

RESEARCH ARTICLE

Role of the lateral line mechanosensory system in directionality of goldfish auditory evoked escape response

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SUMMARY

Goldfish (*Carassius auratus*) escape responses to sudden auditory stimuli are mediated by a pair of reticulospinal neurons, the Mauthner (M-) cells, which integrate mechanosensory inputs from the inner ear and the lateral line (LL) to initiate a fast directional response away from the aversive stimulus. This behavior is context dependent; when near an obstruction the fish may rather turn towards the sound to avoid hitting the object. Mechanisms underlying this directionality remain unknown. Here we investigate the contribution of the LL system to auditory evoked escapes and provide behavioral evidence that it transmits stimulus – and environmental-dependent information that determines the initial response direction of the escape. We quantified escape latency, probability and directionality following abrupt sound stimuli before and after removal of the entire LL with 0.03 mmol l^{-1} cobalt chloride (CoCl_2), 0.002% gentamicin or selective posterior LL nerve (pLLn) transection. CoCl_2 significantly increased escape onset latency without affecting probability and reduced open field directionality from 77% to chance, 52%. This effect on directionality was also observed with gentamicin. Transection of the pLLn had no effect on directionality, indicating the anterior LL nerve (aLLn) afferents are more likely to transmit directional information to the M-cell. When the fish were near a wall, the error rate was quadrupled by both CoCl_2 and pLLn transection. Visual elimination had no influence on directionality unless combined with LL elimination.

Key words: startle, escape, Mauthner, lateral line.

INTRODUCTION

An organism's ability to successfully escape a predatory strike requires that its nervous system detects and integrates sensory cues to make appropriate and timely behavioral decisions that will guide the correct response. Historically, vertebrate and invertebrate escape behaviors have served as ideal models to address questions related to sensory motor integration as these systems have quantifiable behaviors and are under the command of tractable networks, sometimes containing identifiable neurons (Eaton, 1984). In teleost fish, abrupt and unexpected stimuli trigger a short latency escape behavior, the C-start, which is under the command of a pair of 'decision-making' reticulospinal neurons called the Mauthner (M-) cells (Eaton et al., 1977; Eaton et al., 1991; Zottoli, 1977). Following an abrupt auditory stimulus, the M-cell on one side is activated, sending a downstream signal that overrides all other swimming motions to contract the muscles along the length of the body on the opposite side (Fetcho, 1991).

A striking feature of this behavior is that in an unobstructed open field it is appropriately directional, thus serving as an important predatory evasion mechanism (Blaxter et al., 1981). This behavior is modifiable and context dependent. When the escape path is obstructed, the fish is most likely to turn to avoid hitting the obstacle, which means a directional override (Eaton and Emberley, 1991). This implies that the M-cell system is capable of localizing the sound source and that the two M-cells are differentially excited or inhibited. This computational mechanism is likely to be complex and require contributions from more than one component of the octavolateralis system. Indeed, the cellular mechanisms whereby fish can achieve

directional escapes and suppress it are not yet understood. It has been proposed that fish localize sounds by comparing the phase of pressure and particle motion of a sound source (Schuijf and Buwalda, 1975; Schuijf and Buwalda, 1980). Eaton and colleagues (Eaton et al., 1995) proposed that the phase model can be applied to Mauthner-mediated escape responses, whereby the M-cell encodes sound direction by comparing the non-directional, sound pressure generated by the swimbladder and the directional particle motion (acceleration) resulting from the displacement of otoliths (Popper and Fay, 1993). However, attempts to validate this hypothesis by stimulating the different components of the inner ear have not revealed directional information detected by the M-cell (Canfield and Eaton, 1990).

Because both the mechanosensory lateral line (LL) organ and the inner ear are responsive to many of the same stimulus fields (Braun and Coombs, 2000), we investigated the possibility that the LL may be contributing to the directional nature of the escapes. In a series of behavioral studies where components of the LL system were selectively removed, we observed that the LL system is capable of encoding directionality in the escape response. The data also indicate a potential role of the LL in the decision-making process of the M-cell when there is an obstruction in the escape path. Increase in onset latency of the escape responses after eliminating LL input suggests that this modality can also affect the behavioral threshold of the escape.

MATERIALS AND METHODS

Experimental animals

Goldfish [*Carassius auratus* (Linnaeus 1758), length 4–6 inches] were obtained commercially from Huntington Creek Fisheries (Thurmont,

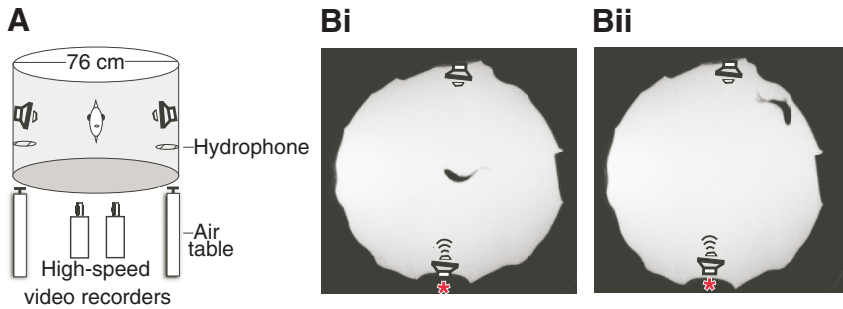


Fig. 1. (A) Experimental setup for recording auditory evoked escape responses in goldfish. Sound stimuli were generated randomly through one of a pair of underwater speakers positioned at opposing sides of the tank. Responses were captured at 1000 frames per second by high-speed video recorders. (B) Directional escapes were elicited in individual goldfish. Top view of the tank showing (Bi) an aversive C-start when the fish is in the center of the tank and (Bii) directional override when the fish is within two-thirds of its body length near the wall of the tank. Red asterisks mark the speaker that generated the sound in each case.

MD, USA) and EECHO Systems (North Kansas City, MO, USA) and maintained as previously described (Preuss and Faber, 2003). The goldfish were acclimated to our holding system for at least 2 weeks to allow recovery from stresses caused during their transportation and the new housing conditions in our laboratory environment.

Behavior setup and testing

Escapes were evoked in a circular aquarium with a 72 cm diameter; water temperature was maintained at 19°C (Fig. 1A). The testing tank was mounted on an anti-vibration table (TMC 63-530; Technical Manufacturing Corp., Peabody, MA, USA) to minimize vibrations and external mechanosensory cues. The walls of the tank were lined with polyurethane foam, which encased two underwater loud speakers (UW-30; University Sound, Buchanan, MI, USA) on opposite sides of the tank, to minimize speaker vibrations as well as visual cues from the outside environment. Illumination was provided through a 3 mm thick translucent tank cover by a single 100 W floodlight centered above the tank and from below by three 40 W lights with translucent covers.

Short-latency C-start escapes were triggered by single sinusoidal waves of 200 Hz produced by a digital waveform generator (Model 39; Wavetek Ltd, Norwich, Norfolk, UK) in combination with an audio power amplifier (Servo 120; Samson, Syosset, NY, USA). The direction of the sound source varied randomly, as did the stimulus amplitude and the time intervals between trials, from 130 to 170 dB re. 1 μ Pa (which translates to 60–110 dB in air) and 1–20 min, respectively. A high-speed color video camera recorded ventral views of the behavior at 1000 frames⁻¹ at a resolution of 512 \times 384 pixels (Kodak Ekta Pro 1000 HRC; Eastman Kodak, San Diego, CA, USA), which allowed us to analyze escape kinematics with a 1 ms temporal resolution. Data were initially stored on an internal magneto optical drive and then re-recorded onto a VCR, preserving temporal resolution. A 1 ms LED stimulus marker outside of the tank marked the onset of the sound stimulus and served as a reference point for latency measurements. In addition, two hydrophones (SQ05; Sensor Technology, Collingwood, ON, Canada), one positioned close to the underwater speaker and the other on the opposite border, recorded the waveform and amplitude of the auditory stimulus (30 μ s sampling rate).

The absence or presence of the escape response and its latency and direction were noted following each sound pulse. Because response directionality has been shown to be influenced by nearby obstacles (Foreman and Eaton, 1993; Preuss and Faber, 2003), center-field responses (where the fish was in the center of the tank, unobstructed) and near-wall responses (where the fish was at the periphery of the tank within two-thirds of its body length from the wall) were analyzed separately (Fig. 1B). The fish were tested successively, with control trials performed initially and experimental conditions performed on a subsequent day, after the fish were allowed to recover from the appropriate treatment. Therefore, each

fish served as its own control. A minimum of 15–20 trials were performed for each fish, and only one fish was tested at a time.

Kinematic analysis

To confirm that escapes triggered following complete LL elimination were M-cell initiated, we measured two of the kinematic parameters that have been shown to be directly governed by M-cell activation: namely, stage 1 turn angle peak velocities and escape latency (Eaton and Emberley, 1991; Nissanov et al., 1990). For each escape sequence, 180 frames (starting 10 frames before the stimulus) were digitized and the *x*- and *y*-positions of the head and the center of mass (COM) were tracked manually using ImageJ Software (NIH, Bethesda, MD, USA). The vector connecting these two points was used to quantify fish location and orientation, as shown in Fig. 2A,B for different stages of the response. The COM was defined as a midline point located between the pectoral fins of the animal. Escape trajectories (Fig. 2Bii) were analyzed as described by Preuss and Faber (Preuss and Faber, 2003), using macros developed for Igor Pro. Instantaneous velocity and acceleration of the COM, and the total distance traveled by the COM at various time intervals (i.e. 50, 100 and 150 ms after stimulus onset) as well as angular velocity and acceleration were calculated from smoothed (10 factor binomial; Igor Pro; Wavemetrics, Lake Oswego, OR, USA) *x,y* position data. Response latencies were measured at the first detectable movement of the head after the stimulus onset, and the direction of the C-start was recorded relative to the location of the stimulus. Stage 1 was defined as the interval beginning with movement of the head and ending with forward propulsion of the COM by more than 5 mm (Eaton and Emberley, 1991; Nissanov et al., 1990).

Elimination of LL inputs

Several methods were used to either eliminate the LL organ completely or to selectively remove distinct LL components.

Cobalt treatment

Pharmacological blockade of lateral-line receptors using cobalt has been well established for use in freshwater species (Karlsen and Sand, 1987). Fish were exposed to 0.03 mmol⁻¹ cobalt chloride [CoCl₂.6H₂O] (Sigma-Aldrich, St Louis, MO, USA) made in calcium free solution of Mili-Q water plus (in mmol l⁻¹): NaCl 0.2; KCl 0.025; Na₂HPO₄ 0.05; KH₂PO₄ 0.05; MgSO₄ 0.1. NaOH was added to maintain a pH of 7.5. We used the lowest concentration of cobalt that has been reported to effectively block the LL (Karlsen and Sand, 1987), since higher concentrations have been shown to result in behavioral changes in the fish that could otherwise confound the results (Casagrand et al., 1999; Janssen, 2000).

Gentamicin treatment

Gentamicin has been traditionally thought to selectively damage the canal neuromasts of the lateral line (Song et al., 1995) while leaving

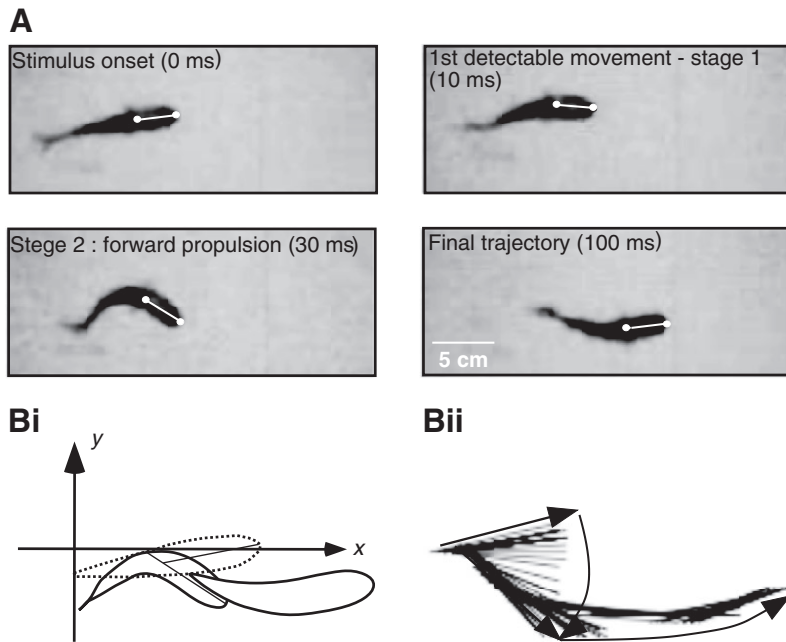


Fig. 2. Stages of the C-start escape and measurements of escape kinematics (A) Video picture showing selected frames of the stages of a goldfish escape behavior filmed from below at 1000 frames per second. Heavy lines represent anterior midline from head to center of mass (COM). (Bi) The x - and y -positions of the head and COM were measured and the vector connecting these two points was used to measure the angle of orientation, taking the position at the first frame as the reference point. (Bii) Computer-generated plot of the trajectory in A at 2 ms intervals.

the superficial neuromasts intact, which was the rationale to use this treatment in our studies. However, recent studies by van Trump et al. (van Trump et al., 2010) suggest that gentamicin damage to LL hair cells may not be as specific *in vivo*. In light of these recent results our gentamicin treatment can be still understood as a second independent means means of eliminating the entire LL system. Fish were immersed in aerated conditioned water containing 0.002% gentamicin sulfate (Sigma-Aldrich, St Louis, MO, USA) for 3 days. The water was changed daily and new gentamicin added each day. Fish were tested on the 4th day after treatment.

Posterior LL nerve transection

The posterior LL nerve (pLLn) runs rostro-caudally along the lateral side of the trunk of the fish and innervates the neuromasts on the body of the fish. It lies very close to the surface of the skin and is easily visible with the naked eye after removal of superficial scales just below the operculum. The nerve was transected after initially anesthetizing the fish with a continuous perfusion of ice water through the mouth containing 60 mg l^{-1} MS222 (3aminobenzoic acid ethyl ester; Sigma, St Louis, MO, USA). Lidocaine was added to the exposed area before a small incision was made. A cut was made into the nerve and a small piece (2–3 mm) was removed to guarantee separation of the two ends. The fish were then removed from the surgery chamber and, using a small Pasteur pipette, water was perfused through the mouth as the fish was floated in the tank until it was able to breath and subsequently swim on its own. For the entire procedure, gloves were worn, to minimize infection and removal of the slime coat of the fish. Following the surgical procedure, fish were allowed to recover for 24 h prior to testing.

Elimination of visual inputs

Fish were anesthetized as above and placed in the surgical chamber. The area around the eye was completely dried using kimwipes, taking care not to damage the eye. Eye caps were prepared from the end of plastic Pasteur pipettes, painted with dark nail varnish to minimize light penetrating, and small holes were punctured into them to allow water to circulate through. The eye caps were allowed

to dry overnight and were then glued on to the area around the eye using non-toxic tissue adhesive glue (Vetbond, n-butyl cyanoacrylate; 3M, St Paul, MN, USA). After the eye caps were completely dry, the fish were allowed to recover and testing began 24 h later. In cases where vision and the LL were both eliminated, we transected the nerve as described above at the same time as the caps were placed. Another group of animals was treated with cobalt 24 h recovery from the visual elimination.

Behavioral observations after treatments

The fish were monitored during the recovery to ensure that they fed and swam normally. There were no noticeable changes in the swimming behavior of the fish following treatment except for the blindfolded fish, which were observed to spend a greater amount of time in the center of the tank swimming in a distinct circular motion also observed following bilateral optic nerve transection (Kato et al., 1999).

Statistical analysis

All statistical tests were carried out using Statview 5.0 (SAS Institute, Cary, NC, USA) and Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). In the first series of experiments, we tested each animal (unless otherwise stated) before and after treatment to get a baseline response in controls, to ensure the effect was due to treatment and not another variable, for example, health of the fish. For these experiments escape probabilities, latencies and the directionality of eliciting a C-start were calculated for each fish before and after treatment. Paired t -tests were used to determine differences in the mean latencies between treatments for each animal and the Wilcoxon signed-rank test for non-parametric data was used to determine differences in mean response probability and directionality between treatment for each animal. For our second series of experiments (the visual eliminations studies) the data were not paired, as we compared the treatment group to a control group. Unpaired t -test comparison of individual controls showed no difference in their response probabilities or latencies. We have consolidated all the trials for the fish in each experimental group since after treatment the decrease in response probability reduced

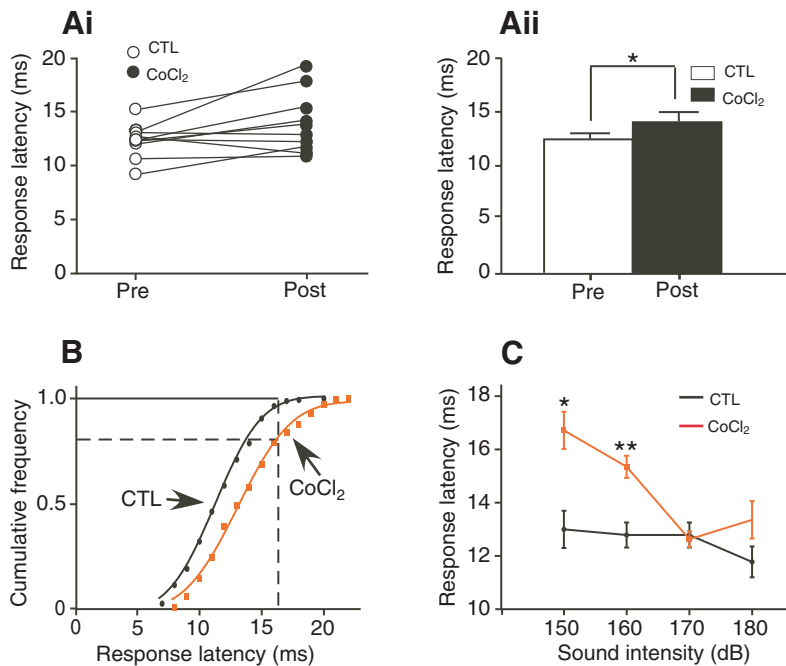


Fig. 3. Response latency following $0.03 \text{ mmol l}^{-1} \text{ CoCl}_2$ treatment. (Ai) Lines join paired data for each fish. (Aii) Bar plots represent mean response latency (ms) from the same data sets \pm s.e.m. for each group pre- and post-treatment. Asterisks represent statistically significant treatment differences (Wilcoxon signed-rank test, $*P < 0.05$). (B) Cumulative frequency graph showing a shift in response latency following treatment with cobalt. (C) Response latency is significantly higher in cobalt treated animals at lower stimulus intensities. Asterisks represent statistical significance as calculated by one-way ANOVA followed by Tukey's pairwise comparison ($*P < 0.05$, $**P < 0.01$). CTL, control.

the number of trials that were available to assess directionality per individual fish. Latencies were compared by repeated-measures ANOVA whereas probability and directionality was assessed with the Fisher's test.

RESULTS

LL elimination increases the onset latency of the escape

Cobalt chloride (CoCl_2) has been shown to reversibly inactivate both the canal and superficial neuromasts of the LL (Karsen and Sand, 1987), thereby resulting in complete inactivation of the sense organ. We found this treatment to significantly increase the onset of the escape, from 12.40 ± 0.50 to 13.97 ± 0.70 ms, as shown in Fig. 3A (Wilcoxon signed-rank test, $P = 0.0371$, $N = 10$). This statistical significance reflects an increase in latency in 7 out of 10 fish. The increase of C-start latencies after treatment likely reflects a delayed firing of the M-cell; however, potentially the response delay can be also explained by triggering of the response by other reticulospinal neurons (Liu and Fetcho, 1999; Zottoli et al., 1999). With the goal of distinguishing between these options we compared the cumulative latency distributions before and after treatment. Both were fit with single Gaussians with a shift to the right after cobalt treatment, supporting a longer mean latency of the M-cell initiated C-starts (Fig. 3B).

A one-way ANOVA was conducted to compare the effect of removing the LL on response latency at different sound intensity ranges. Response latency was independent of sound intensity in control ($F_{3,100} = 0.71$, $P = 0.548$), but without the LL there was a significant effect on response latency for the four different intensity ranges ($F_{3,168} = 15.66$, $P < 0.0001$). Tukey's *post hoc* comparison of the four intensities after LL elimination indicate that the mean latency at 150 dB re. 1 μPa (16.72 ± 0.70) was significantly different than the means at higher intensity of 170 dB re. 1 μPa (12.63 ± 0.30) and 180 dB re. 1 μPa (13.36 ± 0.70) (Fig. 3C). Furthermore at lower stimulus intensities the mean latency after cobalt was significantly higher than mean control latency. Specifically, at 150 dB re. 1 μPa , mean latency increased from 13.0 ± 0.70 ms in the control to 16.72 ± 0.70 ms after cobalt (unpaired *t*-test, $t = -5.33$, d.f. = 87, $P < 0.0001$) and at 160 dB re. 1 μPa , means increased from 12.79 ± 0.47

to 15.35 ± 0.42 ms after cobalt (unpaired *t*-test, $t = -3.46$, d.f. = 26, $P < 0.002$).

Gentamicin sulphate is an ototoxin that has been shown to damage the hair cells of canal and superficial neuromasts of the LL (van Trump et al., 2010). We tested seven fish before and after treatment with 0.002% gentamicin sulphate. Even though there was an increase in response latency after gentamicin treatment from 12.01 ± 0.34 ms before to 12.91 ± 0.36 ms, the result lacked statistical significance (Wilcoxon signed-rank test, $P = 0.1568$). This result suggests that gentamicin is not as affective at blocking the entire LL system as cobalt chloride.

There are two main LL afferent nerves in the goldfish; the pLLn innervates the neuromasts on the trunk of the fish and the anterior LL nerve (aLLn) innervates the neuromasts on the head of the fish (Puzdrowski, 1989). The pLLn runs very close to the surface of the skin rostro-caudally along the lateral side of the trunk. Therefore bilaterally transecting the pLLn is a relatively non-invasive method to distinguish between the contributions of the trunk *versus* head LL receptors. Nine animals were tested before and 24 h after pLLn transection. However, the latency was unchanged, equaling 12.27 ± 0.34 ms before and 12.59 ± 0.45 ms after pLLn transection (Wilcoxon signed-rank test, $P = 0.1094$).

There are two main stages to the C-start. Stage 1 is triggered by the M-cell and is characterized by a rapid and intense C-shaped turn. This is then followed by propulsion away from the initial location (stage 2), which requires the activity of other reticulospinal neurons (Domenici and Blake, 1997; Eaton and Emberley, 1991; Eaton et al., 2001). The kinematic parameters of stage 1 have been shown to be stereotypical for M-cell initiated escapes. We therefore analyzed measurements of these parameters as a means to verify the responses were M-cell initiated. There were no significant differences in any of the stage 1 kinematic parameters between control and cobalt, suggesting that all the responses were M-cell initiated (Table 1). However, there was a significant decrease in the distance travelled at 150 ms (control, 83.29 ± 3.8 mm, $N = 24$; CoCl_2 , 65.20 ± 4.3 mm, $N = 22$) (one-way ANOVA, $F = 47.33$, $R^2 = 0.64$, d.f. = 5, $P < 0.0001$). This suggests that LL input influences the final trajectory of the escape and hence the activity of other reticulospinal motor neurons.

Table 1. Kinematic parameters measured in control and CoCl₂-treated goldfish

Parameter	Control	CoCl ₂
Stage 1 duration (ms)	21.24±0.83	25.28±1.3
Angle at the end of stage 1 (deg)	40.84±2.93	42.62±3.17
Peak angular velocity (deg ms ⁻¹)	4.86±1.57	4.49±1.57
Distance moved after 70 ms (mm)	43.89±0.96	39.51±1.8
Distance moved after 150 ms (mm)	89.97±4.0	68.0±3.6***

Values are means ± s.e.m. for 24 and 22 trials (control and 0.03 mmol l⁻¹ CoCl₂, respectively).
****P*<0.001, calculated by one-way ANOVA.

LL elimination does not affect escape probability

The M-cell is the first reticulospinal neuron to fire in the escape network (Eaton et al., 1991; Weiss et al., 2006; Zottoli, 1977). That is, it triggers the behavior; therefore, the escape probability is a direct indication of M-cell responsiveness. We asked whether afferent input from the LL influences the probability of getting an escape response. In contrast to escape latency, CoCl₂ did not have a significant effect on escape response probability (Fig. 4Ai,ii). Mean response probability was 82.45±6.24% pre-treatment and 83.40±6.84% after CoCl₂ (Wilcoxon signed-rank test, *P*=0.9102, *N*=10). As shown in Fig. 4B, average escape probabilities ranged from 0.5 to 0.7 as intensity increased, and there were no significant differences in the intensity dependence between control and cobalt experiments. Furthermore following transection of the pLLn, escape probabilities did not significantly change from 79.72±5.26% pre-treatment to 85.01±4.76% post-treatment (Wilcoxon signed-rank test, *P*=0.4961, *N*=9). The same result was observed following with gentamicin, from 77.93±4.02% pre-treatment to 74.29±3.35% post-treatment (Wilcoxon signed-rank test, *P*=0.5999, *N*=7).

C-start directionality in the open field is eliminated following LL elimination

Escapes used for center field studies required not only that prior to stimulus onset the fish be located in the center of the tank but also that there be a clear directional choice (i.e. initial body orientation perpendicular to the underwater speaker). Center field percent correct responses are presented for each animal before and after treatment and were determined by expressing the number of escapes away from the stimulus as a fraction of the total number of escapes ×100. Mean % of correct responses was calculated for each fish before and after treatment and the two-tailed Wilcoxon matched pair test was used to analyze the data.

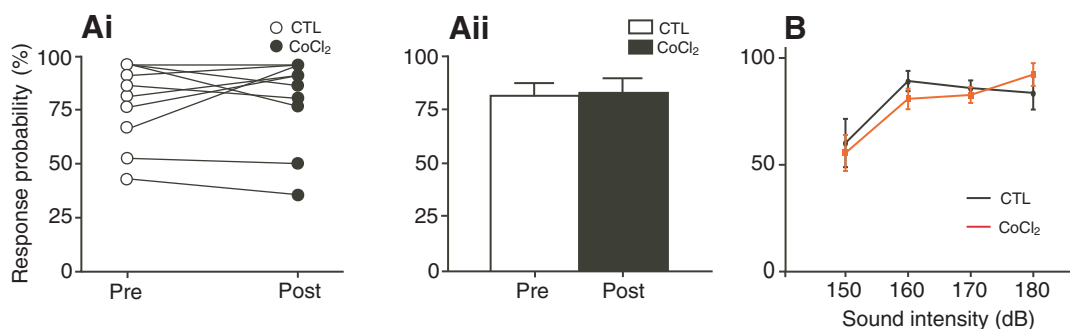


Fig. 4. Probability of eliciting C-starts following 0.03 mmol l⁻¹ CoCl₂ treatment, (Ai) Lines join paired data for each fish. (Aii) Bar plots represent mean response frequencies (%) from the same data sets ± s.e.m. for each group pre- and post-treatment. Means were not statistically different. (B) Escape response frequency at increasing sound intensities in control (CTL) or 0.03 mmol l⁻¹ CoCl₂-treated animals.

Cobalt treatment resulted in a significant decrease in the mean percentage of correct responses from 76.61±5.12 to 52.45±6.02% (*P*=0.0156) (Fig. 5). This reflects a decrease in seven out of nine animals whereas the other two apparently were unaffected. Similarly, with gentamicin treatment the directionality of the escape responses was significantly reduced in five out of six animals tested, with one animal remaining unaffected. Overall, the mean percentage of correct responses dropped from 78.52±7.20 to 42.54±5.10% (*P*=0.0313; Fig. 5). These results closely match those observed in the cobalt treated animals. Although the percentage of correct responses decreased following pLLn transection, this difference was not significant, 70.08±7.94 to 64.38±8.41% (*P*=0.5281).

Directional overrides in the presence of an obstruction may be influenced by the LL

The LL is typically understood as an organ that detects the presence of nearby objects (von Campenhausen et al., 1981). We therefore asked whether this system plays a functional role in the directional overrides observed in the escapes that occur when the fish is close to the wall of the tank (Fig. 6). In such a scenario the wall is an obstruction and hence a turn away from the stimulus (as observed for center tank responses) would turn the fish into the wall, a likely maladaptive behavior. Thus, fish can: (1) turn away from the wall and thus towards the direction of the sound source (directional override); (2) turn towards the wall and then execute a direction change to avoid hitting the wall; or (3) turn towards the wall and hit it (Fig. 6). Because the initiation of the response is under the control of the M-cell, we first asked if LL elimination affects the frequency of escapes away from the wall, i.e. the directional overrides. These were calculated as the percentage of trials where the fish turned away from the wall relative to the total number of responses near the wall. Mean % of directional overrides was calculated for each fish before and after treatment and the two-tailed Wilcoxon signed-rank test was used to analyze the data.

There was no significant difference in the mean percentages of directional overrides observed in any of the treatment conditions: CoCl₂, 67.58±4.96% before and 60.63±6.72% after treatment (*P*=0.4609); gentamicin, 65.44±3.28% before and 71.47±8.23% after treatment (*P*=0.6875); or pLLn transection 82.91±7.07% before to 61.00±6.77% after treatment (*P*=0.0742). However, a closer inspection of responses when the fish turned towards the wall, revealed that cobalt significantly increased the frequency of occurrences of behaviors where the fish was unable to change its trajectory adequately enough to avoid hitting the wall from 3.68±1.90 to 16.34±4.30% (*P*=0.0207). These inappropriate responses also occurred at a significantly higher rate in the pLLn

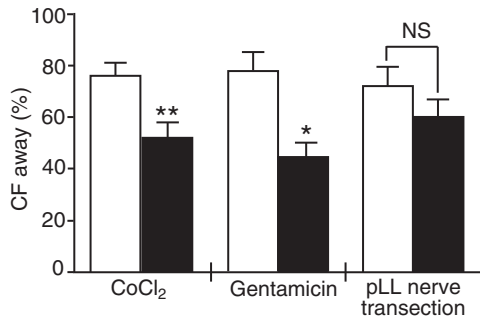


Fig. 5. Comparison of frequency of correct (aversive) escape responses in the center field (CF) following complete lateral line (LL) elimination with 0.03 mmol l^{-1} CoCl_2 or 0.002% gentamicin, or partial LL elimination through pLLn transection. Column graphs represent mean frequency of correct responses \pm s.e.m. for each group pre- and post-treatment. Asterisks represent statistically significant pre- and post-treatment differences in response (Wilcoxon signed-rank test, * $P < 0.05$, ** $P < 0.01$). NS, not significant.

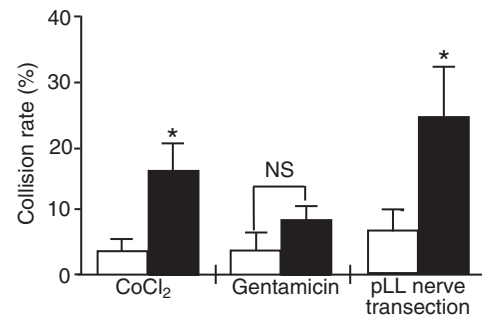


Fig. 7. Comparison of frequency of near wall collisions following complete lateral line (LL) elimination with 0.03 mmol l^{-1} CoCl_2 or 0.002% gentamicin or partial LL elimination through pLLn transection. Column graphs represent mean frequency of collisions (%) from the same data sets \pm s.e.m. for each group pre- and post-treatment. Asterisks represent statistically significant pre- and post-treatment differences in response (Wilcoxon signed-rank test, * $P < 0.05$). NS, not significant.

transection animals from 5.27 ± 3.68 to $22.11 \pm 5.31\%$ ($P = 0.0078$). However, an observed increase after gentamicin treatment from 3.88 ± 2.87 to $8.64 \pm 2.44\%$ was not significant ($P = 0.1563$; Fig. 7).

Visual influences on escape characteristics

It is possible that near wall responses might have been influenced not only by LL inputs but also by visual cues. We therefore compared a control group of fish ($N = 10$) with those that had visual input eliminated alone ($N = 6$) or in combination with the LL through cobalt treatment ($N = 9$). We compared the total number of responses across all trials and observed a significant decrease in response probability

following visual elimination alone or together with cobalt treatment [control, 76.15% (166 out of 218 trials); visual elimination, 47.92% (69 out of 144 trials), Fisher's two-tailed test, $P < 0.0001$; visual and LL elimination, 59.75% (141 out of 236 trials), Fisher's two-tailed test, $P = 0.0002$; Fig. 8A].

Escape latency also increased significantly (Fig. 8B) from 14.05 ± 0.28 ms in controls to 15.68 ± 0.43 ms following visual elimination and 16.58 ± 0.38 ms following both visual and complete LL elimination (one-way ANOVA, $F = 76.93$, $R^2 = 0.31$, d.f. = 2, $P < 0.0001$; Tukey's *post hoc* comparison of control and visual elimination, 95% CI = -3.534 to -1.611 , $P < 0.0001$; control and visual and LL elimination, 95% CI = -4.892 to -3.305 , $P < 0.0001$).

We also analyzed center field and near wall escape directionality for these groups of fish (Fig. 9). In the center field, vision alone did not influence directionality; however, when both vision and LL were removed, escape directionality was diminished to chance level [control, 72.86% (51 out of 70 responses); visual elimination, 75.76% (25 out of 33 responses); visual elimination and cobalt, 55.56% (19 out of 37 responses), Fisher's two-tailed test, $P = 0.033$]. This is consistent that the LL is critical to the correct directional responses in the open field.

Vision also had little influence on directional overrides unless the LL was also eliminated [control, 72.09% (62 out of 86 responses); visual elimination, 60.00% (21 out of 35 responses), Fisher's two-tailed test, $P = 0.2029$; visual elimination and cobalt 57.14% (56 out of 98 responses), Fisher's two-tailed test, $P = 0.0451$; Fig. 9]. There was also a significant increase in the frequency of collisions into the wall following both visual and LL elimination, which was not evident when only vision was eliminated [control: 10.46% (9 out of 86 responses); visual elimination: 20.00% (7 out of 35 responses); Fisher's two-tailed test, $P = 0.2343$; visual elimination and cobalt 34.78% (38 out of 98 responses; Fisher's two-tailed test, $P < 0.0001$) (Fig. 9).

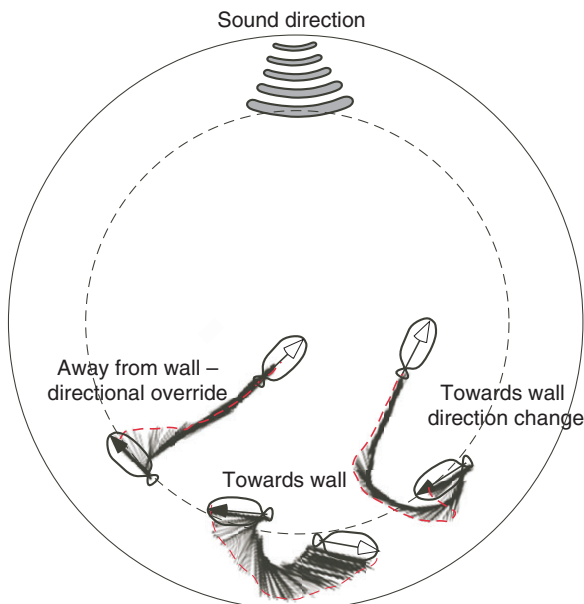


Fig. 6. Computer-generated silhouettes of various escape trajectories when the fish is near the wall. Lines represent x - and y -coordinates at 2 ms intervals. Filled arrows represent the starting position of the fish and open arrows represent the final position of the fish at the end of the C-start. The dashed black line depicts the area where the fish are within two-thirds of their body length away from the wall (solid line). This marks the near wall area in the behavior tank. The trajectories are depicted by the dashed red lines.

DISCUSSION

The aim of this study was to quantitatively determine the contribution of the mechanosensory LL system to the acoustic escape response, especially its directionality. We demonstrate here that in the absence of the LL, escape directionality is diminished to mere chance when the fish is in an open field. Although response probability remained unaffected, latencies increased significantly

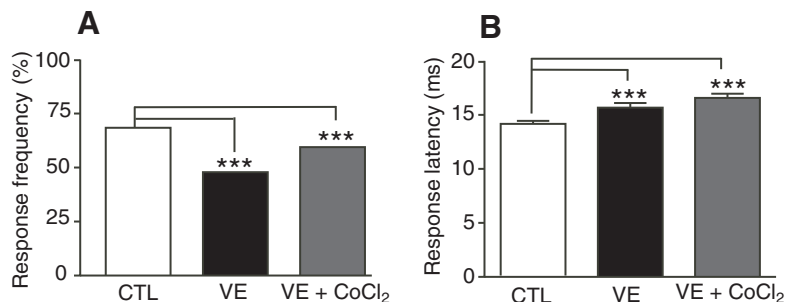


Fig. 8. Visual elimination in combination with lateral line (LL) elimination decreases the occurrence of escapes and increases response latency. (A) Column graphs represent response frequencies calculated as the percentage of escapes recorded out of the total number of trials presented in each experimental group. Asterisks represent statistical significance as calculated by Fisher's two-tailed test ($***P < 0.001$). (B) Column graphs represent mean response latencies for the total number of observed escapes in each experimental group. Asterisks represent statistical significance as calculated by one-way analysis of ANOVA followed by Tukey's pairwise comparison ($***P < 0.001$). CTL, control; VE, visual elimination; VE + CoCl₂, visual elimination + cobalt chloride treatment.

when the LL system was removed with cobalt. In cases where fish were close to the wall, we observed a marked increase in the frequency of events in which fish that turned towards the wall collided with it. When viewed in the context that other sensory inputs likely provide the major contribution for bringing the M-cell to threshold, the present data suggest an essential role of LL inputs for C-start directionality, particularly for open field responses.

LL contribution to the escape behavioral threshold

The M-cells set the threshold for escape. If there is sufficient sensory input, both cells are depolarized close to threshold. However, only one fires an action potential that triggers the escape behavior (Eaton et al., 1991; Faber et al., 1991; Zottoli, 1977). The M-cell has a very low input resistance and a high resting membrane potential, properties that ensure escapes only occur in response to large amplitude sounds. It is certain that (at least in goldfish) acoustic stimuli that trigger an escape engage more inputs than those associated with the LL system. Indeed, inputs from the anterior and posterior eighth nerve afferents have been shown to project to the M-cell lateral dendrite (Lin and Faber, 1988; Szabo et al., 2007; Zottoli and Faber, 1979), and all evidence indicates the fast synaptic inputs from large myelinated club endings of afferents in the posterior branch of the eighth nerve are required for M-cell firing, in the case of abrupt sound stimuli applied in air (Canfield and Eaton, 1990). The issue here is whether input from the LL, acting in concert

with the other sensory organs, can influence the threshold and timing of an escape.

In our experiments with abrupt auditory stimuli we observed that after the elimination of the LL with cobalt, response latencies significantly increased (Fig. 3) whereas escape probability remained unaffected (Fig. 4). We saw similar trends with gentamicin that were however not statistically significant. This may be due to a slight difference between the efficacy of gentamicin and cobalt on the whole or individual components of the LL. Nevertheless, the simplest explanation for the observed change in threshold is that there was at least a 1.5 ms longer time window in which the response could be evoked after LL elimination. Indeed at sound intensities where 100% of control animals respond, only approximately 80% of cobalt-treated animals respond (Fig. 3B). This implies a decrease in the initial excitation to the M-cell in the time window where threshold is normally reached. The increased time window reflects a reduction in the initial excitation of the M-cell. In addition, the C-start probability has been shown to depend on the intensity of the stimulus (Neumeister et al., 2008). We used relatively high stimulus intensities in our study to ensure sufficient escapes in the control animals, and that protocol may have masked small contributions to threshold from the LL that would have been detected at lower stimulus intensities. Consistent with this notion is the finding that the increase in C-start latency after LL elimination is more pronounced with the weaker stimuli (Fig. 3C).

The latency effect is consistent with a reduction in excitatory input to the M-cell, such that remaining inputs take longer to bring the cell to threshold. This result raised the question of whether the longer latency responses were nevertheless M-cell triggered. The similar kinematics suggested they were. Furthermore, there is sufficient evidence that C-starts are M-cell initiated if the cell is not experimentally eliminated (Weiss et al., 2006; Zottoli et al., 1999). Although escapes triggered in experiments where the M-cell was lesioned only differed from controls in that they had a longer latency, albeit by a greater increment, 7 to 8 ms *versus* 1.5 ms in the present study (Eaton et al., 1988; Zottoli et al., 1999). In addition, the cumulative frequency distribution of the latencies (Fig. 3B) indicates that the responses are all from one population. Thus, we conclude that C-starts before and after LL elimination are M-cell generated.

The conclusions concerning threshold are consistent with electrophysiological data: Faber and Korn (Faber and Korn, 1975) reported that stimulation of the pLLn produces only weak excitation of the proximal region of the lateral dendrite, although it remains uncertain whether these inputs are mono- or polysynaptic (Zottoli

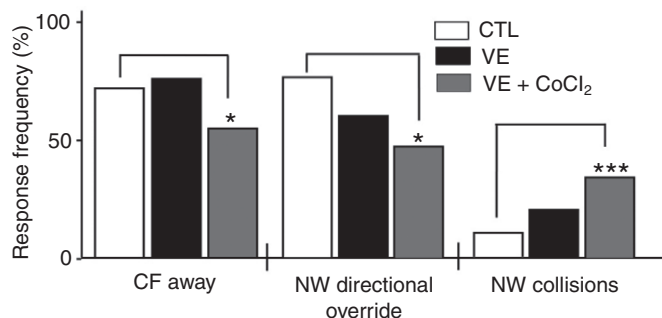


Fig. 9. Visual elimination is not required for directional responses in the open field (CF) or near wall (NW). Column graphs represent the frequency of responses (out of the total number of response). Asterisks represent statistical significance as calculated by Fisher's exact probability test ($*P < 0.05$, $***P < 0.001$).

and Van Horne, 1983). It should be noted that more recently, we found that the aLLn also projects to the Mauthner cell, with a synaptic delay that is short enough for this input to fall within the processing window of the M-cell, approximately 2 ms (Mirjany and Faber, 2011).

The LL influences the directionality of the escape

Many behavioral studies have shown that fish are capable of localizing a sound source (Buwalda et al., 1983; de Munck and Schellart, 1987; Fay, 1984; Rogers et al., 1988; Schellart and de Munck, 1987; Schuijf and Buwalda, 1980). However, an understanding of the cellular mechanisms by which fish achieve directional hearing is unclear. Although the Mauthner cell initiated escape behavior has been shown to be appropriately directional (Blaxter et al., 1981; Eaton and Emberley, 1991; Preuss and Faber, 2003), to date the source of the directional input and the mechanism whereby the Mauthner cell computes this information is not clear.

The major input to the M-cell lateral dendrite, the electrically coupled afferents of the saccular otoliths, ensures speed and reliability of the escape (Canfield and Eaton, 1990; Lin and Faber, 1988; Lin et al., 1983). However, this input lacks directional information since it depends on vibration in the central swimbladder, which responds to sound pressure changes and by design equally activates the left and right sacculi and M-cells. Although the utricle is specialized to detect sound acceleration, which does carry directional information, there is no conclusive evidence that either Mauthner cell is activated differentially following utricular stimulation (Casagrand et al., 1999; Eaton et al., 1995). This may be due to the limitations of the experimental paradigms and that these sensory channels have to be co-activated to encode directionality. However, this information might be encoded in feedforward inhibition of the M-cell, activated by the same sensory afferent pathway (Casagrand et al., 1999; Guzik et al., 1999; Weiss et al., 2008). Alternatively, these inputs may not be the source of the decision concerning which M-cell fires and therefore sets the response direction. Our data nevertheless indicate that although these systems may potentially contribute to directionality (Eaton et al., 1995; Eaton and Emberley, 1991; Eaton and Popper, 1995; Weiss et al., 2008), the Mauthner cell networks cannot solve the underlying computational task without the LL.

Previous results have shown that the directionality of C-starts depends upon where the fish is located in the experimental tank, i.e. in the open field or close to an obstacle that blocks an appropriate escape trajectory (Foreman and Eaton, 1993; Preuss and Faber, 2003). Therefore, we analyzed the directional nature of the two classes of escapes separately. We discuss the open field C-starts in this section and leave the near wall escapes for the next section. Complete LL elimination with cobalt resulted in a non-directional response (Fig. 6). The same result was obtained following gentamicin treatment, confirming that the detection of acceleration signals that influence directionality may be the function of the LL. Indeed, canal neuromasts of the LL are designed to detect pressure differences that are generated by acceleration of fluid at the surface of the fish (Kelly and van Netten, 1991; Kroese and Schellart, 1992; van Netten and Kroese, 1987). They are therefore capable of detecting the acceleration (directional) component of a sound wave as it travels through the medium (Kalmijn, 1988a). This is consistent with the notion that the LL is typically considered to be a 'near field' detector, and near field sources include a major acceleration component (Dijkgraaf, 1963; Kalmijn, 1988; Kalmijn, 1989). Transection of the pLLn did not produce similar effects, which may reflect a longer conduction time required for the pLLn than the anterior branch. Since

the processing time window of the M-cell is at most 2–4 ms, only fast synaptic inputs can contribute to the outcome of the escape response. And, the pLLn may influence later features of the behavior, such as direction change.

It would be interesting to study the interaction at the M-cell level of the LL inputs and those from the inner ear, in order to understand the mechanisms by which the network combines information about the sound source from the different sensory systems and uses it to elicit the proper escape responses. Such an approach would allow a distinction between two alternative explanations for the observed loss of directionality: (1) the preferred concept that the LL encodes direction, and (2) the alternative that the loss of short latency input alone increased the temporal processing window and secondarily degrades directionality. It should be noted that the second alternative would also account for the lack of any change in response probability – in fact, if response rates are compared for the same duration time window, removing the LL input does result in a decreased probability.

Tonic inputs from the LL contribute to the modulation of the escape behavior

A striking feature of the escape response is that the directionality is reversed if the fish is close to a physical barrier (Eaton and Emberley, 1991). This is an indication that the M-cell may process information concerning the physical environment prior to stimulus onset and this information may bias the firing of one cell in favor of the other, for example, by a bias in tonic inhibition. The superficial neuromasts of the LL are specialized velocity detectors that can detect minor changes in the flow field and hence are capable of informing the fish of its physical surroundings such as the presence of stationary objects (Montgomery et al., 1997; Montgomery et al., 1994). We therefore expected these receptors to contribute to the directional override that is seen in the near wall responses. The only possible indication in our results (albeit not significant) for tonic modulation related to the directional override comes after the elimination of the pLL. Furthermore, contrary to the center field responses where no role of the posterior LL was detected, near the wall it plays a significant role in transmitting tonic input to the M-cell. This may be both due to a high preponderance of these receptors on the trunk of the fish (Puzdrowski, 1989) as well as a decrease in the time constraint for tonic inputs. These results and direct measurements of the acoustic signals in different regions of the experimental tank indicate that the directional override itself is not due to a change in the stimulus caused by the obstruction.

The frequency of events when the fish turned and hit the side of the tank increased considerably after LL elimination (Fig. 7). While the M-cell is responsible for the initiation of the escape and its initial direction, the ultimate trajectory of the fish is under the control of a much larger 'brainstem escape network (BEN)' that involves other reticulospinal neurons and Mauthner homologues (Eaton et al., 2001; O'Malley et al., 1996). Although all the kinematics parameters associated with firing of the Mauthner cell are unaffected, the distance that the fish travels following complete LL elimination is significantly shorter (Table 1). These results imply that the LL may not only contribute to the role exerted by the M-cell in the initiation of the escape but may also activate other reticulospinal neurons that determine the variability of the subsequent trajectory.

The contribution of visual inputs to escape directionality

Ultimately, successful behaviors rely on the integration of information from different sensory modalities. Particularly in near wall responses where the response is more complex, we would

expect the fish to rely on not only the LL system but also visual and tactile senses to elicit an effective escape response. These sensory systems may function either independently or synergistically or they may be capable of compensating for inactivation of other elements. Our results indicate that visual information is not required for the directionality of the sound evoked escape response in the open field (Fig. 9). These results indicate that visual inputs seem not to bias the M-cell system. In this context the fish seems to rely primarily on acoustico-lateralis inputs for the decision-making processes related to directionality. Response probability and latency were on the other hand affected (Fig. 8), suggesting that the M-cell indeed integrates multiple sensory modalities for the decision related to whether or not to trigger an escape.

We expected visual cues to play a part in the directional overrides observed in escape behavior when the fish was near the wall, but their removal alone did not reveal such a role (Fig. 9). Directional override was reduced to chance only after the elimination of both vision and LL suggesting that either modality alone is sufficient for the expression of this property of the escape. This observation is consistent with reports that the LL can function independently to provide blind fish with sufficient directional information to navigate around nearby objects (Burt de Perera, 2004; von Campenhausen et al., 1981).

In summary, we found that the short latency C-start that is triggered by abrupt sounds is influenced by multiple sensory systems, some of which contribute phasic information that influence the escapes when the fish is in the center of the tank, with an unobstructed path. Of these, the LL seems to be required for open field directionality.

On the other hand, our results also lead us to postulate that tonic information, from the LL and from the visual system, can bias the M-cell system and specifically modulate the near wall directional override. These results constitute one of the clearest demonstrations of the function of the LL in auditory evoked escape behaviors. Although this study did not attempt to demonstrate a selective role for either LL submodality, it may be highly possible that the specialized neuromasts of the LL have differential roles in these two behaviors. The Mauthner system is an ideal system to analyze electrophysiologically the integrative properties of the LL with other components of the octavolateralis system in an attempt to understand the mechanism whereby this input can modulate the C-start.

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