biol. Med.* **22**, 17-19.

Phelps, C. H. (1969). Comparative ultrastructure of spinal cord regeneration in teleosts and mammals. *Anat. Rec.* **163**, 244.

Schwab, M. E. and Bartholdi, D. (1996). Degeneration and regeneration of axons in the lesioned spinal cord. *Physiol. Rev.* **76**, 319-370.

Sharma, S. C., Jadhao, A. G. and Prasada Rao, P. D. (1993). Regeneration of supraspinal projection neurons in the adult goldfish. *Brain Res.* **620**, 221-228.

Titmus, M. and Faber, D. S. (1990). Axotomy-induced alterations in the electrophysiological characteristics of neurons. *Prog. Neurobiol.* **35**, 1-51.

Titmus, M., Faber, D. S. and Zottoli, S. J. (1986). Altered excitability of the goldfish Mauthner cell following axotomy. I. Characterization and correlations with somatic and axonal morphological reactions. *J. Neurophysiol.* **55**, 1424-1439.

Tuge, H. and Hanzawa, S. (1935). Physiology of the spinal fish, with special reference to the postural mechanism. *Sci. Rep. Tohoku Imp. Univ. Biol.* **10**, 589-606.

Tuge, H. and Hanzawa, S. (1937). Physiological and morphological regeneration of the sectioned spinal cord in adult teleosts. *J. Comp. Neurol.* **67**, 343-365.

Van Raamsdonk, W., Maslam, S., de Jong, D. H., Smit-Onel, M. J. and Velzing, E. (1998). Long-term effects of spinal cord transection in zebrafish: swimming performances and metabolic properties of the neuromuscular system. *Acta Histochem.* **100**, 117-131.

Wieland, C. M. and Eaton, R. C. (1983). An electronic cine camera system for the automatic collection and analysis of high-speed movement of unrestrained animals. *Behav. Res. Meth. Instr.* **15**, 437-440.

Yamada, H., Miyake, T. and Kitamura, T. (1995). Regeneration of axons in transection of the carp spinal cord. *Zool. Sci.* **12**, 325-332.

Zottoli, S. J. (1977). Correlation of the startle reflex and Mauthner cell auditory responses in unrestrained goldfish. *J. Exp. Biol.* **66**, 243-254.

Zottoli, S. J. (1978). Comparative morphology of the Mauthner cell in fish and amphibians. In *Neurobiology of the Mauthner Cell* (ed. D. S. Faber and H. Korn), pp. 13-45. New York: Raven Press.

Zottoli, S. J. and Faber, D. S. (1977). Lack of functional Mauthner cell recovery following spinal cord regeneration in the goldfish. *27th Proc. Int. Union Physiol. Sci., Congr. Paris* **13**, 839.

Zottoli, S. J. and Faber, D. S. (2000). The Mauthner cell: what has it taught us? *Neuroscientist* **6**, 25-37.

Zottoli, S. J. and Freemer, M. M. (1991). Behavioral recovery of the goldfish startle response after spinal cord crush. *Soc. Neurosci. Abstr.* **17**, 942.

Zottoli, S. J., Agostini, M. A., Danielson, P. D., Lee, E. J., Laidley, T. L., Markstein, E. A. and Scalise, T. L. (1988). Pathway selection by regenerating Mauthner axons of the adult goldfish. *Soc. Neurosci. Abstr.* **14**, 54.

Zottoli, S. J., Bentley, A. P., Feiner, D. G., Hering, J. R., Prendergast, B. J. and Rieff, H. I. (1994). Spinal cord regeneration in adult goldfish: implications for functional recovery in vertebrates. *Prog. Brain Res.* **103**, 219-228.

Zottoli, S. J., Hangen, D. H. and Faber, D. S. (1984). The axon reaction of the goldfish Mauthner cell and factors that influence its morphological variability. *J. Comp. Neurol.* **230**, 497-516.

Zottoli, S. J., Marek, L. E., Agostini, M. A. and Strittmatter, S. L. (1987). Morphological and physiological survival of goldfish Mauthner axons isolated from their somata by spinal cord crush. *J. Comp. Neurol.* **255**, 272-282.

Zottoli, S. J., Newman, B. C., Rieff, H. I. and Winters, D. C. (1999). Decrease in occurrence of fast startle responses after selective Mauthner cell ablation in goldfish (*Carassius auratus*). *J. Comp. Physiol. A* **184**, 207-219.

Zottoli, S. J., Northen, S. C. and Scalise, T. L. (1989). Regeneration of the goldfish Mauthner cell does not underlie behavioral recovery of the startle response. *Soc. Neurosci. Abstr.* **15**, 512.