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Chemical modulation of memory formation in larval zebrafish

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Whole organism–based small-molecule screens have proven powerful in identifying novel therapeutic chemicals, yet this approach has not been exploited to identify new cognitive enhancers. Here we present an automated high-throughput system for measuring nonassociative learning behaviors in larval zebrafish. Using this system, we report that spaced training blocks of repetitive visual stimuli elicit protein synthesis–dependent long-term habituation in larval zebrafish, lasting up to 24 h. Moreover, repetitive acoustic stimulation induces robust short-term habituation that can be modulated by stimulation frequency and instantaneously dishabituated through cross-modal stimulation. To characterize the neurochemical pathways underlying short-term habituation, we screened 1,760 bioactive compounds with known targets. Although we found extensive functional conservation of short-term learning between larval zebrafish and mammalian models, we also discovered several compounds with previously unknown roles in learning. These compounds included a myristic acid analog known to interact with Src family kinases and an inhibitor of cyclin dependent kinase 2, demonstrating that high-throughput chemical screens combined with high-resolution behavioral assays provide a powerful approach for the discovery of novel cognitive modulators.

Results and Discussion

Zebrafish Larvae Demonstrate Long-Term Habituation to Visual Stimuli. To determine whether larval zebrafish have the capacity to acquire, store, and later recall learned information, we exposed larvae at 6 d postfertilization (dpf) to repetitive visual stimulation and then tested for responsiveness to the trained stimulus. Equilibrating larvae to a uniformly illuminated testing chamber and then abruptly extinguishing the light for 1 s (dark flash) elicits a unique turning maneuver called the O-bend (28). Larvae were exposed to 120 total min of dark flashes with ISIs ranging from 15 s to 60 s in either a massed or spaced dark flash-training format, and then tested for O-bend responsiveness to 10 dark flashes delivered at a 60-s ISI (Fig. L4). Maximal long-term habituation was observed when dark flashes were delivered at a 15-s ISI during training. Consistent with habituation paradigms for other organisms (29), a spaced training protocol yielded a longer-lasting response decrement, up to 24 h postraining, compared with only 1 h using a massed training protocol (Fig. LB).

Unlike short-term habituation, long-term habituation requires protein synthesis (29, 30). To determine whether O-bend habituation after a spaced training protocol requires protein synthesis, we bathed larvae in 10 μM cyclohexamide (CHX) during a spaced training protocol, washed out the CHX after the fourth training session, and tested for O-bend responsiveness 4 h later. As expected, CHX treatment prevented long-term habituation, indicating that protein synthesis is required for O-bend habituation.

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The authors declare no conflict of interest.

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later by delivering 10 dark flashes at a 60-s ISI. CHX treatment significantly reduced the degree of O-bend response attenuation, suggesting that protein synthesis is required for the observed habituation (Fig. 1C). Notably, CHX treatment did not reduce intra-training session habituation, suggesting that short-term habituation is unaffected by CHX treatment (Fig. S1). These results demonstrate that larval zebrafish can store and retrieve visual memory information up to 24 h.

Previous studies in larval zebrafish have shown that retinal circuits can memorize stimulus time intervals; however, the time scale for this memory is ~20 s (27), and thus this property likely does not account for the memory recall that we observed up to 24 h after training. Here, we demonstrate that 6-dpf larval zebrafish exhibit long-term habituation, consistent with the idea that the neural circuits regulating O-bend behavior are competent for memory storage and retrieval at the larval stage.

**Repetitive Acoustic Stimulation Decreases Short-Latency C-Start Responses.** We next asked whether larval zebrafish also exhibit short-term habituation of a simple sensorimotor behavior. Best et al. (25) previously used low–temporal resolution motion analysis to measure a gradual decrement in larval spatial displacement in response to repetitive acoustic stimuli. However, zebrafish larvae demonstrate multiple kinematically different responses in response to acoustic stimuli (18), necessitating the use of high-resolution motion and kinematic analyses to distinguish between these different responses and to focus on a single sensorimotor behavior with known underlying circuitry (24).

Five-dpf zebrafish larvae exhibit a highly stereotyped acoustic startle response characterized by a kinematically unique maneuver known as short-latency C-start (SLC), which is maintained through adulthood (18, 31, 32). To execute the larval and adult SLC behavior, acoustic or tactile sensory input is transmitted directly to the hindbrain, where the Mauthner cells, the command neurons of the SLC response, process the input and subsequently activate contralateral motor neurons and muscle contraction (18, 33–36). Despite the circuit’s simplicity, the larval zebrafish’s SLC response shows remarkable capacity for sensorimotor-gating processes, such as prepulse inhibition, indicating that the circuit can modulate responsiveness based on experience (18). Moreover, repeated delivery of acoustic stimuli at short ISIs attenuates the SLC response, suggesting that the simple, Mauthner-driven SLC response circuit may have the capacity for nonassociative learning (18).

To examine key kinematic parameters associated with startle attenuation, 5-dpf larvae were exposed to a series of 60 acoustic stimuli of varying intensity and ISI (Fig. 2A). Responses of individually housed larvae were recorded at millisecond resolution and analyzed with automated tracking software for SLC responsiveness (Fig. 2B and C and Movie S1) (18). First, larvae were exposed to 10 “subthreshold” stimuli (SI Experimental Procedures), delivered at a 20-s ISI, to test startle sensitivity. Stimuli 11–20 (prehabitation phase) consisted of “above-threshold” stimuli (SI Experimental Procedures) with a 20-s ISI to determine various kinematic parameters of the SLC response (including latency, turning angle, and velocity) under nonhabituating conditions. Stimuli 21–50 (habitation phase), consisted again of above-threshold stimuli, now delivered with a 1-s ISI. As shown in Fig. 3A, stimulation at a 1-s ISI elicited a robust SLC response decrement, reaching an asymptotic level by the 10th–15th stimulus at a 1-s ISI (the 30th–35th overall stimulus), suggesting the larvae were habituated. After the 50th stimulus, larvae were given a 3-min rest period, after which they were exposed to an additional set of 10 above-threshold stimuli with a 20-s ISI (recovery phase). During this last phase, larvae had recovered and resumed execution of SLCs at a frequency identical to that observed during the prehabitation phase, consistent with the observation that animals should spontaneously recover from short-term habituation (Fig. 3A) (9).

Quantitative analysis of 3,590 responses from more than 180 larvae demonstrated that the kinematics of the SLC response, including latency, duration, turning angle, and angular velocity of the C-bend, as well as the overall distance the fish travels as a result of performing a SLC, were identical at all phases of the assay (Fig. S2). To quantify the degree of habituation for each individual tested, we calculated the ratio of the average SLC responsiveness during the last 10 habituation phase stimuli (i.e., stimuli 41–50) to the 10 prehabitation phase stimuli (i.e., stimuli 11–20). The extent of habituation was similar in animals at 5, 10, and 14 dpf (Fig. 3B), demonstrating that our behavioral assay measures be-
larvae to three separate sessions of 20 stimuli delivered at a 1-s ISI with 3–60 min between sessions. Quantitative analysis revealed a potentiated increase in the rate of habituation (Fig. 3E). For example, larvae exposed to only one training session reached 80% habituation after 20 stimuli, whereas larvae exposed to two training sessions exhibited 90% habituation already after 13 stimuli, and larvae exposed to a third session reached this level of habituation after only 5 stimuli. Treatment with CHX before and during the assays (as outlined in Figs. 2A and 3E) did not influence SLC attenuation or potentiation, respectively, suggesting that SLC short-term habituation and potentiation do not require protein synthesis (Fig. S3). Notably, further increasing the ISI and/or using a spaced training protocol did not increase SLC habituation or potentiation beyond a 60-min period. This result may not be surprising, given that the innate function of the startle circuit is to mediate escape from predators, and that habituating to a threatening stimulus for an extended period likely constitutes an evolutionary disadvantage.

Finally, to confirm that SLC response attenuation is indeed learning and not a result of sensory or motor fatigue, we tested whether SLC response attenuation is instantly reversible through cross-modal stimulation (9). To test for cross-modal dishabituation, we presented control larvae with 30 acoustic stimuli at a 1-s ISI and then delivered a 31st acoustic stimulus at 3 s after the 30th acoustic stimulus (Fig. 3F). As expected, control larvae displayed SLC response attenuation at the 31st acoustic stimulus. To provide a cross-modal stimulus, between the 30th and 31st acoustic stimuli we applied a brief tactile stimulus that efficiently elicits an SLC response (36). In contrast to control larvae, application of a tactile stimulus with a handheld poker to the larval head during the 3-s period restored SLC responsiveness to the 31st acoustic stimulus, demonstrating that the attenuated SLC response reflects habituation, not fatigue. Consistent with the defined parametric habituation criteria, larvae also habituated to the dishabituating tactile stimulus. Interestingly, replacing the dishabituating tactile stimulus with a visual, dark-flash stimulus was not sufficient to dishabituate acoustic startle habituation; larvae failed to respond to the 31st acoustic stimulus nearly identically to when no dishabituating stimulus was given (Fig. 3F). Dishabituation to acoustic stimulation via dark-flash stimulation may be unlikely, given that the dark-flash–induced O–bend behavior is Mauthner-independent (37), whereas the tactile dishabituating stimulus elicits a Mauthner-mediated response, and thus a tactile stimulus “resets” the appropriate, habituated circuit. Thus, examination of several hallmark criteria for habituation, including modulation of habituation intensity and spontaneous recovery by varying stimulation frequency (Fig. 3C and D), habituation potentiation (Fig. 3E), and cross-modal dishabituation (Fig. 3F), provides compelling evidence that zebrafish larvae display nonassociative learning. Moreover, these results demonstrate that our assay readily measures short-term habituation at the level of an individual, kinematically distinct behavior.

**NMDA-Type Glutamate Receptor Antagonists Reduce Startle Habituation.** Pharmacologic manipulation of glutamate neurotransmission has been shown to modulate habituation in various model systems, and also has proven effective in treating human neuropsychological disorders that manifest with habituation deficits (38–43). To investigate whether key pharmacologic substrates of mammalian habituation are conserved in zebrafish, we tested the effects of two NMDA-type glutamate receptor antagonists, MK-801 and ketamine, on SLC habituation. A 15-min incubation in either MK-801 or ketamine did not alter the kinematic performance of the SLC behavior (Movie S2), nor did it affect the spontaneous initiation of turning or swimming bouts (Fig. S4A). However, MK-801 and ketamine each caused a dose-dependent and reversible reduction (via washout) in startle habituation and increased startle sensitivity (Fig. 4A and B; data not shown).

**Fig. 3.** Larval zebrafish SLC habituation to acoustic stimuli fits nonassociative learning parametric criteria. (A) Mean SLC response trend of 180 5-dpf larvae to the acoustic stimulation protocol described in Fig. 2A, showing exponential response decrement during habituation phase and spontaneous recovery after a 3-min rest period between stimulus 50 and stimulus 51. (B) Mean degree of SLC habituation is equivalent in 5- to 14-dpf larvae. N larvae shown within each bar in graph. (C and D) Mean SLC response trend (C) and degree of habituation (D) of 30 5-dpf larvae exposed to acoustic stimuli at a 1-s, 5-s, or 20-s ISI during the habituation phase. Mean SLC responses are binned by sets of 10 successive stimuli. SLC habituation is greater and spontaneous recovery is more robust after a 3-min rest period when larvae are stimulated more frequently. *P < 0.001 vs. 20-s ISI group, Student t test. (E) Mean SLC habituation trend of 48 5-dpf larvae during three sets of 20 acoustic stimuli delivered at a 1-s ISI, separated by 3-min rest periods, indicates potentiation of habituation. The degree of habituation at stimuli 2–7 and 15–17 of set 2 and at stimuli 2–12 and 16–18 of set 3 differed significantly from that at the corresponding stimulus during the initial set of 20 stimuli (P < 0.01, Student t test). (F) Mean SLC responsiveness of 10 5-dpf larvae to acoustic stimuli. Larvae were subjected to 30 acoustic stimuli at a 1-s ISI, then a 3-s window during which either no stimulus or a head touch with a hand-held poker was given, followed by a final acoustic stimulus. Larvae dishabituated to the acoustic stimulus via cross-modal, tactile stimulation. Error bars indicate SEM.

**havioral responses of a mature nervous system. Thus, we observe a robust attenuation of a kinematically distinct, simple behavior to acoustic stimulation that is consistent with the idea that our assay measures short-term habituation.**

**SLC Response Attenuation Assay Measures Nonassociative Learning.** To further validate that the SLC response attenuation assay measures nonassociative learning, we examined additional hallmark criteria of habituation. One such criterion is that shortening the ISI should increase habituation and decrease the time required for the animal to spontaneously recover (9). Indeed, stimulating the larvae at a 1-s ISI elicited a significantly greater degree of habituation, with a shorter time required for complete recovery, compared with stimulation with an ISI of 5 s (Fig. 3 C and D). Habituation also potentiates with repeated sessions of stimulus exposure (9). To validate this criterion, we exposed larvae to three separate sessions of 20 stimuli delivered at a 1-s ISI with 3–60 min between sessions. Quantitative analysis revealed a potentiated increase in the rate of habituation (Fig. 3E). For example, larvae exposed to only one training session reached 80% habituation after 20 stimuli, whereas larvae exposed to two training sessions exhibited 90% habituation already after 13 stimuli, and larvae exposed to a third session reached this level of habituation after only 5 stimuli. Treatment with CHX before and during the assays (as outlined in Figs. 2A and 3E) did not influence SLC attenuation or potentiation, respectively, suggesting that SLC short-term habituation and potentiation do not require protein synthesis (Fig. S3). Notably, further increasing the ISI and/or using a spaced training protocol did not increase SLC habituation or potentiation beyond a 60-min period. This result may not be surprising, given that the innate function of the startle circuit is to mediate escape from predators, and that habituating to a threatening stimulus for an extended period likely constitutes an evolutionary disadvantage.

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These results demonstrate that MK-801- and ketamine-treated larvae were able to execute a normal response, but were unable to modulate their responsiveness properly, providing strong evidence that the lack of SLC response attenuation was not attributable to hyperactivity. Thus, brief exposure to known chemical modulators of habituation influences the modulation of the larval zebrafish SLC response without disrupting SLC response performance.

Conservation of Neural Substrates of Learning Between Zebrafish and Mammals. To test the feasibility of our habituation assay for large-scale systematic approaches, we screened two small bioactive compound libraries consisting of 1,760 compounds with defined targets. Five-dpf larvae were incubated in each compound for 15 min before and during the acoustic stimulation assay described in Fig. 2A. Among the 1,760 compounds screened, 11 compounds reduced startle habituation and 19 compounds increased startle habituation (Fig. 4 C–G and Tables S1 and S2). Overall, compounds with similar or common targets often had a comparable influence on habituation rate, whereas compounds with converse effects on identical targets usually caused opposing effects on habituation. Consistent with their high representation in the two chemical libraries, the majority of the compounds affecting habituation were those targeting neurotransmitter systems, including those previously identified to affect mammalian startle modulation (44, 45). For example, compounds antagonizing 5HT-2 serotonin receptors (e.g., pirenperone, ritanserin) or L-type calcium channels (e.g., verapamil, nimodipine) increased habituation, whereas compounds antagonizing glutamate receptors (e.g., MK-801, ketamine, t-701,324) or potassium channels (e.g., lino-

The ability to rapidly evaluate phenotypic specificity at the overall activity level, behavioral execution, and behavioral modulation is critical to teasing apart the relationship between molecular and cellular mechanisms underlying behavior. Importantly, none of the compounds reported to affect habituation altered the kinematic performance of the SLC response, including response latency, C-bend turn duration, turning angle, angular velocity, or the distance moved as a result of performing a SLC (selected compounds, Fig. S4 D and E). We noted that many of the compounds reducing habituation also increased startle sensitivity, but did not cause hyperactivity (Table S1). The phenotypic overlap between hypersensitivity and a habituation deficit is consistent with the notion that neural targets and substrates for startle sensitivity and habituation are intricately linked, and that the identification of targets specific for habituation requires behavioral screens designed to instantly distinguish between both processes in vivo. Indeed, we identified several compounds that reduced habituation without increasing startle sensitivity (i.e., hydralazine, SU-9516, and butaclamol; Fig. 4 C and D and Table S1). Finally, our screen was completed in 25 experimental days, which included simultaneous testing of 32 larvae with two behavioral apparatuses, confirming the scalability of our learning assay to large-scale genome-wide or systematic approaches. Thus, using a high-throughput chemical screening assay for short-term habituation modifiers, we have demonstrated a high degree of overlap between the substrates underlying nonassociative learning in larval zebrafish and adult mammals.

Identification of Compounds Regulating Nonassociative Learning. Our screening identified two classes of compounds previously not known to modulate learning behaviors. First, we identified three compounds targeting cell cycle regulators that modulate SLC habituation. SU-9516, kenpaullone, and indirubin-3’-monoxime...
are ATP-competitive inhibitors of serine/threonine cyclin-dependent kinase (Cdk) (47–49). SU-9516, an inhibitor of Cdk2 and, to a lesser extent, of Cdk1 and Cdk4 (48), reduced SLC habituation without altering sensitivity, reducing baseline motor activity, or affecting SLC performance kinematics (Table S1 and Fig. S4). In contrast, both indirubin-3′-monoxime and kenpaullone, which inhibit Cdk1, Cdk2, and Cdk5 (47, 49, 50), increased SLC habituation during the prehabituation phase (Table S2). Indirubin-3′-monoxime and kenpaullone also have been reported to inhibit glycolgen synthase kinase 3 beta (GSK3B) (51–53), which has known effects on learning as well as on prepulse inhibition of the mammalian acoustic startle response (54). GSK3B hyperactivity is thought to impair memory formation in neuropsychiatric conditions such as Alzheimer’s disease (55). Consistent with this idea, indirubin-3′-monoxime has been shown to reduce learning deficits in Alzheimer’s disease models (56), but neither indirubin-3′-monoxime nor kenpaullone has been shown to increase short-term learning in WT animals.

Despite the potential promiscuity of small-molecule kinase inhibitors, the brief exposure to SU-9516, indirubin-3′-monoxime, and kenpaullone during a period in which all neurons of the SLC circuit are postmitotic suggests a possible cell cycle–independent role for these compounds in mediating learning. Consistent with this hypothesis, Cdk6 are expressed in terminally differentiated neurons (57–61), and furthermore, Cdk5, the sole non–cyclin-activated member of the Cdk family, has been shown to regulate synaptic plasticity and learning (62–67). Notably, neither SU-9516 nor its primarily characterized target, Cdk2, has been implicated in synaptic plasticity or learning. However, without direct evidence that SU-9516 is inhibiting Cdk5 within the SLC circuit, we cannot exclude the possibility that SU-9516 influences learning through Cdk-independent targets.

Second, we identified the myristic acid analog 12-methoxydodecanoic acid (12-MDA), which reduced SLC habituation and increased SLC sensitivity without affecting hyperactivity or SLC kinematic performance (Fig. 4E and F and Fig. S4). Myristic acid compounds are 13- or 14-carbon saturated fatty acids that are cotranslationally added to the N terminus of membrane-associated proteins and are also common food and cosmetic additives (68). The libraries tested contained two other myristic acid analogs, 4-oxatetradecanoic acid and 2-hydroxymyristic acid, which are structurally similar to 12-MDA (Fig. 4G). Interestingly, neither of these related compounds altered SLC habituation or sensitivity (Fig. S4), suggesting that the position of the oxygen residue within the 12-MDA backbone is important for conferring substrate specificity. 12-MDA has been investigated primarily for its action in inhibiting virus replication (69, 70), although this is unlikely to be the mechanism underlying its effects on habituation.

12-MDA also has been shown to bind and redistribute Src family kinases (SKFs) from the membrane to the cytosol (71). The SKFs Src and Fyn contribute to the scaffolding of the NMDA receptor complex, and modulate synaptic efficacy by regulating postsynaptic glutamate receptor expression (72). Thus, it is conceivable that 12-MDA alters SKF localization, thereby affecting NMDA receptor signaling and thus reducing habituation. To further explore this potential functional link between 12-MDA and NMDA receptor signaling during habituation, we tested whether 12-MDA can modulate the function of NMDA receptors in vivo. We found that coincubation of larvae in 100 μM NMDA with 50 μM 12-MDA reversed the SLC habituation deficit observed after incubation in 50 μM 12-MDA alone (Fig. 4H).

Furthermore, coincubation of larvae in subeffective concentrations of 12-MDA (5 μM) and ketamine (50 μM) produced a significantly greater attenuation of SLC habituation than the additive effect of each individual compound at these doses (Fig. 4H). Similarly, the startle hypersensitivity phenotype was suppressed by coincubation of 100 μM NMDA and 50 μM 12-MDA and enhanced by treatment with 5 μM 12-MDA/50 μM ketamine (Fig. S5). Taken together, these results reveal a role for a myristic acid analog in learning, and may suggest new therapeutic approaches to regulating postsynaptic glutamate receptors.

In summary, our high-resolution behavioral assay has shown that larval zebrafish robustly exhibit nonassociative learning, with landmark parametric criteria and conserved pharmacologic characteristics. By scaling our behavioral assay to screen small-molecule libraries with high throughput, we have demonstrated the feasibility of this approach for large-scale genome-wide or systematic approaches that can identify new compounds with specific effects on nonassociative learning in vivo. Several small-molecule screens for basic behaviors, such as sleep/resting and phototactic responses, have been performed in zebrafish (21, 23). Importantly, our assay distinguishes between the effects of a compound on behavioral modulation (e.g., habituation, sensitivity) and alterations in kinematic performance. Given that deficits in modulation of the mammalian acoustic startle response represent an endophenotype common to many neuropsychiatric disorders (73–75), future screening with the assay described here can be applied to distinguish between many kinematic and behavioral processes as the primary target of already available drugs, and also can be applied toward the systematic identification of more “behavior-specific” compounds.

Experimental Procedures

All experiments were performed on zebrafish larvae between 5 and 7 days post fertilization. Fish maintenance, behavioral assays, testing apparatuses, pharmacologic applications, and behavioral scoring methods have been described previously (18, 28, 76). Details and any variations in these methods are provided in SI Experimental Procedures.

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Supporting Information

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SI Experimental Procedures

Fish Maintenance. The zebrafish (Danio rerio) larvae used in this study were from intercrosses of Tuebingen long-fin strain parents. Embryos were collected in the morning and raised at 28 °C on a 14-h:10-h light:dark cycle. Larvae were raised as described previously (1). Behavioral experiments were conducted at 5–14 dpf. Larvae tested at 5–6 dpf were raised only in E3, whereas larvae tested at >7 dpf were fed paramecia beginning on 6 dpf.

Behavioral Assays, Video Recording, and Behavioral Analysis. Dark-flash–induced O-bend responses were elicited and measured as described previously (1). Larvae were trained and tested at a density of 20 larvae per 9 mL E3 in 6-cm Petri dishes and kept in this dish during training or testing. Larvae were maintained on a 200-μW/cm² white light box when not training or testing. For CHX treatment experiments, 90 μL of 1 mM CHX dissolved in 100% DMSO was added to each dish of 20 larvae in 9 mL E3. Control dishes received 90 μL of 100% DMSO. CHX/DMSO or DMSO-only medium was washed out between 15 and 30 min after the final training session by removing and then replacing 6 mL of the medium with fresh E3 medium 10 times. To evaluate O-bend responsiveness, images were recorded for the initial 800 ms of each 1-s dark flash.

Acoustic startle responses were elicited and measured as described previously (2), with the following modifications. All startle stimuli were of 3-ms duration, with 1,000-Hz waveforms of variable intensity. Stimulus intensity was calculated by measuring the approximate displacement of the testing arena caused by vibration. Subthreshold, low-acoustic stimuli were designed to elicit a response of ~5–20% SLC responses, whereas above-threshold stimulation typically yielded >75% SLC responses without causing image distortion from excessive water disruption. To evaluate SLC behavior, images were recorded 30 ms before and 90 ms after the delivery of the 3-ms acoustic stimulus. All acoustic behavioral analyses were performed in the testing arena shown schematically in Fig. 2 B and C, so that larvae could be tracked and analyzed individually. The 4 × 4 testing grids were laser-cut from acrylic by Pololu Corporation, and then glued to a circular acrylic base plate (56-mm diameter, 1.5-mm thick; Pololu) with thin acrylic cement (Weld-On #3; IPS). The base plate was affixed to the inside of a 6-cm Petri dish lid with a viscous acrylic cement (Weld-On #16; IPS). The Petri dish lid was then attached to the metal ring with modeling clay. For image capture purposes, a 96-bulb infrared LED array (IR100 Illuminator removed from its housing; YYtrade) was positioned below the testing arena (not shown in Fig. 2). A 3-mm-thick sheet of white acrylic was positioned ~3 cm below the testing arena to diffuse the IR light. A white LED bulb (PAR38 LED light; LEDlight.com) was positioned above the testing arena to illuminate the testing arena with white light. Acoustic startle dishabituation was elicited by gently applying a handheld poker to an individual larval head while the larva was in the 4 × 4 testing grid.

To evaluate the spontaneous initiation of turning and swimming movements (1), larval behavior was recorded for 60 trials of 400-ms duration at 5-s intervals in the acoustic startle testing arena, without stimulation.

For all behavioral assays, high-speed video imaging was recorded using a Motionpro camera (Redlake) at 1,000 frames/s and a 512 × 512 pixel resolution, using a 50-mm macro lens. Behavioral analysis was carried out with the FLOTE software package (1–3).

Pharmacology. For acoustic startle and spontaneous movement pharmacologic experiments, larvae were preincubated in each compound for 15 min before and throughout the testing paradigm. MK-801 (M107; Sigma-Aldrich), N-methyl-D-aspartate (M3262; Sigma-Aldrich), and ketamine (K-2753; Sigma-Aldrich) were dissolved in 100% DMSO and administered in a final concentration of 1% DMSO. The small molecule libraries screened were the LOPAC-1280 (Invitrogen) and ICCB-BIO-MOL (Enzo Life Sciences) libraries. For first-pass analysis of the libraries, we tested eight larvae per compound (two compounds per 16-well grid) at a 1:100 dose from the stock concentration (usually 1 mM in 100% DMSO). To increase throughput, larvae were preincubated in 24-plate wells (eight larvae per well) while other larvae were being tested. Testing arenas were rinsed repeatedly with E3 between testing sessions. The sealed 4 × 4 grid walls ensured that compound mixing did not occur during testing. To wash out compounds and retest larvae, 50% of the medium in each well was removed with a Pipetman (Eppendorf) and replaced with fresh E3 a total of 10 times over the course of 10–20 min. SLC response curves for each compound screened were compared with the SLC curve for 48 larvae treated with 1% DMSO on the same experimental day to identify an increase or decrease in SLC habituation by each compound tested. Increased SLC habituation was evaluated during the prehabitation phase. If at least six of eight treated larvae performed an SLC to the first two prehabituating stimuli (stimuli 11 and 12) and then showed a robust response decrement to continued acoustic stimulation at a 20-s ISI (stimuli 13–20), then the compound was categorized as increasing SLC habituation.

To determine a reduction in SLC habituation, we calculated the ratio of the SLC responsiveness during the last 10 habituating stimuli (stimuli 41–50) to the responsiveness during the 10 prehabituating stimuli (stimuli 11–20) and compared this ratio to that in DMSO-treated larvae. Larvae not demonstrating an SLC response to at least 4 of the 10 prehabituating above-threshold acoustic stimuli (likely due to toxicity of the compound) were eliminated from the habituation analysis.

Fig. S1. CHX treatment does not alter intrasession, short-term habituation to visual stimuli. Six-dpf larval zebrafish were incubated in 10 μM CHX/1% DMSO or 1% DMSO only for 4 h and then exposed to a spaced training regime of dark-flash stimuli. Mean O-bend responsiveness shown for initial (1–10) and final (111–120) dark-flash stimuli during each session of a spaced, long-term habituation training paradigm. n = 3 dishes of 20 larvae for each experimental group. Error bars denote SEM. *P < 0.001 compared with initial (1–10) responses for similar treatment, same training session; Student t test.

Fig. S2. SLC startle kinematic properties are consistent throughout startle sensitivity and habituation assays. (A) Mean latency of the onset of the initial C-bend after delivery of an acoustic stimulus. (B) Mean duration of the initial C-bend. (C) Mean turning angle during the initial C-bend. (D) Mean maximum angular velocity of the initial C-bend. (E) Mean distance traveled over 90 ms after delivery of the acoustic stimulus, as a result of SLC behavior. n = 180 5 dpf larvae performing 3,590 SLC responses. Error bars denote SD.
Fig. S3. CHX treatment does not affect startle sensitivity, short-term habituation, or potentiation of short-term habituation. The 5-dpf larval zebrafish were incubated in 10 μM CHX/1% DMSO (red line) or 1% DMSO (black line) only for 4 h and then exposed to the startle sensitivity and habituation assay (A) or the short term habituation potentiation assay (B and B'). (A) Mean SLC response trend to startle sensitivity and habituation assay. (B and B') Mean SLC habituation trend during three sets of 20 acoustic stimuli delivered at a 1-s ISI, separated by 3-min rest periods. Similar increases in the rate of SLC habituation during sets 2 and 3 were observed in DMSO-treated and CHX-treated larvae. n = 32 larvae per group for all experiments. Error bars denote SEM.
Fig. S4. Small molecule compounds affecting SLC habituation do not cause spontaneous hyperactivity (A), enhance startle sensitivity (B), or alter SLC behavioral kinematic properties (D–G) in 5-dpf larvae after a 15-min incubation in each compound. (A) Mean initiation of spontaneous turning and swimming bouts. Spontaneous movements captured by video recording 40 trials of 400-ms duration every 5 s. (B) Mean SLC responsiveness to 10 low-level, subthreshold acoustic stimuli. (C) Mean SLC responsiveness of myristic acid analogs during the startle habituation assay. (D–F) Mean SLC kinematic properties. The following concentrations were used: 12-MDA, 50 μM; 2-hydroxymyristic acid (2-HMA), 20 μM; 4-oxatetradecanoic acid (4-OTDA), 20 μM; DMSO, 1%; MK-801, 100 μM; ketamine, 500 μM; SU-9516, 50 μM. Each concentration represents the optimal dose to modulate habituation or highest, subtoxic dose for compounds not influencing habituation (2-HMA and 4-OTDA). n = 32 larvae per group for all experiments. Error bars denote SEM.
**Fig. S5.** The increased SLC response sensitivity to 12-MDA is modulated by an agonist and antagonist of NMDA receptor function. Mean SLC responsiveness to 10 low-level, subthreshold acoustic stimuli after a 15-min incubation in combinations of varying concentrations of NMDA, ketamine, and/or 12-MDA is shown. n = 32 larvae per group. *P < 0.01; **P < 0.001, Student t test vs. DMSO group or indicated control group. #P < 0.05 vs. additive effect of 5 μM 12-MDA and 50 μM ketamine groups, Student t test. Error bars indicate SEM.

**Table S1. Compounds reducing short-term SLC habituation**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sensitivity</th>
<th>Spontaneous movement</th>
<th>Description/target/function</th>
</tr>
</thead>
<tbody>
<tr>
<td>MK-801</td>
<td>Increased</td>
<td>No effect</td>
<td>NMDA receptor antagonist</td>
</tr>
<tr>
<td>Ketamine</td>
<td>Increased</td>
<td>No effect</td>
<td>NMDA receptor antagonist</td>
</tr>
<tr>
<td>l-701,234</td>
<td>Increased</td>
<td>Increased</td>
<td>NMDA receptor antagonist</td>
</tr>
<tr>
<td>BAY K-8644</td>
<td>Increased</td>
<td>No effect</td>
<td>L-type calcium channel agonist</td>
</tr>
<tr>
<td>Hydrastine</td>
<td>No effect</td>
<td>Decreased</td>
<td>GABA-A receptor antagonist</td>
</tr>
<tr>
<td>N-phenylanthranilic acid</td>
<td>Increased</td>
<td>Increased</td>
<td>Chloride channel antagonist</td>
</tr>
<tr>
<td>Linopirdine</td>
<td>Increased</td>
<td>No effect</td>
<td>Potassium channel antagonist</td>
</tr>
<tr>
<td>Meclofenamic acid</td>
<td>Increased</td>
<td>No effect</td>
<td>Potassium channel antagonist</td>
</tr>
<tr>
<td>Butaclamol</td>
<td>No effect</td>
<td>Decreased</td>
<td>Dopamine receptor antagonist</td>
</tr>
<tr>
<td>Phenoxybenzamine</td>
<td>Increased</td>
<td>No effect</td>
<td>α-Adrenergic receptor antagonist; calmodulin antagonist</td>
</tr>
<tr>
<td>Etazolate</td>
<td>Increased</td>
<td>No effect</td>
<td>Phosphodiesterase 4 inhibitor</td>
</tr>
<tr>
<td>SU-9516</td>
<td>No effect</td>
<td>Decreased</td>
<td>Cdk1, Cdk2, and Cdk4 inhibitor</td>
</tr>
<tr>
<td>12-MDA</td>
<td>Increased</td>
<td>No effect</td>
<td>Myristic acid analog</td>
</tr>
</tbody>
</table>

Incubation of 5 dpf larvae in each of these drugs for 15 min before and during the acoustic startle sensitivity and habituation assay significantly reduced SLC habituation. Each compound also was evaluated for its influence on SLC sensitivity to low-level, subthreshold stimuli as well as the spontaneous initiation of turning and swimming bouts to evaluate baseline activity levels.

**Table S2. Compounds enhancing short-term SLC habituation**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Compound target/function</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis-4-Aminocrotonic acid</td>
<td>GABA-C receptor agonist</td>
</tr>
<tr>
<td>5-α-THDOC</td>
<td>Positive allosteric modulator of GABA-A receptors</td>
</tr>
<tr>
<td>Allopregnan-3α-ol-20-one</td>
<td>Positive allosteric modulator of GABA-A receptors</td>
</tr>
<tr>
<td>Citalopram hydrobromide</td>
<td>Selective serotonin reuptake inhibitor</td>
</tr>
<tr>
<td>Pirenperone</td>
<td>5-HT2 serotonin receptor antagonist</td>
</tr>
<tr>
<td>Ritanserin</td>
<td>5-HT2 serotonin receptor antagonist</td>
</tr>
<tr>
<td>Urapidil</td>
<td>Selective α1A adrenoceptor antagonist</td>
</tr>
<tr>
<td>BMY 7378 dihydrochloride</td>
<td>Partial 5-HT1A serotonin receptor agonist; α1D adrenoceptor antagonist</td>
</tr>
<tr>
<td>Prazosin hydrochloride</td>
<td>α1 adrenoceptor antagonist</td>
</tr>
<tr>
<td>Yohimbine hydrochloride</td>
<td>α2 adrenoceptor antagonist</td>
</tr>
<tr>
<td>Verapamil</td>
<td>Potent L-type calcium channel antagonist; adrenoceptor antagonist</td>
</tr>
<tr>
<td>Nimodipine</td>
<td>Potent L-type calcium channel antagonist</td>
</tr>
<tr>
<td>cis[α(+)-8-OH-PBZI hydrobromide</td>
<td>D3 dopamine receptor agonist</td>
</tr>
<tr>
<td>Trihexyphenidyl hydrochloride</td>
<td>Muscarinic acetylcholine receptor antagonist</td>
</tr>
<tr>
<td>Olprinone hydrochloride</td>
<td>Phosphodiesterase 3 inhibitor</td>
</tr>
<tr>
<td>Ammonium pyrrolidinedithiocarbamate</td>
<td>Nitric oxide synthase inhibitor</td>
</tr>
<tr>
<td>Chelerythrine chloride</td>
<td>Protein kinase C inhibitor</td>
</tr>
<tr>
<td>Indirubin-3′-oxime</td>
<td>Cdk inhibitor; GSK3B inhibitor</td>
</tr>
<tr>
<td>Kenpaullone</td>
<td>Cdk inhibitor; GSK3B inhibitor</td>
</tr>
</tbody>
</table>

Incubation of 5-dpf larvae in each of these drugs for 15 min before and during the acoustic startle sensitivity and habituation assay caused robust habituation under nonhabituating conditions (prehabituation phase).
Movie S1. SLC startle responses during startle sensitivity and habituation assay. The 5-dpf larvae were exposed to 60 repetitive acoustic stimuli at varying intensities and ISIs, described in the top panel. Images were captured at 1,000 frames/s, and the video was captured at 10 frames/s. The video shows movement of 16 larvae from 30 ms before to 30 ms immediately after exposure to the acoustic stimulus. The time bar represents relative time within this 60-ms period, and “bump” indicates delivery of a 2-ms long stimulus. Arrow on top indicates stimulus within assay.

Movie S2. SLC startle responses after incubation in DMSO or MK-801. The 5-dpf larvae were incubated in either 1% DMSO (four larvae on the left) or 100 mM MK-801/1% DMSO (four larvae on the right) for 15 min, and then exposed to an above-threshold, loud acoustic stimulus. Images were captured at 1,000 frames/sec, and the video was captured at 10 frames/s. The video shows movement of eight larvae from 30 ms before to 30 ms immediately after exposure to the acoustic stimulus. Time bars represent relative time within this 60-ms period, and “bump” indicates delivery of a 2-ms stimulus.