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RESEARCH ARTICLE

Prolonged exposure to stressors suppresses exploratory behavior in zebrafish larvae

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ABSTRACT

Environmental stressors induce rapid physiological and behavioral shifts in vertebrate animals. However, the neurobiological mechanisms responsible for stress-induced changes in behavior are complex and not well understood. Similar to mammalian vertebrates, zebrafish adults display a preference for dark environments that is associated with predator avoidance, enhanced by stressors, and broadly used in assays for anxiety-like behavior. Although the larvae of zebrafish are a prominent model organism for understanding neural circuits, few studies have examined the effects of stressors on their behavior. This study examines the effects of noxious chemical and electric shock stressors on locomotion and light preference in zebrafish larvae. We found that both stressors elicited similar changes in behavior. Acute exposure induced increased swimming activity, while prolonged exposure depressed activity. Neither stressor produced a consistent shift in light–dark preference, but prolonged exposure to these stressors resulted in a pronounced decrease in exploration of different visual environments. We also examined the effects of exposure to a noxious chemical cue using whole-brain calcium imaging, and identified neural correlates in the area postrema, an area of the hindbrain containing noradrenergic and dopaminergic neurons. Pharmaceutical blockade experiments showed that α-adrenergic receptors contribute to the behavioral response to an acute stressor but are not necessary for the response to a prolonged stressor. These results indicate that zebrafish larvae have complex behavioral responses to stressors comparable to those of adult animals, and also suggest that these responses are mediated by similar neural pathways.

KEY WORDS: *Danio rerio*, Noradrenergic neurons, Area postrema, Calcium imaging, Stress

INTRODUCTION

A wide variety of environmental stimuli induce stress in adult fishes, including toxic or noxious chemicals (Pratap and Bonga, 1990; Thomas et al., 1981; Yeh et al., 2013), crowding (Montero et al., 1999; Pickering and Pottinger, 1989; Ramsay et al., 2006), handling (Barton, 2000; Ramsay et al., 2009) and exposure to predators (Barcellos et al., 2007; Woodley and Peterson, 2003). The behavioral response of adult fish to stressful stimuli varies by species, but often includes avoidance, freezing, aggression, learning depression and decreased feeding (Schreck et al., 1997; Stewart et al., 2012). The response to stressors has been especially well studied in adult zebrafish, in which anxiety-like states are associated with increased time spent at the bottom of the tank, wall-following, erratic swimming and freezing (Bencan et al., 2009; Egan et al., 2009; Grossman et al., 2011; Maximino et al., 2010b). Adult zebrafish also display a robust preference for dark environments (Serra et al., 1999) that has been associated with predator avoidance (Maximino et al., 2007) and is enhanced by stressors (Chakravarty et al., 2013). The light–dark box assay quantifies this preference by recording the relative time spent by individuals in well-lit and darkened areas of a tank, has been widely adopted by the toxicology and pharmacology communities (Baiamonte et al., 2016; Blaser and Peñalosa, 2011; Caramillo et al., 2015; Facciol et al., 2017; Grossman et al., 2011; Maximino et al., 2010a, 2012, 2011; Piato et al., 2011) and is similar to the light–dark box assay used with mammalian vertebrates (Bourin and Hascoët, 2003; Crawley and Goodwin, 1980; Imaizumi et al., 1994).

The larval stage of zebrafish is a prominent model organism for identifying the function and architecture of neural circuits (Ahrens et al., 2012; Clark et al., 2013; Vanwalleghem et al., 2018), but less is known about the effects of stressors on their behavior. Some studies have found that acute exposure to osmotic, pH and noxious chemical stressors induces hyperactivity (Prober et al., 2008; Steenbergen and Bardine, 2014; Vom Berg-Maurer et al., 2016). However, other studies have found that comparable stressors have complex dose-dependent effects on locomotor activity (Diggles et al., 2017; Ko et al., 2019; Lopez-Luna et al., 2017; Ryu and De Marco, 2017). Similarly, electric shock has time-dependent effects, eliciting a transient increase in activity followed by a longer period of depressed activity (Andalman et al., 2019; Duboué et al., 2017; Steenbergen, 2018). Similar to adult zebrafish, larvae discriminate between light and dark environments, but they display a light rather than a dark preference (Burgess and Granato, 2007). Recent studies have found that this preference is enhanced by temperature and ultraviolet stressors (Bai et al., 2016), is decreased by anxiolytic pharmaceuticals (Chen et al., 2015; Steenbergen et al., 2011) and has a heritable component (Wagle et al., 2017). As multiple effects have been observed, it is not yet clear whether stressors induce a characteristic behavioral phenotype in zebrafish larvae.

The neurobiological mechanisms responsible for stress-induced changes in vertebrate behavior are immensely complex and not fully understood. These processes have been most extensively studied in mammalian models, where stress induces changes in the activity of a diversity of brain regions (Arnsten et al., 2015; Bullitt, 1990; Hunt et al., 1987). In particular, the nucleus of the solitary tract (NTS) plays a central role in autonomic regulation, transmission of autonomic stressors to other brain regions, and control of sympathetic activity (Andresen and Kunze, 1994; Saper, 2002; Zoccal et al., 2014). Stress also induces elevated activity in catecholaminergic (CA) neurons of the locus coeruleus (LC) (Chen

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and Sara, 2007; Passerin et al., 2000), which have widespread projections throughout the central nervous system (CNS) (Jones et al., 1977; Sara, 2009), and are associated with anxiety-like behaviors (McCall et al., 2017). CA neurons from both the NTS and LC also project to the paraventricular nucleus (PVN), where they modulate the activity of corticotrophin-releasing (CRH) neurons that control the hypothalamic–pituitary–adrenal (HPA) axis (Herman, 2018; Moore and Bloom, 1979).

These pathways appear to be broadly conserved across vertebrates. Similar catecholaminergic projections in the CNS are observed in zebrafish early in their development (Kastenhuber et al., 2010; Ma, 1994a,b; Rink and Wullimann, 2002; Tay et al., 2011), the anatomy of the sympathetic nervous system is comparable (Nilsson, 1976, 1983), and the hypothalamic–pituitary–interrenal (HPI) axis of fishes parallels the HPA axis of mammals (Schreck et al., 1997; Wendelaar Bonga, 1997). Recent studies in zebrafish have found that stressors acting via different sensory modalities induce elevated activity in a diversity of neuronal populations, including noradrenergic neurons, oxytocin neurons, CRH neurons, radial astrocytes and the habenula (Andalman et al., 2019; Mu et al., 2019; Vom Berg-Maurer et al., 2016; Wee et al., 2019). Transitions between active and passive behavioral responses to stressors are also correlated with changes in activity in noradrenergic neurons, radial astrocytes and habenula neurons (Andalman et al., 2019; Duboué et al., 2017; Lee et al., 2010; Mu et al., 2019). Furthermore, the choice between light and dark environments by adult zebrafish correlates with activity in the dorsal telencephalic region and ventral telecephalic region (Lau et al., 2011), and manipulation of the activity of serotonergic neurons alters the light preference of larval zebrafish (Cheng et al., 2016). However, the cellular-level architecture of the neural circuits that integrate sensory information about potential stressors and modulate behavioral responses are still not well understood.

In this study, we addressed three questions: (1) how does prolonged exposure to noxious chemical and electric shock stressors affect locomotion and exploratory behavior in zebrafish larvae?; (2) which neuronal populations show selective responses to noxious chemical cues and how does activity change during prolonged exposure to this stressor?; and (3) what is the contribution of adrenergic pathways to the behavioral response to a noxious chemical stressor?

MATERIALS AND METHODS

Experimental animals

Adult and larval *Danio rerio* (F. Hamilton 1822) were housed under standard conditions (28°C, 1000 µS, 14 h:10 h light:dark cycle) and fed a mixture of hatched Artemia and commercial fish feed (API Tropical Fish Flake Food). Larvae were reared in static tanks containing bicarbonate-buffered E3 medium (5 mmol l^{-1} NaCl, 0.17 mmol l^{-1} KCl, 0.33 mmol l^{-1} $CaCl_2$, 0.33 mmol l^{-1} $MgSO_4$, 0.27 µmol l^{-1} Methylene Blue, 0.35 mmol l^{-1} $NaHCO_3$, titrated to neutral pH; modified from Nusslein-Volhard and Dahm, 2002), received daily water changes to avoid build-up of nitrogenous waste products, and were given dry feed twice daily (Skretting Gemma Micro 75) starting at 5 days post-fertilization (dpf). Unless otherwise noted, all zebrafish were from the AB strain. All animal experiments and procedures were approved by the Oregon State University Institutional Animal Care and Use Committee (protocols 4715, 5025 and 5149).

Behavioral assays: experimental design

The behavioral response of zebrafish larvae (7 dpf) to noxious chemical and electric shock stressors was recorded using a custom-made apparatus constructed inside a temperature-controlled enclosure (28°C regulated with Fisher Scientific IsoTemp 6200 R28). For each trial, a single larva was placed in an acrylic experimental arena (35 mm diameter) positioned over an opal glass diffuser. The chamber was illuminated from above with white light (ST-WP-5050-DL-RL, TheLEDLight.com), backlit using near-infrared (NIR) light (ThorLabs M850L3, 850 nm), and imaged from above using a NIR-sensitive camera (Point Grey GS3-U3-41C6NIR-C; 30 frames s^{-1}) equipped with a visible light blocking filter (Lee Filter no. 87). To improve throughput, a single camera was used to simultaneously image multiple experimental arenas (up to six) with results being analysed independently.

The response to a noxious chemical cue was recorded by introducing the irritant mustard oil (allyl isothiocyanate; AITC) into the experimental arena. AITC is responsible for the pungent odor of wasabi and horseradish (Terada et al., 2015) and is a potent agonist of TrpA1 channels (Bandell et al., 2004; Jordt et al., 2004). TrpA1 channels are associated with sensing of noxious chemicals in a broad diversity of vertebrate animals (Kwan et al., 2006; Prober et al., 2008) and in zebrafish are expressed in the epibranchial sensory ganglia and Rohon–Beard neurons by 30 hours post-fertilization (hpf) (Prober et al., 2008). The experimental arena was filled with E3 medium (5 ml), a single larva was transferred into the arena, and the larva was allowed to acclimate for 10 min. At the start of the trial, the baseline behavior of the larva was recorded for 5 min, then 1 ml of AITC solution was added to the arena (10 µmol l^{-1} AITC in 0.05% DMSO final concentration), and the response was recorded for 30 min. Preliminary experiments with dye solutions indicated that injection of the AITC solution produced rapid mixing and a uniform concentration throughout the arena. Preliminary experiments were also performed with a range of AITC concentrations (1, 10 and 100 µmol l^{-1}; ∼60 hpf larvae), and 10 µmol l^{-1} was used for subsequent experiments as it was the lowest concentration that produced robust effects and is consistent with the concentration used in prior studies (Prober et al., 2008).

The response to electric shock was recorded using similar procedures. Animals were allowed to acclimate for 10 min, baseline activity was recorded for 5 min, and an electric shock stressor was introduced via stainless-steel electrodes placed on opposite ends of the experimental arena (Mauldin Products, 316 stainless-steel sheet metal, 0.004 inches thick, 20 mm wide, full arena height). Electric shocks were presented as 5 s bursts of 1 ms 6 mA pulses at 40 pulses s^{-1}, delivered with one burst per 60 s (Grass S88 stimulator). The response was recorded for 20 min, the electric shock was then stopped, and recovery behavior was recorded for 10 min.

To record the effect of stressors on exploratory behavior, we next examined the movement of larvae (7 dpf) in experimental arenas (35 mm diameter) with light and dark halves. For the bottom-shaded arenas, a visible light-absorbing optical filter (Lee Filter no. 87) was placed between the opal glass and the experimental arena so that it obscured one-half of the circular arena. As the optical filter transmits NIR wavelengths, this filter did not impede imaging of the animals. For the top-shaded arenas, the optical filter was placed immediately above the experimental arena. The effects of AITC and electric shock stressors were examined as in prior experiments. For electric shock experiments, the electrodes were centered on the light–dark edge so that non-uniformity in the electric field would not produce a light–dark bias. To determine if observed shifts in light–dark preferences resulted from learning effects, we next performed a series of experiments in which animals were allowed to acclimate to the split-bottom arena; the bottom was then switched to all-light or all-dark, AITC was introduced, after ∼30 s the bottom was replaced with a

split-bottom stimulus, and the light preference and exploratory behavior of the animals was recorded as above, with larvae of different ages (4, 5, 6 and 7 dpf). Furthermore, to confirm that baseline light–dark preferences were robust to minor variations in the experimental configuration, we also examined behavior in experimental arenas of different sizes (35, 55 and 75 mm), and in larvae that were not fed. No stressors were employed for these.

The contribution of adrenergic pathways to the observed changes in exploratory behavior was determined by treating larvae with the α-adrenergic receptor blocker phenoxybenzamine hydrochloride (PB; 16211, Cayman Chemical). The experimental arena (35 mm diameter) was filled with 5 ml of E3 medium, a single larva (7 dpf) was transferred to the arena, the animal was allowed to acclimate for 10 min, 1 ml of treatment solution was then added to the arena producing either control (0.05% DMSO final concentration) or PB-treatment (10 µmol l^{-1} in 0.05% final concentration) medium, the response was recorded for 45 min, 1 ml of AITC solution was then added to the arena (10 µmol l^{-1} in 0.05% DMSO final concentration), and the response was recorded for an additional 45 min. Preliminary experiments were also performed with a range of PB concentrations (3, 10 and 15 µmol l^{-1}), and 10 µmol l^{-1} was used for subsequent experiments as it was the lowest concentration that produced robust effects.

Behavioral assays: statistical analysis

Images of zebrafish larvae were processed with MATLAB R2018b using custom-written MATLAB scripts (available at https://bitbucket.org/jastrother/larval_proving_grounds). Images from the camera were compressed in real time and stored as UFMF files. In subsequent offline processing, template matching was used to identify the position of the larva in each frame, and frames with low signal-to-noise ratio matches were discarded. Computed trajectories were manually curated to ensure that tracking was successful, filtered to remove digitization noise (fourth order Butterworth, 5 Hz cut-off), and fitted with a cubic interpolating spline. The instantaneous velocity was calculated from the derivative of the spline, and swimming speed was taken as the magnitude of the velocity. The heading direction was taken as the direction of the velocity vector, fitted with a cubic spline, and angular speed was calculated as the absolute value of the derivative of the spline. The distance to the nearest wall was computed by calculating the Euclidean distance transform of a binary image representing the walls of the tank and sampling this distance map for each position of the trajectory. The light–dark preference of the animal was calculated as:

$$\mathrm{PI} = \frac{T_\mathrm{L} - T_\mathrm{D}}{T_\mathrm{L} + T_\mathrm{D}}, \quad (1)$$

where T_L is the time spent on the light side of the experimental arena and T_D is the time spent on the dark side. This light–dark preference is equal to +1 when the animal remains exclusively on the light side of the arena and is equal to −1 when the animal stays exclusively on the dark side. The exploration index was calculated as:

$$E = 1 - |\mathrm{PI}|. \quad (2)$$

This exploration index is equal to 0 when the animal remains on one side of the arena (light or dark) and is equal to 1 when the animal spends equivalent amounts of time on each side of the arena. Time series for swimming speed, angular speed, wall distance and light preference were plotted as the time average over 60 s intervals. In order to reduce the dependence of the exploration index on the amount of locomotion performed, the time series for the exploration index were computed using 5 min intervals, over which time a typical larva swimming normally would traverse the experimental arena numerous times. The statistical methods used for hypothesis tests are described with the results below.

Whole-brain calcium imaging: experimental design

Neurons with selective responses to acute and prolonged exposure to a noxious chemical cue were identified using whole-brain calcium imaging. Transgenic zebrafish larvae (5–7 dpf; HuC:GCaMP6F$^{cy14/+}$; roy$^{-/-}$; nacre$^{-/-}$) that pan-neurally express the fluorescent calcium indicator GCaMP6F (Ahrens et al., 2012; Chen et al., 2013) were immobilized by immersion in the paralytic α-bungarotoxin (1 mg ml^{-1}, Tocris 2133), embedded in low-melting point agarose gel (3%), and immersed in aerated bicarbonate-buffered E3 medium (5 mmol l^{-1} NaCl, 0.17 mmol l^{-1} KCl, 0.33 mmol l^{-1} $CaCl_2$, 0.33 mmol l^{-1} $MgSO_4$, 0.27 µmol l^{-1} Methylene Blue, 0.35 mmol l^{-1} $NaHCO_3$, titrated to neutral pH; modified from Nusslein-Volhard and Dahm, 2002). A slab of agarose around the tail was dissected away, so that the head remained embedded but the tail was exposed to the perfusate. The preparation was maintained in a custom-made anodized aluminium perfusion bath kept at 28°C by circulating coolant (Thermotek T255P) through a heat exchanger integrated into the perfusion bath. The preparation was continuously perfused (3 ml min^{-1}) with E3 medium using a syringe pump (Harvard Apparatus Model 44). Fluorescence of the indicator was recorded using a custom-built multiphoton microscope configured for fast z-scanning (Thorlabs MPM-SCAN4 resonant galvo, Zeiss 20X/1.0 421452-9601 objective, Thorlabs PFM450E objective scanner). The field of view was ∼950 µm×450 µm×250 µm *xyz*, volumes were captured at ∼0.5 Hz, and excitation was provided with a pulsed femtosecond laser turned to 950 nm (Coherent Ultra II) (Svoboda and Yasuda, 2006). Optical filters included a dichroic mirror for combining excitation and emission paths (Chroma T610LPXR), a dichroic mirror for splitting green and red emitted light (Chroma T550LPXR), and filters for green and red channels (Chroma AT525/30 and AT585/30). Animals were allowed to acclimate to the imaging apparatus for 5 min and baseline activity was recorded for 10 min. The perfusate was then rapidly switched to an AITC solution (25 µmol l^{-1} AITC in 0.1% DMSO) by disabling the E3 medium pump and enabling a second syringe pump loaded with the AITC solution (Harvard Apparatus Model 44). The volume of the fluid contained in the perfusion bath was estimated as 750 µl, resulting in approximately one water change every 15 s. The response to perfusion with AITC was recorded for 15 min.

Whole-brain calcium imaging: statistical analysis

Fluorescence images were processed with MATLAB R2018b using custom-written MATLAB scripts (available at https://bitbucket.org/jastrother/neuron_image_analysis). To construct neural activity maps, volumes were downsampled spatially (3×) in the *xy* dimensions to reduce computational requirements, volumes were labeled according to the experimental condition (baseline activity: 2–10 min pre-stimulus, immediate response: 0–5 min post-stimulus, delayed response: 10–15 min post-stimulus), and a support vector machine (SVM) classifier (Schölkopf et al., 2002) was constructed for immediate and delayed response conditions using a one-versus-all strategy. The beta coefficients from the SVM classifier were then transformed into images by scaling positive-valued coefficients to the pixel intensity range, and placing the immediate and delayed activity in the red and blue channels, respectively.

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The responses of the area postrema neurons were measured by manually selecting regions of interest for every clearly identifiable cell body within the area postrema using time-averaged GCaMP6F images. For each cell, $\Delta F/F$ was calculated as the difference between the instantaneous and baseline fluorescence, taking the baseline fluorescence as the fifth percentile over the entire time series. Unresponsive neurons ($\Delta F/F$<20%, <5% of total) were removed from the dataset. The remaining neurons showed similar temporal patterns of activity, rising rapidly with the AITC injection and then decaying gradually. The half-life for the decay was computed by filtering the $\Delta F/F$ (0.5 min^{-1} cut-off frequency, fourth order Butterworth filter), finding the maximum $\Delta F/F$, and performing a least-squares fit of the response to an exponential decay function. A histogram was then constructed from all the measured half-life values. Responses were then categorized by their half-life, and the median responses of cells within representative categories (<3 min, 5–6 min, >9 min) were plotted.

RESULTS

Open field behavioral responses

Many stressors have direct physiological effects that may complicate the interpretation of observed changes in behavior. In order to determine the effect of a stressor that is sensed but has few acute physiological effects, we first examined the behavioral response to the noxious chemical allyl isothiocyanate (AITC). We found that AITC induced a brief increase in locomotor activity and wall-following, reflected by an increase in the median swimming speed to 163% of the pre-stimulus baseline value (P<0.001; two-tailed t-test, N=23) and a decrease in the median wall distance to 40% of the baseline value (P<0.001, two-tailed t-test, N=23, Fig. 1C–E). This transient increase in locomotor activity is consistent with AITC-induced hyperactivity observed in younger zebrafish larvae confined to 96-well plates (Ko et al., 2019; Prober et al., 2008), and hyperactivity in freely swimming zebrafish larvae in which TrpA1 is transiently stimulated optogenetically (Wee et al., 2019). This behavior was short-lived and quickly transitioned into more sedentary behavior. By 10 min, the median swimming speed had decreased to 16% of the baseline value (P<0.001, two-tailed t-test, N=23) and the median angular speed had increased to 249% of the baseline value (P<0.001, two-tailed t-test, N=23), indicating that the animals had adopted a more spatially constrained swimming pattern. Afterwards, the swimming speed gradually returned to normal levels, and by 25 min post-exposure the median swimming speed was 81% greater than the speed at 10 min post-exposure (P<0.001, two-tailed t-test, N=23).

To determine if other stressors elicited a similar behavioral response, we next examined the effect of electric shock on swimming behavior. In order to reduce the potential for physiological damage from the electric shocks, preliminary experiments were performed to select the smallest current that still produced a robust behavioral response and electric shocks were delivered intermittently (5 s burst of 1 ms pulses at 40 Hz, delivered once per 60 s). Each burst elicited a brief increase in the median swimming speed (<1 s, 225% increase for first burst) that subsided when the electric shock ceased (Fig. 1F, inset). After 20 min of exposure to an electric shock stressor, the median swimming speed had decreased to 67% of the baseline value (P=0.024, two-tailed t-test, N=28) and the median angular speed had increased to 134% of the baseline value (P=0.026, two-tailed t-test, N=28, Fig. 1F–H). These responses are consistent with the results of recent studies using different electric shock protocols (Andalman et al., 2019; Duboué et al., 2017; Steenbergen, 2018). Furthermore, our results indicate that noxious chemical and electric shock stressors induce comparable behavioral responses, which suggests that these responses may be mediated by similar pathways.

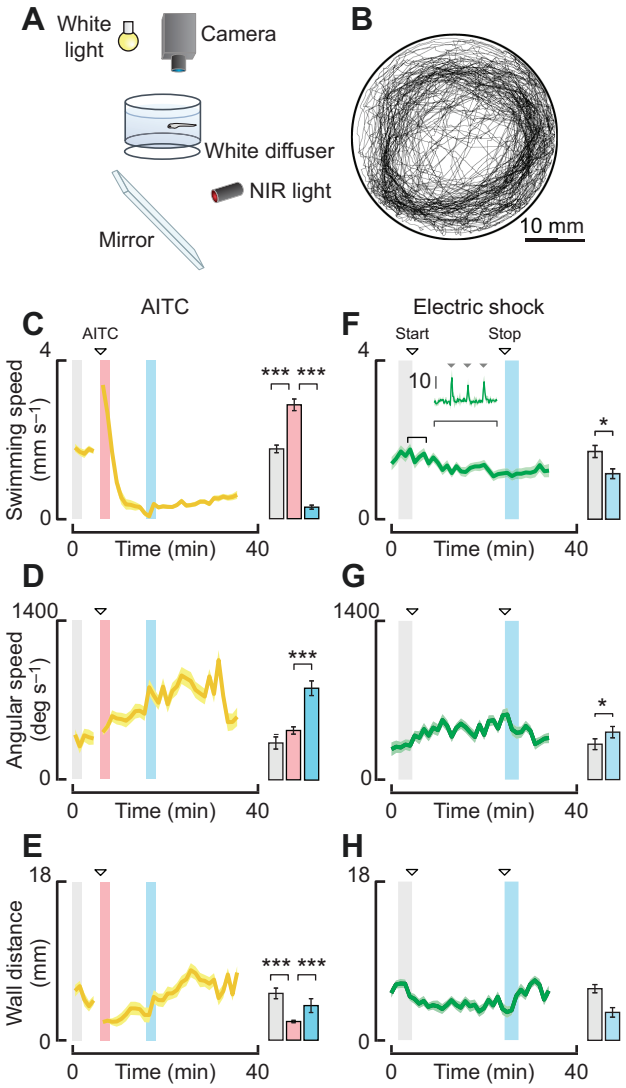

Fig. 1. Zebrafish larvae exhibit characteristic behavioral responses to stressors. (A) Schematic of experimental apparatus used to record the behavioral response of zebrafish larvae to stressors. (B) Typical trajectory of a zebrafish larva recorded under control conditions. (C–E) Behavioral response of zebrafish larvae to injection of TrpA1 agonist AITC into the water of the experimental arena (10 µmol l^{-1} in 0.05% DMSO final concentration). Measured parameters include swimming speed, angular speed and distance to the nearest wall. Time series (left) show values averaged over 60 s periods and bar plots show time average over pre-stimulus (gray), immediate response (pink) and delayed response (blue) intervals (median across individuals; error bars are s.e.m.; N=23). Significant differences in pre-stimulus versus immediate response values and immediate response versus delayed response values are indicated with asterisks (*P<0.05, **P<0.01, ***P<0.001, two-tailed t-test). (F–H) Behavioral response of zebrafish larvae to electric shocks (5 s burst of 1 ms pulses at 40 Hz, delivered once per 60 s) plotted similarly to panels C–E. Bar plots (right) show time average over pre-stimulus (gray) and post-stimulus (blue) windows. Significant differences between pre-stimulus and post-stimulus behavior are indicated with asterisks (*P<0.05, **P<0.01, ***P<0.001, two-tailed t-test, N=28). Inset trace in panel F shows transient response to electric shocks, plotted as maximum swimming speed over 3 s intervals for the first three bursts.

Light–dark behavioral responses

To explore the effect of stressors on the light–dark preference of the animals, individual zebrafish larvae were placed in a circular arena in which half of the bottom was light (white) and half was dark (black). We found that under these conditions, zebrafish larvae do

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not display a statistically significant preference for the dark side of the tank (P>0.05, two-tailed t-test, N=30, Fig. 2A). Furthermore, neither the AITC nor electric shock stressor produced a statistically significant shift in preference (P>0.05, two-tailed t-test, N=30 for AITC, N=28 for electric shock; Fig. 2A,B). These results contrast with prior studies that found that zebrafish larvae prefer light environments (Steenbergen et al., 2011) and this preference is enhanced by certain stressors (Bai et al., 2016). However, previous studies have also employed varying combinations of bottom, side and top shading. To determine if these factors contribute to the observed differences, we next examined light–dark preference in an environment in which the dark side was produced with an overhang at the top of the tank. Under these conditions, we observed a moderate light preference of 0.51±0.08 (mean±s.e.m.) under unstressed conditions (P<0.001, two-tailed t-test for null hypothesis of zero mean, N=33; Fig. 2C). However, the AITC stressor decreased mean light preference (79% decrease, P<0.001, two-tailed t-test, N=33) and electric shock did not have a significant effect on the mean preference (P>0.05, two-tailed t-test, N=28; Fig. 2C,D). Although AITC and electric shock had mixed effects when averaged across many individuals, we observed that individual animals developed strong preferences for either the light or dark side following the stressor presentation. To capture this effect, we computed an exploration index for each animal that is 1 when the animal spends equal amounts of time on both sides of the arena and 0 when the animal remains entirely on one side. The exploration index was computed for 5 min intervals, during which time even slow-moving animals typically traverse the entire arena. We found that both AITC and electric shock stressors induced significant decreases in the exploration index for both bottom-shaded and top-shaded environments. The exploration index was high prior to the stimulus, remained high for the first several minutes following the stressor presentation, but had significantly decreased by 10 min post-exposure [AITC–bottom: 66% decrease (P<0.001, N=30); shock–bottom: 38% decrease (P<0.01, N=28); AITC–top: 77% decrease (P<0.001, N=33); shock–top: 33% decrease (P<0.001, N=28); two-tailed t-test; Fig. 2E–H]. These results suggest that zebrafish larvae do not show a stress-induced shift in light preference. Instead larvae appear to exhibit a stress-induced decrease in the exploration of different visual environments with individual larvae developing a preference for either the light or dark side of the arena.

For the above experiments, animals were allowed to move freely throughout the experimental arena and typically experienced both the light and dark environments during the stressor presentation. We next examined whether the light preference displayed by individual larvae resulted from associative learning during this period or innate inter-individual differences. Zebrafish larvae were allowed to acclimate in an experimental arena with a half-light and half-dark bottom. The visual stimulus under the larvae was then changed to all-light or all-dark and AITC was introduced into the arena. Afterwards, the visual stimulus was returned to the half-light and half-dark bottom display, and the larvae were allowed to explore the arena. We found that zebrafish larvae that experienced an all-light bottom during the stressor presentation tended to show a dark preference in the test period (PI=−0.36±0.21, mean±s.e.m.), larvae that experienced an all-dark bottom during the stressor tended to display a subsequent light preference (PI=0.25±0.24), and this difference in light preference was statistically significant (P=0.032, one-tailed t-test, N=14 light, N=12 dark; Fig. 3A, B). These results support the conclusion that AITC-induced stress produces a decrease in exploratory behavior that is realized through a learned light–dark preference. The emergence of a

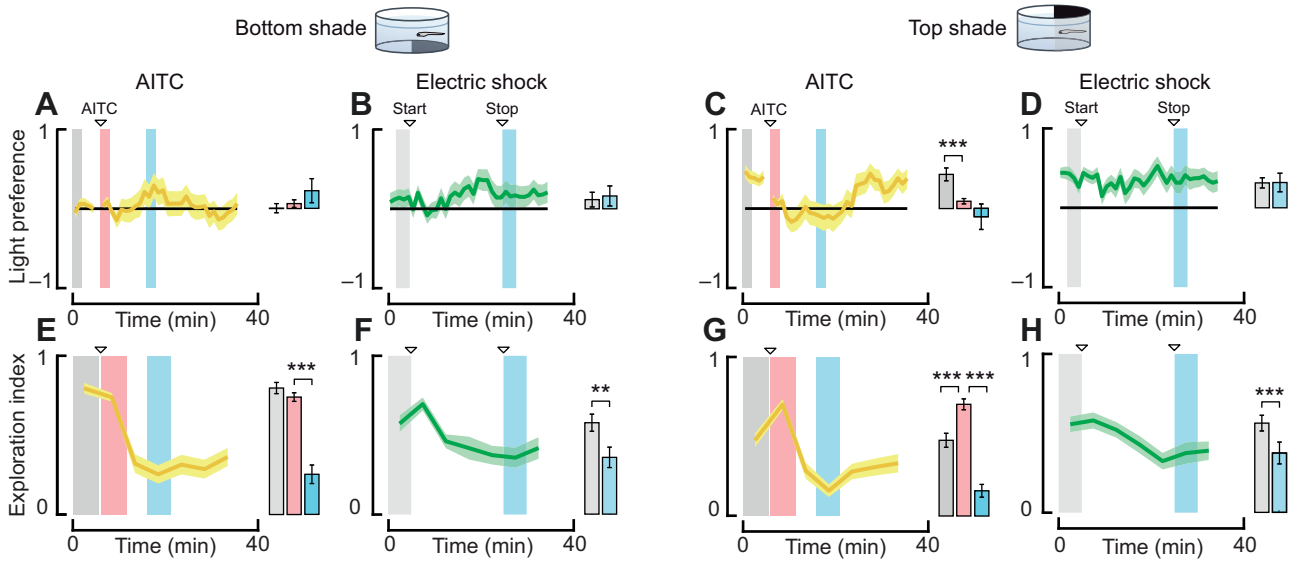

Fig. 2. Prolonged exposure to stressor decreases exploratory behavior. (A) Light preference of zebrafish larvae (7 dpf) swimming in a circular arena with half-light and half-dark bottom exposed to a TrpA1 agonist AITC (10 µmol l^{-1} in 0.05% DMSO final concentration). Time series (left) displays preference averaged over 60 s periods, and bar plots (right) show time average over pre-stimulus (gray), immediate response (pink) and delayed response (blue) intervals (median across individuals; error bars are s.e.m.; N=30). Significant differences in pre-stimulus versus immediate response values and immediate response versus delayed response values are indicated with asterisks (*P<0.05, **P<0.01, ***P<0.001, two-tailed t-test). Light preference was not significantly different from 0 during the pre-stimulus period (P>0.05, two-tailed t-test). (B) Similar to panel A except that the larvae were exposed to an electric shock stressor (5 s burst of 1 ms pulses at 40 Hz, delivered once per 60 s). Bar plots (right) show time average over pre-stimulus (gray) and post-stimulus (blue) windows (N=28). (C,D) Similar to panels A,B except that the experimental arena was half-light and half-dark as a result of a top overhang (C and G, N=33; D and H, N=28). Light preference was significantly greater than 0 during the pre-stimulus period (P<0.001, two-tailed t-test). (E–H) Exploration index of zebrafish larvae exposed to AITC stressor. Data are plotted as in panels A–D, except that the time series and bar plots use averages over 5 min intervals.

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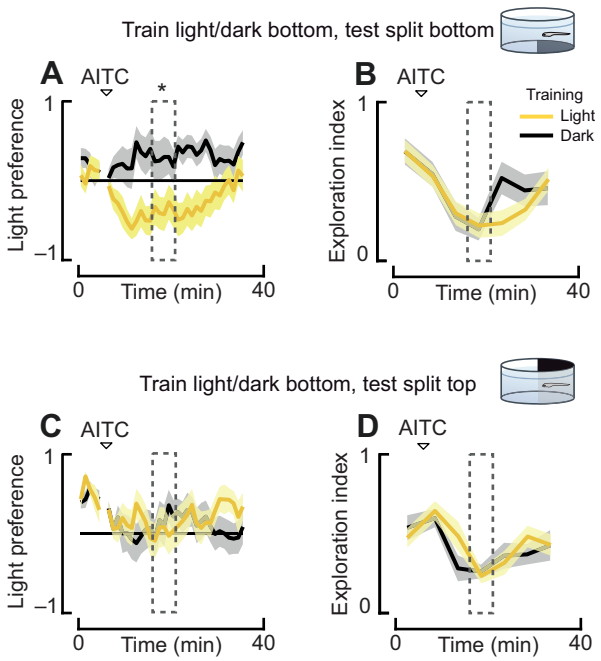

Fig. 3. Larval zebrafish learn light–dark preference. (A,B) Zebrafish larvae were presented with an all-light (yellow line) or all-dark (black line) bottom during exposure to an AITC stressor (10 µmol l^{-1} in 0.05% DMSO final concentration), and then light preference and exploration index were measured in an experimental arena with a half-light and half-dark bottom. Time series displays preference averaged over 60 s periods (median across individuals; error bars are s.e.m.; all-light, *N*=14; all-dark, *N*=12). Significant differences in the average response over an interval of 15 min post-stimulus (dashed box) are indicated with asterisks (**P*<0.05, ***P*<0.01, ****P*<0.001, one-tailed *t*-test). (C,D) Similar to panels A and B except that zebrafish larvae were exposed to the AITC stressor in an arena with an all-light (yellow line) or all-dark (black line) bottom and then light preference and exploration index were measured in an experimental arena that was half-light and half-dark as a result of a top overhang (all-light, *N*=13; all-dark, *N*=12).

light–dark preference even when the stressor is presented in a half-light and half-dark arena may reflect an element of superstitious learning (Skinner, 1948). We next examined the degree to which these learned responses are generalized. Animals were placed into an experimental arena with an all-light or all-dark bottom, AITC was introduced into the arena, and then the preference of the animals was recorded in an experimental arena that was shaded from the top. We did not observe a significant learning effect under these conditions (*P*>0.05, two-tailed *t*-test, *N*=13 light, *N*=12 dark), which suggests that learned responses to one light–dark environment are not necessarily generalized to other light–dark environments (Fig. 3C,D).

To confirm that the behavioral metrics we utilized were robust measures, we also examined the light–dark preference of zebrafish larvae in experimental arenas of different sizes and the preference of larvae of different ages. We found that the light preference and exploration index were substantially consistent between different experimental arenas and larval ages, although the swimming and angular speeds were variable and the youngest larvae (4 dpf) differed from the other ages (Fig. 4). In addition, as larval zebrafish are visual predators (Bianco et al., 2011), we also explored whether hunger alters the light preference of the animals. We found no significant differences in the light preference of fed and unfed zebrafish larvae (*P*>0.05, two-tailed *t*-test, *N*=26 fed, *N*=41 unfed; Fig. 5), which suggests that this behavior is not associated with prey tracking.

Whole-brain calcium imaging

In order to examine the neural pathways associated with stress-induced changes in exploratory behavior, we next used two-photon whole-brain calcium imaging to identify neuron populations with selective responses to a noxious chemical stressor (Fig. 6A). The fluorescent calcium indicator GCaMP6F (Chen et al., 2013) was expressed pan-neurally, a two-photon microscope (Svoboda and Yasuda, 2006) was used to record activity throughout the CNS, the response to addition of AITC to the perfusion bath was recorded (25 µmol l^{-1} in 0.1% DMSO), and neural activity maps were constructed using a SVM classifier (Schölkopf et al., 2002) (Fig. 6B). As these maps are based on classifier models, they highlight neuronal populations with selective responses to one experimental condition and tend to ignore neuronal populations with non-selective responses. A neural activity map was constructed for each individual, and inspection of these maps revealed several common features. For every individual, the neural activity map indicated the presence of separate cell bodies in the area postrema that were selectively active during either the immediate or delayed response period. As we observed the most robust responses from the area postrema, we next computed the normalized fluorescence intensity ($\Delta F/F$) for each responsive cell body located within the area postrema. We found that AITC perfusion produced a significant increase in the activity ($\Delta F/F$) of area postrema neurons (3 min pre-stimulus compared with 3 min post-stimulus, *P*<0.001, two-tailed *t*-test, *N*=65 cells; Fig. 6C–F). Interestingly, the half-life of the response varied substantially, ranging from less than 3 min to greater than 10 min. These results suggest that neurons in the area postrema integrate noxious chemical cues, and that there are multiple functional classes that may be distinguished by their rate of adaptation.

Adrenergic contribution to behavior

The area postrema contains a large number of noradrenergic neurons (Ma, 1997; McLean and Fetcho, 2004), adrenaline and noradrenaline have critical roles in the primary stress response (Schreck et al., 2016; Wendelaar Bonga, 1997), and α-adrenergic receptors are highly expressed throughout the CNS of larval zebrafish (Ruuskanen et al., 2005). Consequently, we next examined the contribution of adrenergic pathways to stress-induced changes in behavior. Zebrafish larvae were allowed to freely move in an experimental arena with a half-light and half-dark bottom. An irreversible blocker of α-adrenergic receptors, phenoxybenzamine (PB) (Frang et al., 2001), was then introduced into the bath and the behavioral response was recorded for 45 min. After this period, AITC was introduced into the bath and the response was recorded for an additional 45 min. Similar to previous experiments, in untreated animals the AITC stimulus induced an immediate increase in locomotor activity, followed by a period of decreased locomotion and exploratory behavior, followed by a gradual return to baseline levels consistent with adaptation (Fig. 7). PB-treated animals exhibited similar behaviors, except the median swimming speed prior to the stressor decreased by 85% (*Q*<0.05, two-tailed *t*-test with Benjamini–Hochberg false discovery rate correction across all measures and time intervals, *N*=30 control, *N*=21 PB), and the median speed immediately following the AITC stressor decreased by 41% (*Q*<0.05). The long-term recovery to baseline levels was also delayed and by 25 min the median swimming speed of PB-treated animals was 65% lower than that of untreated larvae (*Q*<0.05). Notably, both untreated and PB-treated animals showed decreases in swimming speed and exploration index during the intermediate period (10–12 min post-AITC

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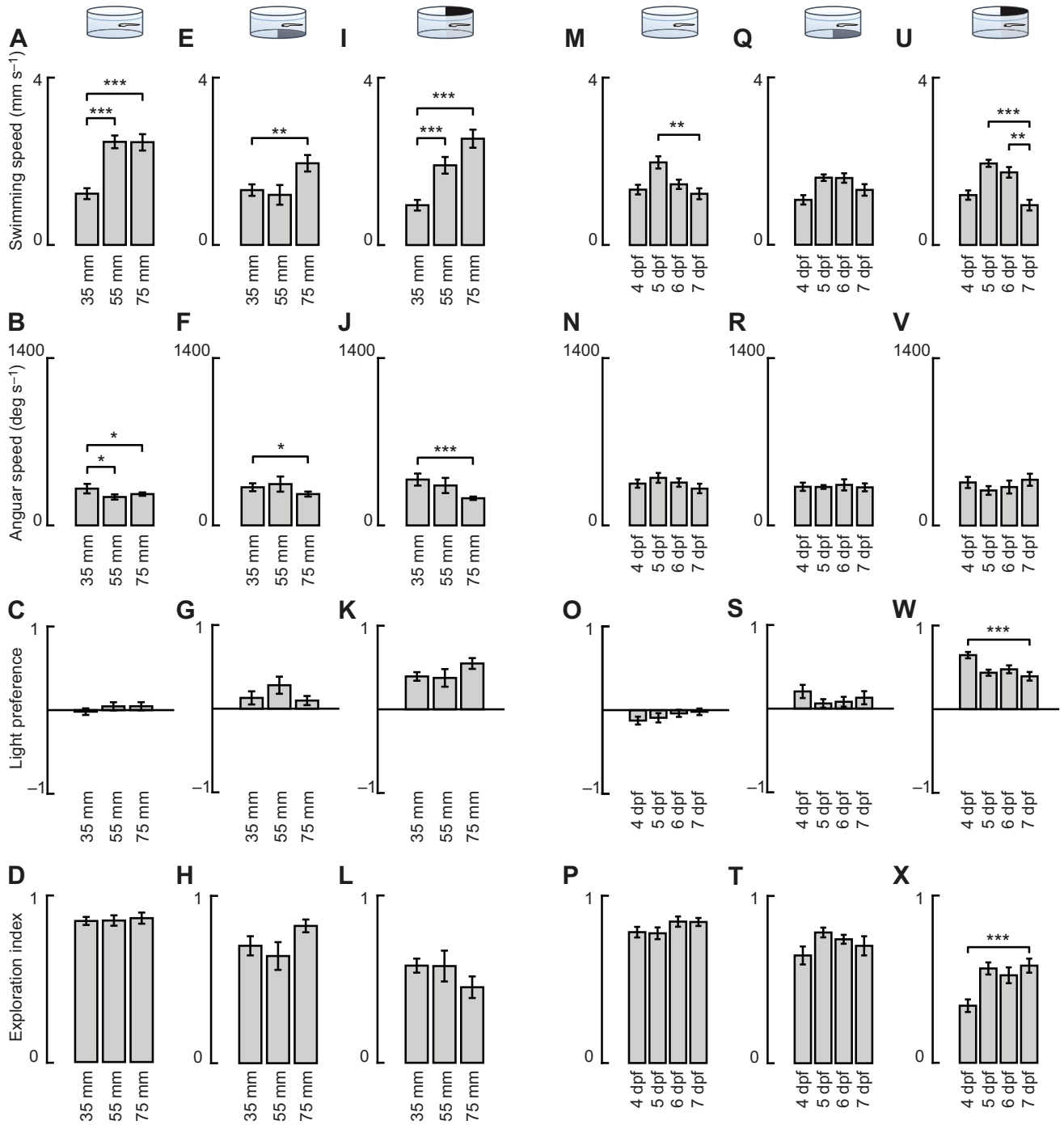

Fig. 4. Behavioral metrics are robust to differences in experimental arena and larval age. (A–D) Behavior of zebrafish larvae (7 dpf) swimming in circular experimental arenas of varying diameters (35, 55 and 75 mm) with an all-light bottom. Bar plots show the time average over a 25 min interval (median across individuals; error bars are s.e.m.; 35 mm, *N*=26; 55 mm, *N*=16; 75 mm, *N*=15). Measured parameters include swimming speed, angular speed, light preference and exploration index. The values observed in the 35 mm arena were independently compared with the values from the 55 and 75 mm arenas, and significant differences are indicated with asterisks (**P*<0.05, ***P*<0.01, ****P*<0.001, two-tailed *t*-test). (E–H) Similar to panels A–D except that the experimental arena had a half-light and half-dark bottom (35 mm, *N*=26; 55 mm, *N*=15; 75 mm, *N*=19). (I–L) Similar to panels A–D except that the experimental arena was half-light and half-dark as a result of a top overhang (35 mm, *N*=25; 55 mm, *N*=15; 75 mm, *N*=16). (M–P) Behavior of zebrafish larvae of varying ages (4–7 dpf) swimming in an experimental arena with an all-light bottom (4 dpf, *N*=23; 5 dpf, *N*=24; 6 dpf, *N*=28; 7 dpf, *N*=26). Data are presented as in panels A–D. (Q–T) Similar to panels M–P except that the experimental arena had a half-light and half-dark bottom (4 dpf, *N*=24; 5 dpf, *N*=26; 6 dpf, *N*=23; 7 dpf, *N*=26). (U–X) Similar to panels M–P except that the experimental arena was half-light and half-dark as a result of a top overhang (4 dpf, *N*=23; 5 dpf, *N*=25; 6 dpf, *N*=26; 7 dpf, *N*=25).

addition), and there were no significant differences in their responses (Q>0.05, two-tailed *t*-test). These results suggest that adrenergic pathways contribute to hyperactivity that occurs immediately following a stressor presentation, but are not necessary for the decrease in exploratory behavior that occurs after sustained stressor presentation.

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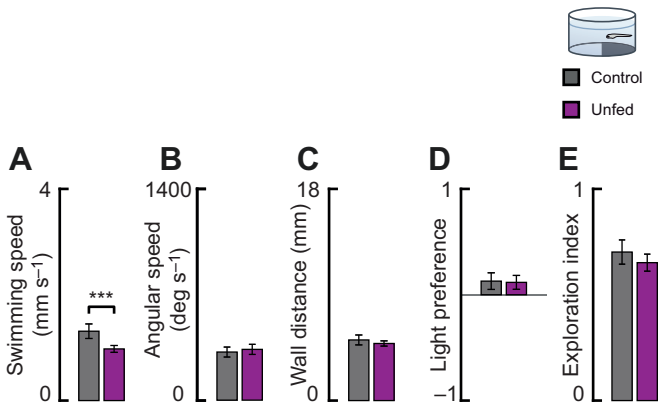

Fig. 5. Behavior of animals in light–dark environments was similar with and without feeding. Behavior of zebrafish larvae (7 dpf) that were fed normally (fed twice per day on days 5–7) and that were unfed (subsisted on yolk sac), swimming in an experimental arena that had a half-light and half-dark bottom (control, *N*=26; unfed, *N*=41). Bar plots show the time average over a 25 min interval and significant differences are indicated with asterisks, and error bars are s.e.m. (**P*<0.05, ***P*<0.01, ****P*<0.001, two-tailed *t*-test).

DISCUSSION

We found that noxious chemical and electric shock stressors elicited robust and similar behavioral responses. Acute exposure to either stressor induced elevated locomotor activity, while prolonged exposure suppressed swimming activity (Fig. 1). Although enhancement of light–dark preference has been broadly associated with anxiety-like states in both adult mammals (Bourin and Hascoët, 2003; Crawley and Goodwin, 1980; Imaizumi et al., 1994) and adult zebrafish (Chakravarty et al., 2013), we found that neither noxious chemical nor electric shock stressors enhanced the light preference of larval zebrafish. Instead, we found that both stressors produced a substantial and consistent decrease in the exploration of available visual environments (Fig. 2). The exploration index used in this study decreases if the animal remains on one side of the arena, increases if the animal spends equal amounts of time in all parts of the area, and is not explicitly sensitive to the swimming speed of the animal. However, it is difficult to determine if a slow-moving animal would have explored a different visual environment more often if it had encountered it more frequently. To determine if the larvae were distinguishing between the visual environments, we examined the behavior of larvae that were first presented with a noxious stressor in an all-light or all-dark environment were then allowed to explore a split light–dark arena. We found that larvae displayed a learned preference for the non-stressor environment, supporting the conclusion that the larvae distinguish between the visual environments during exploratory behaviors. As stress-induced decreases in exploratory behavior would be expected to enhance even moderate pre-existing preferences, these effects may explain why other stressors have been observed to shift the light preference of larval zebrafish (Bai et al., 2016). In addition, we observed substantial changes in the behavior of these animals with time, which suggests that future pharmacological studies may benefit from measuring treatment effects as explicit functions of time. Such complications may also help to explain why some prior studies of nociception in zebrafish have found conflicting or complex dose-dependent effects (Diggles et al., 2017; Ko et al., 2019; Lopez-Luna et al., 2017; Steenbergen and Bardine, 2014; Taylor et al., 2017). Furthermore, our results generalize recent findings that electric shock initially increases and then decreases locomotor activity of zebrafish larvae (Andalman et al., 2019; Duboué et al., 2017; Steenbergen, 2018). It is possible that this transition from hyperactivity to hypoactivity results from muscular fatigue rather than a change in motivational state. However, prior studies have found that zebrafish larvae are able to maintain similarly elevated swim speeds for >24 h (Bagatto et al., 2001), so it appears likely that this shift in behavior is driven by a

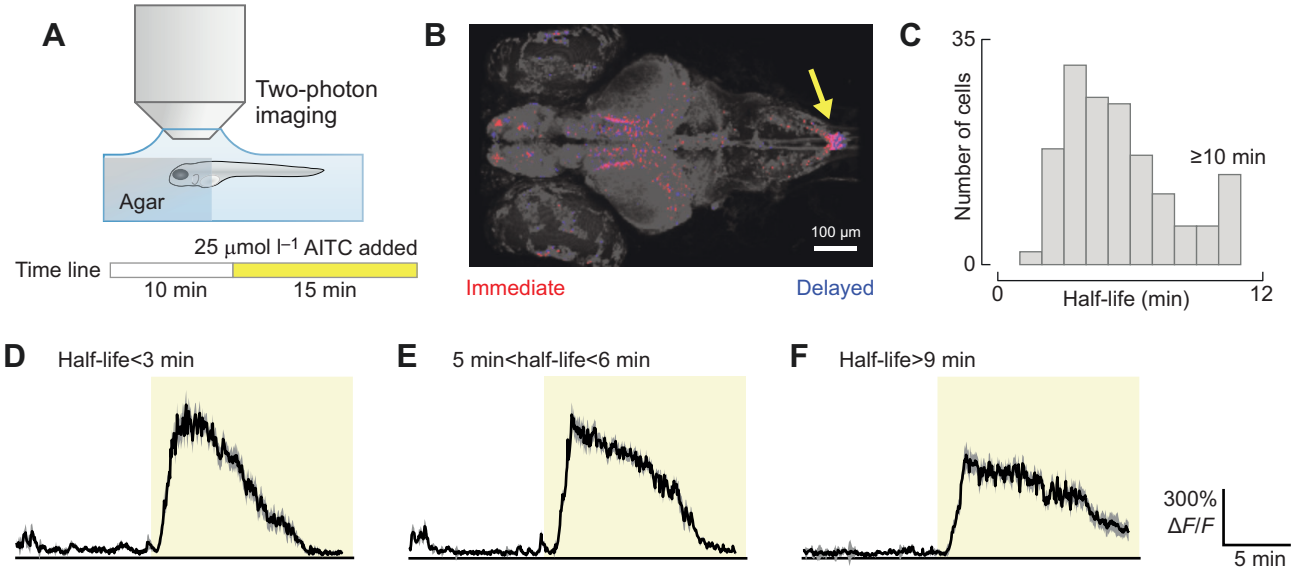

Fig. 6. A noxious chemical cue drives elevated activity in fast and slow adapting neurons of the area postrema. (A) Schematic of the whole-brain calcium imaging experiment used to record neural response to TrpA1 agonist AITC stressor (25 µmol l^{-1} AITC in 0.1% DMSO). (B) Typical neural activity map for the response to the TrpA1 agonist AITC stressor. Red pixels indicate cells that are selectively active immediately after introduction of AITC (0–5 min post-injection), and blue pixels show cells that are selectively active after a delay (10–15 min post-injection). The area postrema (yellow arrow) contains a mixture of red and blue labeled cells. (C) The calcium response of individual area postrema neurons to the introduction of AITC was recorded and the duration of the response (half-life) was computed. The histogram shows the distribution of the measured half-life values. (D–F) Response of area postrema neurons to the introduction of AITC, sorted by their computed half-life (yellow background shows AITC introduction; medians±s.e.m.; <3 min, *N*=20 cells; 5–6 min, *N*=25 cells; >9 min, *N*=20 cells).

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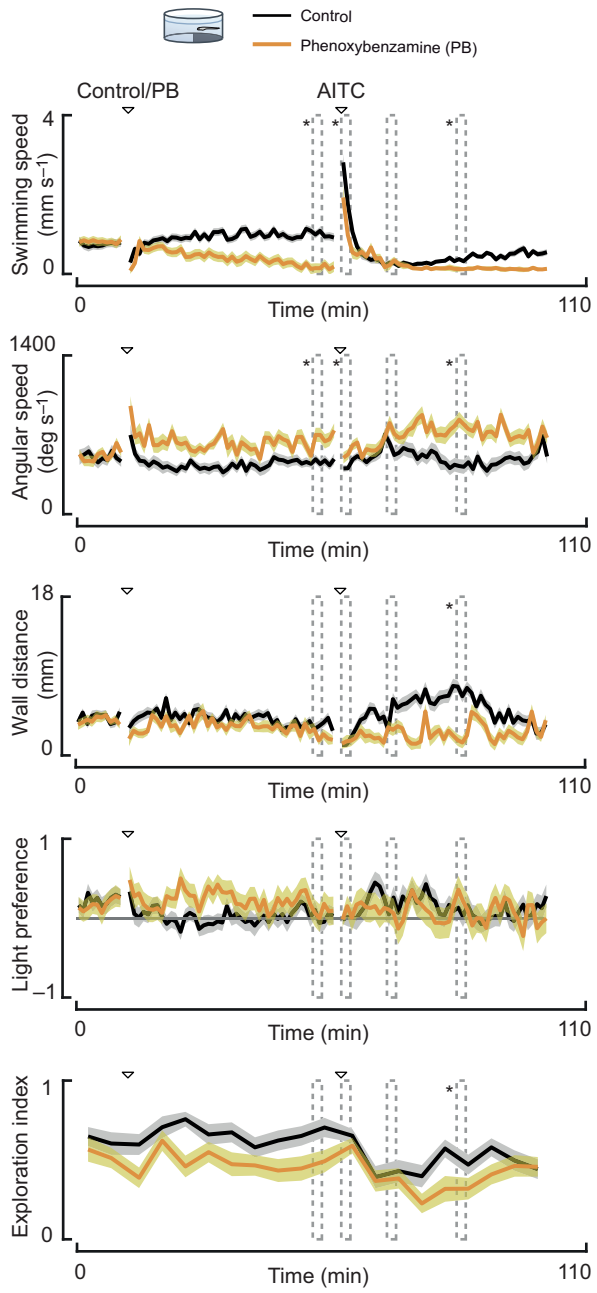

Fig. 7. α-Adrenergic receptors contribute to transient behavioral responses to stressors. Behavior of zebrafish larvae (7 dpf) treated with control solution (0.05% DMSO final concentration) or α-adrenergic antagonist phenoxybenzamine (PB, 10 µmol l^{-1} in 0.05% DMSO final concentration). Larvae were incubated in treatment solution for 45 min, then presented with AITC stressor (10 µmol l^{-1} in 0.05% DMSO final concentration), and the response was recorded for 45 min. Measured parameters included swimming speed, angular speed, distance to the nearest wall, light preference and exploration index (plotted as medians±s.e.m.; control, *N*=30; PB, *N*=21). Behavior of untreated and PB-treated animals was compared during intervals pre-stimulus, immediately after the AITC injection, 15 min following the AITC injection, and 30 min after injection; asterisks indicate significant differences (*Q<0.05 using two-tailed *t*-test with Benjamini–Hochberg false discovery rate correction).

change in neural state and is not a product of physiological limitations. This transition from hypoactivity to hyperactivity has also been compared to the switch between active and passive coping strategies exhibited by rodents (Andalman et al., 2019; Koolhaas and de Boer, 2008; Koolhaas et al., 1999), and is consistent with the conclusion that changes in behavior induced by noxious chemical and electric shock stressors are mediated by similar pathways.

Using whole-brain calcium imaging, we found that the noxious chemical AITC induced a rapid increase in the activity of area postrema (AP) neurons. With prolonged exposure, AP neurons exhibited an adaptation-like decline to baseline activity. Interestingly, the rate of adaptation varied widely between AP neurons, with fast-adapting neurons exhibiting a half-life of <3 min and slow-adapting neurons displaying a half-life of >10 min. The half-life of the fast-adapting neurons is similar to the time period over which zebrafish larvae exposed to the same stimulus transition from hyperactive to sedentary behavioral states. It may be interesting for future studies to determine if this represents a causal mechanism or coincidence through loss-of-function experiments. It is also possible that the immobilization used in our imaging experiments itself acts as a mild stressor, although larvae in similar preparations appear to acclimate quickly and perform complex behaviors including prey tracking and navigation (Ahrens et al., 2012; Dunn et al., 2016; Muto et al., 2017). Our results are also in agreement with recent studies in zebrafish larvae that found that AITC induces increased time-averaged pERK activity in freely swimming animals throughout the brain and especially in the area postrema (Wee et al., 2019).

Although the functional role of the area postrema is not well understood in teleost fishes, the area postrema contains noradrenergic and dopaminergic neurons (Ma, 1997; McLean and Fetcho, 2004), has broad projections throughout the central nervous system (Tay et al., 2011), has been implicated in feeding behaviors (Randlett et al., 2015), and plays an important role in sensing noxious chemicals in mammalian vertebrates (Miller and Leslie, 1994; Price et al., 2008; Tsukamoto and Adachi, 1994). Notably, prior whole-brain studies that examined neural activity during changes in locomotor state did not identify correlates in the area postrema (Ahrens et al., 2012), which suggests that the AITC-induced increases in area postrema activity observed in this study are more likely to be associated with sensing of noxious stimuli than a resulting motor response. We further found that α-adrenergic receptors contribute to hyperactivity induced by an acute stressor, but are not necessary for sedentary behaviors following prolonged stressor exposure. Together, these findings are consistent with a model in which functionally diverse neurons within the area postrema integrate noxious chemical cues, directly or indirectly modulate the activity of downstream targets throughout the CNS, and drive the observed stress-induced changes in behavior. The identity of the downstream targets and their role in mediating stress-induced changes in behavior is not yet clear. However, prior studies in zebrafish have found that activity in the habenula is associated with transitions from active to passive coping states (Andalman et al., 2019; Duboué et al., 2017; Lee et al., 2010). The area postrema does not appear to have substantial direct projections into the region of the dorsal thalamus containing the habenula (Tay et al., 2011). However, the area postrema does project to the lateral diencephalon, which densely innervates the habenula (Hendricks and Jesuthasan, 2007), and it is possible that this pathway plays an important role in this behavior. There is also evidence that the serotonergic neurons of the raphe nuclei are associated with transitions from passive to active coping states in zebrafish, as inhibition of serotonergic neurons reduces swimming speed (Andalman et al., 2019; Cheng et al., 2016) and abolishes alert states induced by flow stimuli (Yokogawa et al., 2012). However, activation or inactivation of serotonergic neurons has a range of

effects on light preference, anxiety-related behaviors and sleep/wakefulness (Cheng et al., 2016; Maximino et al., 2013; Oikonomou et al., 2019). These findings may suggest that diverse serotonergic neuron sub-types have different functional roles. It is unclear if the neurons of the area postrema have relevant projections into the raphe nuclei (Ma, 1997; Tay et al., 2011), although indirect connectivity cannot be ruled out. Lastly, the contribution of the HPI axis to these behavioral transitions is not yet clear, although prior studies have found that cortisol levels peak approximately 10–20 min post-stressor exposure (De Marco et al., 2014). Future studies examining changes in neural activity as the animals interact with different visual environments are likely to produce substantive advances in our understanding of the diversity of neuron types in these pathways and the organization of the neural circuits mediating these behaviors.

Our results indicate that zebrafish larvae display complex behavioral responses to stressor stimuli that are comparable to those observed in adult vertebrates and that involve similar neuromodulatory pathways. These findings suggest that continued advances in our understanding of the neural circuits responsible for stress-induced changes in the behavior of zebrafish larvae may contribute to general insights into the processes by which neuromodulation alters complex behaviors.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: W.A.H., J.A.S.; Methodology: W.A.H., J.A.S.; Software: J.A.S.; Validation: W.A.H., J.A.S.; Formal analysis: W.A.H., B.M., J.A.S.; Investigation: W.A.H., B.M., J.A.S.; Resources: J.A.S.; Data curation: W.A.H., B.M., J.A.S.; Writing - original draft: W.A.H., J.A.S.; Writing - review & editing: W.A.H., J.A.S.; Visualization: W.A.H., J.A.S.; Supervision: J.A.S.; Project administration: J.A.S.; Funding acquisition: J.A.S.

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