A Cyfip2-Dependent Excitatory Interneuron Pathway Establishes the Innate Startle Threshold

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Graphical Abstract

Highlights

- Genome-wide screen reveals novel set of mutants with heightened startle sensitivity
- cyfip2 is a novel regulator of the acoustic startle threshold
- Loss of cyfip2 specifically enhances spiral fiber neuron activity
- Inducing cyfip2 expression after phenotype onset restores normal startle threshold

Authors

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In Brief

Using forward genetics, electrophysiology, and combined behavior and Ca²⁺ imaging in zebrafish, Marsden et al. show that cyfip2 regulates the acoustic startle threshold by controlling the activity of excitatory spiral fiber interneurons.
A Cyfip2-Dependent Excitatory Interneuron Pathway Establishes the Innate Startle Threshold

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SUMMARY

Sensory experiences dynamically modify whether animals respond to a given stimulus, but it is unclear how innate behavioral thresholds are established. Here, we identify molecular and circuit-level mechanisms underlying the innate threshold of the zebrafish startle response. From a forward genetic screen, we isolated five mutant lines with reduced innate startle thresholds. Using whole-genome sequencing, we identify the causative mutation for one line to be in the fragile X mental retardation protein (FMRP)-interacting protein cyfip2. We show that cyfip2 acts independently of FMRP and that reactivation of cyfip2 restores the baseline threshold after phenotype onset. Finally, we show that cyfip2 regulates the innate startle threshold by reducing neural activity in a small group of excitatory hindbrain interneurons. Thus, we identify a selective set of genes critical to establishing an innate behavioral threshold and uncover a circuit-level role for cyfip2 in this process.

INTRODUCTION

A critical function of the nervous system is to detect and respond to threats. The vertebrate auditory system is particularly well adapted to this task. Within milliseconds of an intense and abrupt acoustic stimulus, animals initiate an evolutionarily conserved startle response. From a forward genetic screen, we isolated five mutant lines with reduced innate startle thresholds. Using whole-genome sequencing, we identify the causative mutation for one line to be in the fragile X mental retardation protein (FMRP)-interacting protein cyfip2. We show that cyfip2 acts independently of FMRP and that reactivation of cyfip2 restores the baseline threshold after phenotype onset. Finally, we show that cyfip2 regulates the innate startle threshold by reducing neural activity in a small group of excitatory hindbrain interneurons. Thus, we identify a selective set of genes critical to establishing an innate behavioral threshold and uncover a circuit-level role for cyfip2 in this process.
Forward Genetic Screen

Figure 1. The Startle Threshold Is Reduced in Mutants from the Forward Genetic Screen

(A) Distribution of startle response frequency to 10 low-intensity (13.5 dB) stimuli in 5 dpf wild-type TLF larvae (black bars, n = 110) and larvae from a cross of triggerhappy<sup>p400</sup> carriers (red bars, n = 104).

(B) Startle frequency for 10 trials at each of 6 intensities with sigmoidal fit curves. triggerhappy<sup>p400</sup> and TLF larvae were split into two groups: putative mutants (top 25%; p400, red line; TLF, black line) and putative siblings (bottom 75%; p400, pink line; TLF, gray line) based on their startle response frequency at 13.5 dB (mean ± SEM).

(C) Startle sensitivity indices. The area under the curves in (B) are displayed for the top 25% of WIK and TLF (black circles and squares, mean ± SD) and 7 mutant lines (red triangles; p400-406; ****p < 0.0001, one-way ANOVA with Dunnett’s multiple comparison test).

Type "$\text{TF}$" longfin (TLF) strain larvae initiated a startle response (Figure 1A). To identify recessive mutations, we scored putative mutant clutches as those in which 15%–25% of larvae startled with 40% or higher frequency to these subthreshold stimuli (e.g., mutant line p400; Figure 1A). Larvae with morphological, muscle, or otic vesicle defects were excluded from further behavioral analyses. In total, we screened ~614 genomes or ~1/6 of the genomes screened in one of the previous large-scale morphological screens (Haffter et al., 1996) and identified a set of 7 hypersensitive mutant lines with significantly reduced startle thresholds (Table 1). Mutants were confirmed by testing subsequent generations with an assay consisting of 60 total stimuli, 10 at each of 6 intensities, pseudo-randomized with a 20-s ISI. One line, p400, is shown in Figure 1B, with larvae divided into 2 groups, putative mutants (top 25%) and siblings (bottom 75%), based on their startle frequency at 13.5 dB. Wild-type TLF larvae were similarly divided, highlighting the disparity between putative p400 mutants and the most sensitive wild-type larvae (Figure 1B). After identifying the causative mutation in p400, we confirmed that the top 25% does correspond to the population of p400 homozygous mutants (see below; Figure 1C).

To quantify the severity of the hypersensitivity phenotype, we created a startle sensitivity index by plotting the startle frequency of each larva across the 60-stimulus assay and measuring the area under the resulting curves. We again defined putative mutant larvae as the top 25% of each clutch and compared these to the top 25% of larvae from the wild-type WIK and TLF strains used for the mutagenesis. As shown in Figure 1C, each mutant line exhibited significant hypersensitivity compared with TLF and WIK. Finally, complementation analysis revealed that these 7 mutants represent mutant alleles of 5 different genes (Table 1). Thus, through an unbiased genetic screen, we identified a selective collection of genes critical for establishing the innate startle threshold.

To determine the specificity of the startle threshold phenotype, we subjected the hypersensitivity mutants to a battery of additional behavioral tests (see Table 1 for detailed information). All mutants displayed startle kinematics within the normal range (Burgess and Granato, 2007), indicating normal motor function. All mutants also displayed a normal ability to acutely modulate their startle thresholds (Table 1). Mutants were confirmed by testing subsequent generations with an assay consisting of 60 total stimuli, 10 at each of 6 intensities, pseudo-randomized with a 20-s ISI. One line, p400, is shown in Figure 1B, with larvae divided into 2 groups, putative mutants (top 25%) and siblings (bottom 75%), based on their startle frequency at 13.5 dB. Wild-type TLF larvae were similarly divided, highlighting the disparity between putative p400 mutants and the most sensitive wild-type larvae (Figure 1B). After identifying the causative mutation in p400, we confirmed that the top 25% does correspond to the population of p400 homozygous mutants (see below; Figure 1C).

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Forward genetic screens in both invertebrate and vertebrate systems have identified genes affecting behavioral responses to chemical, thermal, or mechanical stimuli (e.g., Chalfie and Sulston, 1981; Granato et al., 1996; Kernan et al., 1994). These screens exclusively identified mutants with reduced sensitivity because of defects in sensory structures detecting the stimulus (e.g., Nicolson et al., 1998) rather than the central processing of...
### Table 1. Zebrafish Startle Sensitivity Mutants

<table>
<thead>
<tr>
<th>Mutant Allele</th>
<th>Viability</th>
<th>Startle Latency (ms)</th>
<th>Startle Turn Angle (°)</th>
<th>Baseline Activity</th>
<th>Startle Habituation (%)</th>
<th>PPI (%)</th>
<th>PPI Hearing Threshold</th>
<th>Gene Locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>triggerhappyp400</td>
<td>no (7–8 dpf)</td>
<td>6.8 ± 0.4, 83% of sibs, p &lt; 0.00001</td>
<td>96 ± 6.2, 77% of sibs, p &lt; 0.00001</td>
<td>63% ± 4.3% of sibs, p = 0.0002</td>
<td>90 ± 6.3, 90% of sibs, p = 0.15</td>
<td>29 ± 9.3, 56% of sibs, p = 0.027</td>
<td>n/a</td>
<td>chr14 cyfip2</td>
</tr>
<tr>
<td>whisper2000p401</td>
<td>yes</td>
<td>6.2 ± 0.1, 70% of sibs, p &lt; 0.00001</td>
<td>114 ± 4.4, 102% of sibs, p = 0.69</td>
<td>109% ± 6.3% of sibs, p = 0.0012</td>
<td>90 ± 3.9, 111% of sibs, p = 0.24</td>
<td>29 ± 6.0, 39% of sibs, p = 0.00028</td>
<td>n/a</td>
<td>chr7</td>
</tr>
<tr>
<td>detectorp402</td>
<td>yes (weakly dominant)</td>
<td>6.8 ± 0.3, 86% of sibs, p = 0.43</td>
<td>116 ± 6.2, 99% of sibs, p = 0.99</td>
<td>105% ± 6.8% of sibs, p = 0.74</td>
<td>100 ± 0, 100% of sibs, p = 0.46</td>
<td>53 ± 10.4, 91% of sibs, p = 0.33</td>
<td>4.6 dB in muts and sibs</td>
<td>ND</td>
</tr>
<tr>
<td>highstrungp403</td>
<td>no (10–14 dpf)</td>
<td>6.9 ± 0.2, 90% of sibs, p = 0.28</td>
<td>104 ± 3.4, 90% of sibs, p = 0.014</td>
<td>56% ± 5.4% of sibs, p &lt; 0.0001</td>
<td>79 ± 4.5, 89% of sibs, p = 0.14</td>
<td>27 ± 5.1, 41% of sibs, p = 0.017</td>
<td>n/a</td>
<td>chr10</td>
</tr>
<tr>
<td>escapistp404</td>
<td>yes</td>
<td>6.8 ± 0.4, 83% of sibs, p = 0.067</td>
<td>107 ± 3.6, 94% of sibs, p = 0.18</td>
<td>98% ± 11.7% of sibs, p = 0.88</td>
<td>100 ± 0, 102% of sibs, p = 0.31</td>
<td>46 ± 5.5, 78% of sibs, p = 0.35</td>
<td>4.6 dB in muts and sibs</td>
<td>chr25</td>
</tr>
</tbody>
</table>

Summary of behavioral analyses. Viability was determined by raising and crossing phenotypically identified mutants (muts). Three of five mutants survive to adulthood and produce viable offspring. triggerhappyp400 mutants die at 7–8 dpf, likely because their swim bladders do not inflate, preventing them from feeding. It is unclear why highstrungp403 mutants die at 10–14 dpf. detectorp402 is weakly dominant because crosses of identified carriers to wild-type fish produced hypersensitive larvae. Startle kinematics (latency and turn angle) were measured using FLOTE software, and despite some statistically significant differences, all values are within normal parameters (Burgess and Granato, 2007). Total distance traveled over 160 s was normalized to the sibling (sib) average to determine baseline activity. Startle habituation and PPI were calculated as in Wolman et al. (2011) and Burgess and Granato (2007), respectively. PPI hearing threshold was analyzed for mutants without PPI defects by reducing the intensity of the pre-pulse (Bhandiwad et al., 2013). The lowest intensity pre-pulse eliciting significant PPI (p < 0.05) is reported and was unchanged in both mutants tested. Gene loci were determined using whole-genome (triggerhappyp400) or RNA sequencing (whisper2000p401, highstrungp403, and escapistp404-406) to identify highly homozygous genomic regions (Figure S2). See Experimental Procedures for details. All values listed are mean ± SEM with their relation to siblings’ performance (% of sibs). n/a, not applicable; ND, not determined.

*Statistically significant difference (p < 0.05, Mann-Whitney test).
the stimulus. In contrast, we designed our screen to selectively isolate mutants with increased rather than decreased responsiveness. Increased stimulus sensitivity not only reflects an important aspect of several mental health disorders but also provides an opportunity to investigate the molecular mechanisms that regulate the filtering of sensory input into behavioral output. To assay mechanono-acoustic acuity in the hypersensitive mutants, we examined hair cell morphology and function. In zebrafish, hair cells located in lateral line neuromasts and in the otic vesicle (OV) detect water motion induced by acoustic stimuli and connect to the startle circuit’s command-like neurons, the M-cells, via afferent nerves, the anterior lateral line (ALL) and posterior lateral line (PLL), and the auditory (VIII) nerves, respectively (Figure 2A). In all mutants, neuromast numbers and OV hair cell morphology were unaffected (Figures S1B–S1D). In agreement with previous data (Kohashi and Oda, 2014; Pittman et al., 2010), neomycin treatment of wild-type larvae caused a significant decrease in startle sensitivity (Figure S1A), providing evidence that input from OV is necessary for hypersensitivity. In all mutants, we examined hair cell morphology and function. In contrast, we designed our screen to selectively isolate mutants with increased rather than decreased responsiveness. Increased stimulus sensitivity not only reflects an important aspect of several mental health disorders but also provides an opportunity to investigate the molecular mechanisms that regulate the filtering of sensory input into behavioral output. To assay mechanono-acoustic acuity in the hypersensitive mutants, we examined hair cell morphology and function. In zebrafish, hair cells located in lateral line neuromasts and in the otic vesicle (OV) detect water motion induced by acoustic stimuli and connect to the startle circuit’s command-like neurons, the M-cells, via afferent nerves, the anterior lateral line (ALL) and posterior lateral line (PLL), and the auditory (VIII) nerves, respectively (Figure 2A). In all mutants, neuromast numbers and OV hair cell morphology were unaffected (Figures S1B–S1D). In agreement with previous data (Kohashi and Oda, 2014; Pittman et al., 2010), neomycin treatment of wild-type larvae caused a significant decrease in startle sensitivity (Figure S1A), providing evidence that input from OV is necessary for hypersensitivity. In all mutants, hair cell morphology and function were unaffected. The previously identified fmr1hu2787/hu2787 mutants caused hypersensitivity (***p < 0.001, Mann-Whitney test), whereas heat shock reduced the sensitivity of fmr1hu2787/hu2787 mutants by 30% (Figure S3C). Without a heat shock, cyfi2p400/p400 mutants had increased startle sensitivity (***p < 0.001, Mann-Whitney test), whereas heat shock reduced the sensitivity of cyfi2 mutants and siblings with the transgene compared with those without it (**p < 0.01, Mann-Whitney test). (F) Hindbrain expression of Cyfip2 in 5 dpf wild-type cyfi2p400/p400 and mutant larvae using a Cyfip2 antibody (Ab). Membranes of VIII neurons are marked by Tg(SCP1:Gal4FF(y256Et)); Tg(UAS:gap43-citrine) and anti-GFP Ab. Dashed lines indicate the otic vesicles (OVs). Scale bar, 10 μm.
We then focused on the triggerhappyp400 mutants, which represent five genes located on different chromosomes. Using either a previously validated DNA promoter and crossed the Tg(hsp70:cyfip2-GFP) line into the triggerhappyp400 mutant background. Activation of the transgene prior to the onset of the phenotype at 30 hpf restored normal startle sensitivity in genotypically mutant triggerhappyp400 larvae (*p = 0.0036; Figure 2C). Combined, our data provide compelling evidence that the triggerhappyp400 startle hypersensitivity phenotype is caused by mutations in cyfip2.

The Cytoskeletal Regulator cyfip2 Establishes the Innate Startle Threshold Independently of FMRP

Cyfip2 was first identified on the basis of its interaction with FMRP (Schenck et al., 2001) and is thought to interact with FMRP to modulate RNA metabolism (Schenck et al., 2001, 2003). Cyfip2 also functions as a component of the Wiscott-Aldrich syndrome protein/WASP-family verprolin-homologous protein (WAVE) complex that regulates actin nucleation through binding to the GTPase Rac1 (Chen et al., 2010; Eden et al., 2002; Schenck et al., 2004). In several animal models, Cyfip2 has been shown to regulate axon guidance (Pittman et al., 2010; Schenck et al., 2003) as well as synapse formation and function (Schenck et al., 2003). To test whether cyfip2 acts through fmr1 (the gene that encodes FMRP) to establish the startle threshold, we tested startle sensitivity in previously identified fmr1 mutants (den Broeder et al., 2009). We detected no difference in startle frequency across all stimulus intensities (Figure 2E), indicating that cyfip2 acts independently of fmr1 to establish the innate startle threshold.

cyfip2p400 Mutant M-Cells Have Normal Inhibitory and Excitatory Synaptic Connections

To examine Cyfip2 expression in the nervous system, we used a commercially available antibody (Abcam, ab95969) to label triggerhappyp400 (hereafter referred to as cyfip2p400) mutants and siblings at 72 hpf. Siblings showed broad Cyfip2 expression in the neuropil of the olfactory bulb, inner plexiform layer of the retina, tectum, and hindbrain lateral to the M-cell near the VIII ganglion (Figures S3A and S3B). Neuronal staining was absent in mutants, confirming the specificity of the antibody. Using a transgenic line that labels VIII neurons, Tg(SCP1:Gal4FF(y256Et); Tg(UAS:gap43-citrine) (Marquart et al., 2015), we found that, at 5 dpf, when the startle phenotype is observed, Cyfip2 is expressed in and around these neurons in the hindbrain at low levels above the background level observed in mutants (Figure 2F), placing Cyfip2 in a prime location to influence the startle circuit.

We next examined the structural and functional integrity of the startle command-like neurons, the M-cells. We first used the

Sequencing of cyfip2 cDNA from phenotypically identified mutants confirmed a single base pair substitution (nt1024: T to A), causing a premature stop codon in exon 11 at amino acid 343 of 1,253 (Figure 2B). To confirm that triggerhappyp400 startle hypersensitivity is caused by mutations in cyfip2, we performed a genetic complementation assay using a cyfip2 mutant allele previously isolated by a retinotectal axon guidance defect (Pittman et al., 2010; Trowe et al., 1996). Trans-heterozygous larvae displayed increased startle sensitivity compared with siblings (Figure 2C), confirming that mutations in cyfip2 cause startle hypersensitivity. Finally, we created a transgenic line expressing GFP-tagged Cyfip2 under the control of an inducible heat shock promoter and crossed the Tg(hsp70:cyfip2-GFP) line into the triggerhappyp400 mutant background. Activation of the transgene prior to the onset of the phenotype at 30 hpf restored normal startle sensitivity in genotypically mutant triggerhappyp400 larvae (*p = 0.0036; Figure 2C). Combined, our data provide compelling evidence that the triggerhappyp400 startle hypersensitivity phenotype is caused by mutations in cyfip2.

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We next sought to determine the molecular identities of the startle threshold mutants. Using either a previously validated DNA whole-genome sequence (WGS) analysis pipeline (Wolman et al., 2015) or using RNA sequencing (RNA-seq) analysis (Hill et al., 2013), we assigned four of the five mutants to a small genomic interval (Table 1; Figure S2; see Experimental Procedures for details regarding WGS and RNA-seq). This confirmed our complementation analysis showing that the startle threshold mutants represent five genes located on different chromosomes. We then focused on the triggerhappyp400 mutant, in part because our phenotypic analysis suggested that hypersensitivity in this mutant is likely due to improper processing of sensory information downstream of the auditory organs. We mapped triggerhappyp400 to chromosome 14 (Figure S3A), where we identified a single nonsense mutation in the gene cytoplasmic Fragile X mental retardation protein (FMRP)-interacting protein 2 (cyfip2).

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The rheobase, $V_{\text{resting}, R}$, in and $V_{\text{threshold}}$ of M-cells in $R_{\text{in}}$ (Cx35 antibody (Figures S4A and S4B), whole-cell M-cell from VIII afferents. Analysis of club endings using a cyfip2p400 measure to reveal any significant differences between trical stimulation of VIII nerve afferents, and paired-pulse ratio electrophysiology to measure M-cell synaptic responses to electrical stimulation of VIII nerve afferents, and paired-pulse ratio measurements to monitor transmitter release properties failed to reveal any significant differences between wild-type siblings (Figure S6D), consistent with the notion that loss of cyfip2 leads to a lower threshold of the M-cell-dependent startle response. Excitatory VIII nerve afferent inputs form mixed chemical and electrical synapses known as club endings on the M-cell lateral dendrite (Yao et al., 2014; Zottoli and Faber, 1979). Thus, one plausible cause for the hypersensitivity observed in cyfip2p400 mutants might be increased excitatory input to the M-cell from VIII afferents. Analysis of club endings using a connexin 35 (Cx35) antibody (Figures S4A and S4B), whole-cell electrophysiology to measure M-cell synaptic responses to electrical stimulation of VIII nerve afferents, and paired-pulse ratio measurements to monitor transmitter release properties failed to reveal any significant differences between cyfip2p400 mutants and wild-type siblings (Figures S3A–S3E).

We observed that, in cyfip2p400 mutants, the volume of the M-cell body was unaffected, whereas the lateral and ventral dendrites were significantly smaller (**$p \leq 0.0001$, Mann-Whitney test; Figures S4C–S4F), which might cause M-cell hyperexcitability. We therefore measured the M-cell’s rheobase (the current necessary to trigger a spike), resting potential ($V_{\text{resting}}$), input resistance ($R_{\text{in}}$), and action potential threshold ($V_{\text{threshold}}$). The only statistically significant difference we observed was a slight increase in the $V_{\text{resting}}$ from $-81.9 \pm 1.9$ mV in siblings to $-79.0 \pm 2.6$ mV in mutants, a change too small to explain the startle phenotype (Table 2). These findings are consistent with our analysis of sodium channel staining in the M-cell axon cap, in which we found no difference between cyfip2p400 mutants and siblings (Figures S5A and S5B). Thus, we conclude that cyfip2p400 mutants do not exhibit a functional difference in auditory drive onto the M-cell and that loss of cyfip2 does not detectably alter M-cell excitability.

An alternative cause for the hypersensitivity observed in cyfip2p400 mutants might be decreased inhibitory input to the M-cell. Larval zebrafish M-cells receive γ-aminobutyric acid (GABA)ergic (Roy and Ali, 2014) and glycineric inhibitory input from several sources (Koyama et al., 2011; Takahashi et al., 2002), including feedforward neurons (Figure 2A) that have been shown to influence the startle threshold in adult goldfish (Weiss et al., 2008) and African cichlid fish (Neumeister et al., 2010). We therefore measured M-cell glycine receptor (GlyR) expression to assess all glycineric inhibitory inputs and found no difference in the total intensity of GlyR labeling between cyfip2p400 mutants and wild-type siblings (Figures S5C and SSD). Thus, our data strongly suggest that excitatory and inhibitory connectivity onto the M-cell soma is largely unaffected in cyfip2p400 mutants, indicating that cyfip2 likely acts on a different population of startle circuit neurons to establish the innate startle threshold.
observed when the fish did not startle (Marsden and Granato, 2015), specifically ΔF/F₀ > 0.16. The firing probability for each cell was calculated by dividing the number of trials in which the cell fired by the total of 3 trials. By these criteria, SF neurons in cyfip2p400 mutants were more likely to fire following low- and medium-intensity but not high-intensity stimuli (Figures 5A and 5B). Again, these data precisely correlate with the observed behavioral change (Figure S6A). Furthermore, following low-intensity stimuli, of the 6 SF neurons recorded, 3.39 ± 0.36 fired in siblings (****p < 0.0001, Mann-Whitney test). Similarly, after medium-intensity stimuli, 4.4 ± 0.40 SF neurons fired in mutants, whereas just 1.7 ± 0.36 fired in siblings (**p < 0.0001, Mann-Whitney test). These data strongly support a model in which cyfip2 functions primarily to dampen the activity of SF neurons at low stimulus intensities.

Finally, we wondered whether these circuit defects in cyfip2p400 mutants are “hard wired” or reversible. Specifically, we tested whether heat shock-induced expression of Cyfip2-GFP in cyfip2p400 mutants after onset of the hypersensitivity phenotype can restore the wild-type innate startle threshold. Indeed, cyfip2 expression after phenotype onset was sufficient to revert mutants’ hypersensitivity (Figure 5C), revealing a surprising degree of plasticity within the VIII-SF-M-cell circuit mechanisms that establish and maintain the innate startle threshold.

The Role of cyfip2 in Regulating the Innate Startle Threshold

We were initially surprised to identify a cytoplasmic, cytoskeletal regulator rather than a membrane protein such as an ion channel in our screen for genes regulating the innate startle threshold. However, a large body of evidence exists that cyfip2 and cyfip1 are critical regulators of many neural functions (Abekhoucha and Bardoni, 2014). Through their interaction with FRMP, Cyfip1/2 may modulate the translation of cytoskeletal-associated proteins (MAP1B, PP2Ac) (Brown et al., 2001; Castets et al., 2005) or other target RNAs important for synaptic plasticity, such as Arc (De Rubeis et al., 2013; Napoli et al., 2008). Our data indicate that Cyfip2 acts independently of FMRP to establish the innate startle threshold (Figure 2E), suggesting that Cyfip2’s role in the actin-regulating WAVE regulatory complex (WRC) may, instead, underlie this function. Cyfip1 and Cyfip2 both directly interact with Rac1-guanosine triphosphate (GTP), and this binding activates WRC, allowing it to bind Arp2/3 to initiate actin polymerization (Chen et al., 2010; Cory and Ridley, 2002; Derivery et al., 2009). In mice, homozygous mutations in cyfip1 and 2 are lethal (Bozdagi et al., 2012; Han et al., 2014), and, in contrast to our results, cyfip2 heterozygous mice showed decreased startle responsiveness and increased PPI (Han et al., 2014). This discrepancy could be due to differences in gene dosage, although we did not observe any phenotypes in heterozygous cyfip2 larvae. More likely it is due to species differences. The significance of PPI and startle hypersensitivity phenotypes for human disease and the unique opportunity afforded by semiviable homozygous zebrafish mutants, however, makes the zebrafish cyfip2 mutant an important model to better understand the cellular and molecular regulation of these behaviors.

Our circuit analysis reveals that cyfip2 function is dispensable for normal M-cell excitability and, rather, points to a role for cyfip2 in dampening SF neuron excitability or reducing excitatory synaptic input from upstream neurons (Figures 5D and S6E). cyfip2 may act on SF neurons through changes in dendrite morphology (Figure S4) but more likely acts on VIII terminals onto SF neurons or on currently unidentified intermediary neurons to regulate synaptic vesicle trafficking and/or release via the actin cytoskeleton (Hsiao et al., 2016; Schenck et al., 2003). In either scenario, reducing cyfip2 function would cause a weak acoustic stimulus to elicit firing of a larger set of SF neurons, leading to increased transmission onto the M-cell AIS, thereby driving the M-cell to fire and initiate the startle response.

Figure 4. SF Axon Terminal Activity Is Increased in cyfip2 Mutants

(A) Maximum intensity projection of Tg(-6.7FRRhcrTGal4VP16); Tg(UAS:GCaMP5), showing labeled SF neurons (green), M-cells (M) and other reticulospinal neurons were labeled with rhodamine dextran (magenta). Arrowheads indicate SF cell bodies, and asterisks mark SF axon terminals in the M-cell axon cap. Scale bar, 10 μm. (B) Representative pseudocolored images of baseline (F₀) and peak fluorescence in SF axon terminals following a strong acoustic stimulus (13 dB; the color scale denotes fluorescence intensity; black, lowest; white, highest). Scale bar, 10 μm. (C) Averaged traces of SF terminal Ca²⁺ responses following low (−14 dB), medium (−12 dB), and strong (13 dB) acoustic stimuli (n = 42 responses from 10 siblings, blue line; n = 36 responses from 9 mutants, red line; mean ± SEM; *p < 0.05, Mann-Whitney test). (D) Scatterplot of the area under the curve for individual SF axon terminal Ca²⁺ responses (mean ± SD; **p = 0.0049, ***p = 0.0003, Mann-Whitney test).
Independent of the precise mechanism by which cyfip2 regulates the innate startle threshold, given that the human cyfip1 gene is located on 15q11.2, a hotspot for risk factors associated with neuropsychiatric disorders, including schizophrenia, epilepsy, intellectual disability, developmental delay, and autism (reviewed in Cox and Butler, 2015), understanding how Cyfip proteins influence the formation and function of neural circuits underlying whole-animal behavior remains an important question.

**EXPERIMENTAL PROCEDURES**

**Zebrafish Husbandry, Mutagenesis, and Maintenance**

All animal protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC). ENU mutagenesis was performed using TLF and WIK strains as described previously (Wolman et al., 2015). See the Supplemental Experimental Procedures for details.

**Behavioral Assays and Analysis**

Behavioral experiments were performed using 4–6 dpf larvae and analyzed using FLOTE software as described previously (Burgess and Granato, 2007; Wolman et al., 2011). See the Supplemental Experimental Procedures for details.

**WGS, RNA-Seq, and Molecular Cloning of cyfip2**

Pools of 50 behaviorally identified triggerhapyp400 mutant larvae were made, and genomic DNA (gDNA) libraries were created. gDNA was sequenced with 100-bp paired-end reads on the Illumina HiSeq 2000 platform, and homozygosity analysis was done using 463,379 SNP markers identified by sequencing gDNA from ENU-mutagenized TLF and WIK males as described previously (Wolman et al., 2015). Mapping of whisper2000p401, highstrungp403, and escapistp404-6 was performed using RNA-seq. See the Supplemental Experimental Procedures for details.

**Immunohistochemistry, Spinal Backfills, 2-(4-(dimethylamino)styryl)-N-Ethylpyridinium Iodide Staining, and Image Analysis**

Larvae were fixed in either 2% trichloroacetic acid (TCA) for 3 hr or 4% paraformaldehyde (PFA) for 1 hr at room temperature. After washes in PBS + 0.25% Triton X-100, fixed larvae were stained under standard blocking and antibody conditions, dissected, and mounted in Vectashield (Vector Labs). See the Supplemental Experimental Procedures for details.

**Figure 5. SF Neurons Are Hypersensitive by Weak Acoustic Stimuli in cyfip2 Mutants and Reversal of the Hypersensitivity Phenotype**

(A) Representative SF neuron Ca$^{2+}$ responses in cyfip2 siblings and mutants 1 s before (F0) and 150 ms after (peak) medium-intensity (~12 dB) acoustic stimulation. Dashed circles indicate SF cell bodies, arrowheads mark cells that fired according to our criteria (see text). Scale bar, 10 μm.

(B) Distribution of SF neuron firing probability (n = 48 cells from 8 siblings, 36 cells from 6 mutants; ****p < 0.0001, Mann-Whitney test).

(C) Startle sensitivity index of cyfip2p400 siblings and mutants expressing Tg(hsp70:cyfip2-GFP) 4 dpf before 8 heat shock cycles at 37°C separated by 120 min (d4 pre; **p = 0.025, unpaired t test). The same larvae were tested for startle sensitivity after heat shock at 6 dpf (d6 post; **p = 0.0018, paired t test), cyfip2p400 mutants without the hsp70:cyfip2-GFP transgene remained hypersensitive (p = 0.46, paired t test).

(D) Model of Cyfip2’s role in the startle circuit. In cyfip2 mutants, activity is enhanced in the VIII-SF-M-cell pathway, either through a direct VIII-SF connection or through an indirect connection via an unknown cell population (question mark), leading to enhanced M-cell firing and startle behavior. In wild-type fish, Cyfip2 potentially acts at pre- and/or postsynaptic sites, indicated by asterisks, to dampen neural activity.
Combined Ca²⁺ and Behavior Imaging and Analysis

Combined Ca²⁺ and startle behavior experiments were performed as described previously (Marsden and Granato, 2015). See the Supplemental Experimental Procedures for details.

Heat Shock-Induced cyfip2-GFP Rescue

To induce expression of cyfip2-GFP in the Tg(hsp70:cyfip2-GFP) line, 30 hpf larvae were placed in individual wells of a 96-well plate and incubated at 37°C for 40 min in a thermocycler. After heat shock, larvae were returned to Petri dishes, with 4 days of recovery at 29°C. For pre/post heat shock experiments, 4 dpf larvae were tested for startle sensitivity, transferred to 96-well plates, and given 8 heat shock cycles: 37°C for 40 min, 120 min at 28°C. After heat shock, larvae were transferred to individual wells of 24-well plates and kept at 29°C until 6 dpf for post heat shock startle sensitivity testing.

Electrophysiology

Electrophysiological recordings were performed in 5–6 dpf cyfip2+/+ siblings and mutants carrying the Toi056-GFP transgene that labels M-cells (Satou et al., 2009) as described previously (Yao et al., 2014). See the Supplemental Experimental Procedures for details.

Statistics

Statistical analyses, including calculation of means, SD, and SE, were done with Prism (GraphPad). Datasets were tested for normality using the D’Agostino-Pearson omnibus normality test with subsequent t tests, non-parametric (Mann-Whitney) tests, or ANOVA tests for significance, used as indicated in the figure legends.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.03.095.

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AUTHOR CONTRIBUTIONS

K.C.M., R.A.J., M.A.W., A.E.P., and M.G. designed the research. K.C.M., R.A.J., M.A.W., F.A.E., J.C.N., K.E.H., and B.M. performed the research and analyzed the data. K.C.M. and M.G. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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