Cardiac response to startle stimuli in larval zebrafish: sympathetic and parasympathetic components

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Mann KD, Hoyt C, Feldman S, Blunt L, Raymond A, Page-McCaw PS. Cardiac response to startle stimuli in larval zebrafish: sympathetic and parasympathetic components. Am J Physiol Regul Integr Comp Physiol 298: R1288–R1297, 2010. First published February 3, 2010; doi:10.1152/ajpregu.00302.2009.—Central regulation of cardiac output via the sympathetic and parasympathetic branches of the autonomic nervous system allows the organism to respond to environmental changes. Sudden onset stimuli, startle stimuli, are useful probes to study central regulatory responses to the environment. In mammals, startle stimuli induce a transient bradycardia that habituates with repeated stimulation. Repeated presentation of the stimulus results in tachycardia. In this study, we investigate the behavioral regulation of heart rate in response to sudden stimuli in the zebrafish. Larval zebrafish show a stereotyped heart rate response to mild electrical shock. Naïve fish show a significant increase in interbeat interval that resolves in the 2 s following stimulation. This transient bradycardia decreases on repeated exposure to the stimulus. Following repeated stimulation, the fish become tachycardic within 1 min of stimulation. Both the transient bradycardia and following tachycardia responses are blocked with administration of the ganglionic blocker hexamethonium, demonstrating that these responses are mediated centrally. The transient bradycardia is blocked by the muscarinic antagonist atropine, suggesting that this response is mediated by the parasympathetic system, while the following tachycardia is specifically blocked by the beta-adrenergic antagonist propranolol, suggesting that this response is mediated by the sympathetic nervous system. Together, these results demonstrate that at the larval stage, zebrafish actively regulate cardiac output to changes in their environment using both the parasympathetic and sympathetic branches of the autonomic nervous system, a behavioral response that is markedly similar to that observed in mammals to similar sudden onset stimuli.

cardiac regulation; zebrafish

AUTONOMIC DYSREGULATION APPEARS to play a key role in the development of cardiovascular disease (14), and genetic factors are hypothesized to contribute to the variable progression of the disease (30, 34). The startle response can be used as a probe for autonomic regulation of cardiac activity (4, 7). Both parasympathetic and sympathetic responses to the startle stimulus can be observed, which allows for both components of the autonomic nervous system to be probed with a single behavioral manipulation. This study seeks to establish the zebrafish larvae as a model organism to investigate autonomic regulation of cardiac activity by developing a rapid behavioral assay to probe for autonomic regulation and dysregulation of the cardiac output.

In mammals, sudden onset stimuli induce a rapid change in behavior that consists of both locomotor activity and coincident autonomic behavioral changes. The locomotor activity, the startle response, may be characterized by a complex motor response involving contraction of the muscles of the eyelid, neck, and limbs [reviewed in (15)]. In rats, for example, an air-puff stimulus results in a stereotyped motor response and transient bradycardia (7). Repeated exposure to the stimulus results in decreased bradycardia and the appearance of a delayed tachycardia (7). Similar results are observed in mice (3) and in human subjects (13). Likewise, fish have a well-developed and stereotyped locomotor startle response, and this response is well developed at the 5-day-old larval stage in zebrafish (10, 17); however, autonomic components of this reflex behavior have not yet been characterized.

Both branches of the autonomic nervous system contribute to the regulation of cardiac output. The parasympathetic system acts to decrease the heart rate, while the sympathetic system acts to increase the heart rate. Both branches function through ganglionic synapses that are nicotinic, while the postganglionic neuroeffector junctions are muscarinic and adrenergic for the parasympathetic and sympathetic systems, respectively. Transient bradycardia in the air-puff startle response in rats is driven by increased parasympathetic tone, while the delayed tachycardia is driven by decreased parasympathetic tone and increased sympathetic tone (1). Recent work in anesthetized animals has demonstrated that the larval zebrafish heart is responsive to sympathetic and parasympathetic input (29). The function of these components of central cardiac regulation in response to environmental change in the larval stage of the zebrafish has not been investigated.

Using a behavioral assay and pharmacological antagonism, we observe changes in cardiac activity in the zebrafish larva consistent with autonomic regulatory activity. These centrally mediated cardiac behaviors, a transient bradycardia immediately following stimulation and a slowly developing tachycardia, are similar to those observed in mammals. These results suggest that the zebrafish larva may be a good model in which to understand the genetics of the central and peripheral bases of cardiac regulation.

MATERIALS AND METHODS

Zebrafish strains and husbandry. AB and TL strain wild-type zebrafish (Danio rerio) were obtained from our breeding colony and analyzed between 5 days postfertilization (dpf) and 7 dpf. No significant difference was observed in cardiac activity of the two wild-type strains (data not shown), and both sympathetic and parasympathetic behavioral responses are present between 5 and 7 dpf. All experiments were performed with age-matched larvae, reared in common dishes.
from the same clutches. Larvae were raised under standard laboratory conditions in a 28°C incubator with a 14:10-h light-dark circadian cycle (33). Fish larvae were immobilized in low-melt agarose in each experiment, lying on their sides, and maintained at ~26°C during the experiment. The animal protocols were approved by the Institutional Animal Care and Use Committee at Rensselaer Polytechnic Institute.

**Pharmacology and reagents.** Atropine, propranolol, isoproterenol, hexamethonium bromide, and metoprolol (Sigma-Aldrich) were used at the indicated doses in bath application. Animals were acclimated to the drug for a minimum of 15 min before the start of the assay. Hexamethonium and atropine (~1 nl) were injected into the duct of Cuvier using an Applied Scientific Instruments MPPI-2 pressure injector at the indicated doses.

**Imaging system, digital video recording, and stimulus generation.** Cardiac activity was monitored using digital videomicroscopy. A Marlin F-033B (Allied Vision Technologies) IEEE 1394-compliant, machine vision camera capturing video at the full available frame rate was used to capture video of the cardiac behavior. Twenty-second digital video movies were captured on a PowerMac G5 (Apple Computer) using SMI software (Satimage). Each frame of the movie was time-coded by the internal clock with submillisecond precision. Images were postprocessed to analyze cardiac function as described below.

To stimulate the fish, a custom-built stimulator was used to apply 12 V DC/cm for 1 ms across a DIP socket with the pins pressed into the agarose and visualized through the rectangular window between the pin rows. Stimulus was controlled by an iMac computer running Smile (Satimage) to control a serial-controlled custom-built switch regulating the 12 VDC power supply. Control scripts were written in applescript, which controlled both video capture and stimulation.

**Quantification of cardiac activity.** To identify the location of the hearts, the pixels with the highest-intensity variance were identified in the first 50 frames of each movie (Fig. 1C). Pixel intensities for each pixel overlying the heart were then collected in a 2-dimensional array (raster), in which each row represents a time point, and each column represents a pixel over the heart (Fig. 1G, middle, shows the intensity from a single pixel, represented as a column in Fig. 1G, left). In the case of Fig. 1, the number of pixels is 239. This provides an intensity profile for each pixel overlying the heart in the movie. For each heart pixel, the intensity profile was analyzed to find local maxima and minima to identify end diastole and systole. A count of the maxima divided by the length of the movie (in minutes) provides a measure of heart rate. The time between maxima gives a measure of the interbeat interval (IBI). Thus, in the data shown in Fig. 1, 239 measures of the HR are obtained, as well as 239 measures of the IBI. To correct for the small number of hot pixels (camera artifact), misidentified pixels (usually major vessels and melanocytes), and peak detection errors (which result in missed beats in some pixels), the reported HR is the mean HR value for all pixels after outliers are removed. Indicative of the precision of the method, SE for

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Fig. 1. Automated heart rate analysis. The differences in the brightness intensity over the heart was visible in video stills occurring approximately at diastole and systole (arrows in A and B). Pixels with high variance in brightness intensity in the first 50 frames of the movie were found to lie over the heart and were identified algorithmically for further analysis (C and D). The data from these pixels was arrayed as shown (E): one column per pixel, one row per time point. The resulting raster contained between 100 and 250 pixels per larva, depending on the orientation of the larva. In the movie analyzed in this figure, the algorithm collects data from 10 (of a total of 239) pixels that are not contiguous with the heart (pixels 4, 13, 14, 23, 41, 42, 44, 45, 50, and 51). These pixels correspond to melanocytes that move due to beating of the adjacent heart resulting in high variance in their intensity. The resulting raster of pixel intensities (E) reveals the beating of the heart as horizontal bands. Both the beating of the atrial and ventricular chambers is apparent as beats that are out of phase with each other (F). Beats of each chamber are marked with a dot. Analysis of the intensity fluctuation of each individual pixel (G shows pixel 84) allows determination of the heart rate by counting intensity maxima. The mean (less outliers) count of beats for all pixels divided by the length of the movie provides the measure of the heart rate. The heart rate for the raster shown in E and F is 209 beats/min. Temporal distance between maxima, averaged over all heart pixels, after removal of outliers, produces a plot of interbeat intervals.

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all pixels over each heart was generally less than 1 bpm for unstimulated larvae. Manual counting of beats in the movies and in the raster agrees with the algorithm output. This method can be used to measure the HR and IB1 of 1 to 7 larvae per movie with minimal user interaction.

Data are presented as means ± SE. Statistical analysis was performed in Microsoft Excel. Where multiple groups were compared, ANOVA was performed on the data to identify statistically significant differences among groups. Paired t-tests were performed when the comparison was between the same larvae (i.e., between baseline, stimulated, and post-stimulus measures). Comparison between groups was performed with homoscedastic one-tailed t-tests. For Fig. 6, paired t-tests were performed between the same time points in the prestimulus and poststimulus tests. P < 0.05 was considered significant in all experiments.

Surgery. Larvae were anesthetized in 0.16 mg/ml MS-222 and immobilized, dorsal side up, in 2% low-melt agarose with 0.16 mg/ml MS-222. Lesions were made using a flame-sharpened tungsten needle mounted in a glass pipette controlled with an M3301 micromanipulator. Transverse lesions were made posterior to the base of the hindbrain using the posterior edge of the otic vesicle and the base of the fourth ventricle as a landmark. After surgery, fish were gently removed from agarose and anesthesia and allowed to recover for at least 2 h before testing.

RESULTS

Measurement of cardiac activity. To measure cardiac output in larval-stage zebrafish under conditions that allow for manipulation of the environment, we have developed an assay system that allows the fish to be immobilized, and an automated image analysis system to determine heart rate (HR). This system utilizes microvideography with a machine vision (digital) camera to measure the cardiac activity of 1 to 7 fish simultaneously. Movies of agarose-embedded fish larvae are obtained and analyzed off-line. To extract heart rates from the movies, we rely on the change in brightness intensity of the image overlying the heart, as the heart fills and empties with blood (Fig. 1, A and B). To identify the heart algorithmically, we take advantage of the fact that pixels overlying the heart have high brightness variance (Fig. 1C). Few other pixels have high variance. Generation of a variance map of the image over the course of 50 frames allowed identification of those regions of the image that varied in image intensity. Generally, 100 to 250 pixels were found per heart using this method, depending on the orientation of the animal in the image plane (Fig. 1D). For purposes of visual inspection of data quality and gross behavioral responses, we find that presentation of the entire data set as a raster, in which the high-variance pixels from each time point are arrayed as raster rows, each movie frame providing 1 row for the raster (schematized in Fig. 1, D–F). Thus, each row represents the brightness intensity of the heart at each point in time, and the vertical column represents a single pixel overlying the heart (Fig. 1, F and G). We observed that a regular banding pattern occurs in these rasters, resulting from the filling and emptying of the heart and that individual pixels have a similar phase. We also note the presence of populations of pixels in two distinct phases that correlate anatomically with the atrium and ventricle (Fig. 1F). A few high-variance pixels identified in the first step do not correspond to the heart and do not vary in intensity with the heart. These errors have several sources, including hot pixels in the camera, brightness artifacts associated with the eye, melanocytes adjacent to the heart, blood flow in major vessels, and animals that move during the course of the experiment (see asterisks in Fig. 2B). To prevent these artifacts from interfering with the analysis, outlier values are removed algorithmically, and rare movies with large motion artifacts, clearly visible in the raster plots, are not analyzed.

To arrive at an unbiased estimate for HR, each high-variance pixel was analyzed independently by identifying the pixel’s brightness intensity maxima, counting these maxima and dividing them by the duration of the movie. In the case of the raster illustrated in Fig. 1, the HR was 209 bpm. Measurement of the HR over the period following embedding demonstrates that the animal’s HR is elevated immediately after embedding in agarose, but over the course of 30 min, the heart rate decreases to a resting HR consistent with previously reported heart rates (29). We refer to the HR after 30 min of recovery from embedding as the basal HR.

Stimulation. To determine whether zebrafish larvae regulate HR in response to environmental stimulation, we immobilized fish in agarose and presented stimulus trains to the animals to induce centrally mediated changes in cardiac activity. We investigated several stimulus modalities and protocols that induced reproducible and robust changes in cardiac output, which were also mild enough not to induce movement of the fish (twitching) as movement can interfere with image analysis. Dish taps and vibratory stimuli were found to be unsuitable, as they also induced water movement that caused image distortion. We have chosen mild electrical stimulation of the fish as our behavioral stimulus, as it is highly reproducible, rarely induces twitching in the fish, and does not result in movement of the overlying media. Stimulation of the fish at 0.5 Hz for a total of 10 stimuli results in stereotyped changes in cardiac activity. We observed that immediately following the first stimulation, the heart beat paused with a concomitant increase in interbeat interval (Fig. 2, A–C, black curve in 2C). This pause was short in duration and habituated with experience to the stimulus. We refer to these individual stimulus-induced decreases in HR as transient bradycardia events. We define the transient bradycardia response in our assay (TBR10, where the subscript refers to the number of stimuli), as the change in heart rate measured during the 20-s stimulus train (10 stimuli at 0.5 Hz) relative to the basal HR (Fig. 2E). The TBR10 was chosen as the behavioral measure rather than the responses to the individual stimuli (the transient bradycardia events), as it is the most robust measure of the behavior we have found.

In mammalian systems, repeated presentation of a startle-inducing stimulus results in delayed tachycardia (7). To determine whether larval zebrafish show an increase in HR following repeated exposure to the mild-shock stimulus, we observed HR at 60 s, 3 and 5 min following the stimulus train. Following presentation of 10 stimuli at 0.5 Hz, we observed that the heart rate rapidly increases and subsequently returns to baseline over this time course (Fig. 2D). We measure the following tachycardia response at 60-s poststimulus (FTR60, where the superscript refers to the number of seconds between the end of the stimulus and the start of measurement) as the relative change in HR between the basal HR (Fig. 2E). Thus, in our standard protocol, several baseline 20-s HR measurements are made prior to stimulus, the heart rate 1 min prior to stimulation presentation is defined as the basal HR. The TBR10 and FTR60 are similarly measured during and after stimulation.

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Previous studies of parasympathetic and sympathetic tone at the larval stage of zebrafish have used mild anesthesia to allow heart rate measurement (29). We were interested in determining whether the anesthetic used in these experiments affected cardiac activity. Fish were treated at various concentrations of MS-222 during the experiment, and their heart rate was analyzed. Low doses of MS-222 decreased the TBR10, while concentrations commonly used to induce anesthesia ablated the TBR10 (Fig. 3B). No significant change was observed in FTR@60 in response to the stimulus (Fig. 3B), and basal HR showed a biphasic response to the drug, increasing at low concentrations and decreasing at higher concentrations (Fig.
The presence of the FTR\textsubscript{60} in the presence of MS-222 suggests that even at the high doses of MS-222 used in this experiment, larvae are able to sense the stimulus. Because MS-222 anesthesia interfered in regulation of cardiac activity, MS-222 was not utilized in the following experiments.

**Autonomic regulation of heart rate.** To determine whether the HR changes observed are under central nervous system control, pharmacological ganglionic blockade of both the parasympathetic and sympathetic input was used. The nicotinic antagonist hexamethonium bromide (1, 10, and 50 mg/ml, 1 nl each injected into duct of Cuvier) showed a dose-dependent inhibition of both the TBR\textsubscript{10} and FTR\textsubscript{60} (Fig. 4). At the highest dose, both TBR\textsubscript{10} and FTR\textsubscript{60} were absent, while at intermediate doses, only the FTR\textsubscript{60} was significantly reduced. These results are consistent with the hypothesis that nicotinic blockade with hexamethonium blocks both sympathetic and parasympathetic regulation of cardiac activity, suggesting that these cardiac responses to sudden onset stimuli are mediated by the central nervous system. Nicotinic blockade with hexamethonium led to an increase in resting heart rate, which is consistent with a dominant parasympathetic input in fish of this age (Fig. 4C) and consistent with previous results (29).

The TBR\textsubscript{10} is mediated by the parasympathetic nervous system. To determine whether the parasympathetic system was driving the TBR\textsubscript{10} observed, we treated fish with the competitive muscarinic antagonist atropine. We predicted that the muscarinic antagonist would decrease bradycardia. Consistent with our hypothesis, the TBR\textsubscript{10} is strongly affected by a 30-min exposure to 50 \( \mu M \) atropine, more weakly by 15 \( \mu M \) atropine and not significantly by 5 \( \mu M \) atropine (Fig. 5). This result suggests that parasympathetic input is required to mediate the TBR\textsubscript{10}.

If the bradycardia observed during startle stimulation was due to parasympathetically released acetylcholine, then atropine would be expected to decrease the magnitude of the transient bradycardia events. Analysis of the IBI in the 1 min prior to startle stimulation revealed that the IBI varied little over the course of the recording (blue curve shows mean basal IBI \pm SE, data from seven fish from a single movie, Fig. 6A). During mild electrical shock stimulation, the larvae demonstrated large changes in IBI following stimulation (red curve shows mean stimulated IBI \pm SE, Fig. 6A). Treatment of the fish for 30 min prior to beginning the experiment with 5 \( \mu M \), 15 \( \mu M \), and 50 \( \mu M \) atropine, resulted in a decrease in the variability of the IBI in response to stimulation (Fig. 6, B–D, respectively). Upon stimulation of vehicle-treated larvae, we observed a significant increase in IBI compared with the prestimulus IBI values for the same larvae, and this increase was present with a short latency following each stimulus (significance at \( P < 0.05 \) of paired, one-tailed \( t \)-test for each time point is indicated in Fig. 6). In the presence of 5 \( \mu M \) and 15 \( \mu M \) atropine, the transient bradycardia events were diminished, but IBI rapidly rose following the first stimulus and remained significantly elevated throughout the stimulus procedure in the presence of 15 \( \mu M \) atropine. In the presence of 50 \( \mu M \) atropine, the IBI is not significantly different during stimulation from the same fish measured 1 min prior to stimulation (the lowest \( t \)-test value in the time series was 0.202, Fig. 6C). Thus, 30 min of exposure to 50 \( \mu M \) atropine ablates the transient bradycardia events in response to stimuli, while 30 min of exposure to 5 and 15 \( \mu M \) atropine changes the behavioral response from large transient responses to a smaller-magnitude, prolonged response.

**Tachycardia response is driven by the sympathetic system.** To determine whether the increase in HR observed in the FTR\textsubscript{60} was due to increased sympathetic tone, we attempted to inhibit the cardiac adrenergic receptors, which mediate sympathetic activity in the heart (29). The first model can be tested by attempting to antagonize the sympathetic specific receptor, the \( \beta 1 \) adrenergic receptor. To test the hypothesis that FTR\textsubscript{60} is driven by changes in sympathetic activity, the

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**Fig. 3.** The anesthetic MS-222 alters heart rate (HR) regulation. Increasing doses of MS-222 result in modulation of basal heart rate consistent with inhibition of vagal input to the cardiac tissue (A). MS-222 also decreases the TBR\textsubscript{10} but does not significantly alter the FTR\textsubscript{60} response (B). \( P \) value of the \( t \)-test comparing nonnormalized heart rate compared with baseline control is listed where significant.
nonselective adrenergic antagonist propranolol was administered (29). We observed a significant and specific decrease of FTR at 60 on administration of propranolol (Fig. 7). To further confirm the sympathetic origin of the FTR at 60, we performed a surgical lesion to cut the descending projections by severing the spinal column at the base of the hindbrain. Since this manipulation is not expected to affect the vagal nerve, we predicted that vagal tone would be unaffected. As predicted, severing the spinal column immediately posterior to the hindbrain resulted in loss of the FTR at 60 compared with surgical controls (a midsaggital lesion in the forebrain) and sham surgery (Fig. 7E).

Fig. 4. Nicotinic blockade with hexamethonium reduces the TBR10 and the FTR at 60. Fish were injected with -1 nl of hexamethonium or vehicle control (0.5 x PBS) into the duct of Cuvier and tested in the cardiac behavioral response assay. Comparison of the behavioral assay after injection with 1 nl of 50 mg/ml hexamethonium (dashed line) or injection with vehicle control (solid line, A). Basal HR response to injection of 1 nl hexamethonium at 0, 1, 10, and 50 mg/ml (B, two-tailed P values are compared with vehicle). TBR10 is significantly reduced after injection of 1 nl of 50 mg/ml hexamethonium, while the FTR at 60 is decreased after injection of 1 nl of both 10 and 50 mg/ml of hexamethonium (C, two-tailed P values are compared with vehicle, significance upon Bonferroni correction is 0.0167).

Fig. 5. TBR10 is blocked by administration of the parasympatholytic atropine. Treatment of zebrafish with the muscarinic antagonist atropine in bath at 5, 15, and 50 μM (A, left panel) or injection of 1 nl of 5 μM injected into the duct of Cuvier (A, right panel) does not alter basal HR. Similarly the FTR at 60 is not significantly affected by atropine treatment (B, gray bars). Atropine significantly reduces the TBR10 whether applied in bath (B, left panel, black bars) or injected (B, right panel, black bars). One-tailed t-test values are shown compared with the vehicle controls.
DISCUSSION

In this paper, we have established a system in which it is possible to observe changes in cardiac function in immobilized, unanesthetized zebrafish. We find that the commonly used anesthetic MS-222 interferes in sympathovagal function in zebrafish larvae, and therefore, we used agarose rather than anesthesia to immobilize the larvae. We observed environmentally driven changes in cardiac function, including a transient bradycardia and delayed tachycardia that were driven by both the parasympathetic and sympathetic systems, respectively.

The goal of this study has been to develop an assay to measure change in cardiac function in response to environmental change and to determine whether these regulatory systems function through the vagal and sympathetic systems, respectively.

The central result of the current work is that larval zebrafish exhibit regulated cardiac activity that is comparable to that seen in mammals. Specifically, upon presentation of a brief, sudden-onset stimulus, the fish respond with a short latency, brief (less than 2 s duration) bradycardia. Repeated presentation of the stimulus results in an attenuation of the bradycardia, consistent with habituation to the stimulus. This behavioral response is followed by tachycardia, which occurs rapidly following multiple stimuli. Pharmacological blockade of the ganglionic synapses with the nicotinic antagonist hexamethonium, blocked both bradycardia and tachycardia. This result demonstrated that the observed changes in HR were centrally mediated. The muscarinic antagonist atropine was found to block the TBR10, while having little effect on the FTR@60. This result is consistent with a parasympathetically mediated bradycardia. Interestingly, the absence of a strong TBR10 response in atropine-treated animals did not affect the FTR@60, suggesting that the increase in heart rate was not caused by the bradycardia but was mediated independently of cardiac activity.

In mammals, tachycardic responses can result from increased sympathetic activity, as well as altered parasympathetic activity. Vagal release of ACh can reduce noradrenaline release in the atrium (18), resulting in inhibition of sympathetic output (16, 19, 20, 22, 23, 25). To determine the origin of the stimulus results in an attenuation of the bradycardia, consistent with habituation to the stimulus. This behavioral response is followed by tachycardia, which occurs rapidly following multiple stimuli. Pharmacological blockade of the ganglionic synapses with the nicotinic antagonist hexamethonium, blocked both bradycardia and tachycardia. This result demonstrated that the observed changes in HR were centrally mediated. The muscarinic antagonist atropine was found to block the TBR10, while having little effect on the FTR@60. This result is consistent with a parasympathetically mediated bradycardia. Interestingly, the absence of a strong TBR10 response in atropine-treated animals did not affect the FTR@60, suggesting that the increase in heart rate was not caused by the bradycardia but was mediated independently of cardiac activity.

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FTR\textsuperscript{060}, we have used \(\beta\)-adrenergic antagonists to block sympathetic input. We observe a complete block in FTR\textsuperscript{060} with propranolol, which also decreases resting heart rate. These results are consistent with the hypothesis that increased sympathetic activity in response to the shock stimulus results in the FTR\textsuperscript{060}. Overall, the data support the hypothesis that increased sympathetic tone contributes to the FTR\textsuperscript{060}.

We consider the possibility that the TBR\textsubscript{10} that we measure following stimulus represents cardioversion or defibrillation, resulting from the shock stimulus. We consider this interpretation unlikely for four reasons. First, repeat stimulation results in habituation to the stimulus. Cardioversion is unlikely to habituate. Second, the stimulus is insufficient to result in depolarization of the body somitic muscle; twitch is rarely observed in response to the shocks. Third, blocking parasympathetic input to the heart either by blocking the nicotinic or muscarinic receptor systems prevents the bradycardia. This last result demonstrates that the response is mediated centrally and not directly on the cardiac tissue. Finally, the voltages used in these experiments are significantly below those required for cardioversion. In this case, the voltage used is 12 V/cm, which would correspond to a voltage of \(\sim 120\) mV across the heart; this is far below the several hundred volts used therapeutically for cardioversion.

The use of anesthetized animals in behavioral and physiological experiments can be problematic, since the anesthetic may interfere in the behavioral or physiological process under study. We find that the commonly used anesthetic MS-222 interferes in parasympathetic regulation of cardiac activity. MS-222 is known to interfere in INa, and this may affect either sensation of the stimulus or response to it. At commonly used MS-222 dosages (28), we observe a specific effect on the TBR\textsubscript{10}, but do not observe an effect on FTR\textsuperscript{060}. This result is consistent with previous reports of MS-222 affecting activity of respiratory motor output in the bullfrog hindbrain and tench heart rate (12, 26). These results demonstrate the advantage of using unanesthetized animals in behavioral analysis of cardiac function. Measurement of cardiac activity requires the use of immobilized animals in agarose. Similar approaches have been used to immobilize animals in other behavioral assays (5, 11, 21, 27). Although immobilization and restraint can result in stress, and alterations in cardiovascular function in mammalian systems, we find that after a short acclimation period, the immobilized...
larval zebrafish has heart rates that are consistent with previously published results. These results indicate that the unanesthetized, immobilized assay is preferred for the behavioral assay of cardiac function.

It is informative to compare these results with the air-puff startle response in the rat, a system that has been extensively studied by the Printz group (7, 8). In these studies, an air puff elicits a startle response in conscious rats. The cardiac response to this stimulus is an initial bradycardia followed by tachycardia after several stimuli. In this system, cardiac activity is monitored by catheterization, and the stimulus is a single air puff. The cardiac response is characterized in the naïve rat by a transient bradycardia that begins ~1 s after stimulus and recovers within 5 s. With repeated presentation of the stimulus, a tachycardia is observed, which begins ~2 s after stimulus. Conversion from a bradycardia-dominated response to a tachycardia-dominated response occurs after approximately five trials. Tachycardia was driven by both β-adrenergic mediated responses and vagally mediated responses (1). In the present assay, in unanesthetized zebrafish, we find that there is a vagally mediated bradycardia that is present in naïve animals and habituates rapidly. Tachycardia appears somewhat later and is mediated by sympathetic activation. Thus, the larval zebrafish’s cardiac regulation in response to sudden onset stimuli are functionally similar to those of the mammals, suggesting that these responses and their underlying mechanisms are functionally conserved.

The zebrafish has great potential as a model system in which to understand the genetics of cardiovascular physiology and regulation. Forward genetic screens in the zebrafish have been used to identify the genetic pathways that control formation of the heart (31). Cardiac pharmacology (6) and physiology (2) in larval-stage fish has received some attention, but few experiments have studied genetic contributions to cardiovascular control. Mutants that affect cardiac function (contractility and rhythmicity) have been identified together with mutants that affect development of the heart, but these studies have not identified genes that affect the central regulators of cardiac function (9, 32). This assay may provide a foundation for the identification of genes required for the regulation of autonomic function.

Perspectives and Significance

We report here that zebrafish larvae have a well-developed sympathetic and parasympathetic cardiac response to stimulation, consisting of a short latency bradycardia that decreases in intensity on repeated presentation. Repeated presentation of the stimulus also induces a tachycardia response that is temporally delayed and distinct from the bradycardia response. These responses are remarkably similar to those observed in mammalian species; the primary difference in the behaviors being the greatly delayed tachycardia response in fish larvae compared with mammalian species. The presence of sympathetic and parasympathetic activity in fish species has been reported previously, including in larval zebrafish. To our knowledge, however, this is the first report of a behavioral dissection of these responses. The blunting of these responses in MS-222-anesthetized larvae may have prevented observation of this phenomenon previously. It is remarkable that this behavioral response is conserved across such large evolutionary distances.

Fish and mammals differ significantly in metabolism (poikilothermy vs. endothermy) and environment (aquatic vs. terrestrial), and larval zebrafish do not utilize their circulatory system to transport oxygen (24). These physiological differences make it all the more remarkable that these behavioral responses are so well conserved.

REFERENCES


