Neuron

Estrogens Suppress a Behavioral Phenotype in Zebrafish Mutants of the Autism Risk Gene, **CNTNAP2**

Highlights

- Zebrafish mutants of the autism risk gene cntnap2 have GABAergic neuron deficits
- High-throughput behavioral profiling identifies nighttime hyperactivity in mutants
- cntnap2 mutants exhibit altered responses to GABAergic and glutamatergic compounds
- Estrogenic compounds suppress the *cntnap2* mutant behavioral phenotype

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In Brief

Hoffman et al. use zebrafish mutants of the autism risk gene Contactin Associated Protein-like 2 (CNTNAP2) to conduct high-throughput quantitative behavioral profiling and pharmacological screening. This approach reveals dysregulation of GABAergic and glutamatergic systems in mutants and identifies estrogenic compounds as suppressors of the mutant behavioral phenotype.

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Estrogens Suppress a Behavioral Phenotype in Zebrafish Mutants of the Autism Risk Gene, *CNTNAP2*

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SUMMARY

Autism spectrum disorders (ASDs) are a group of devastating neurodevelopmental syndromes that affect up to 1 in 68 children. Despite advances in the identification of ASD risk genes, the mechanisms underlying ASDs remain unknown. Homozygous loss-of-function mutations in Contactin Associated Protein-like 2 (CNTNAP2) are strongly linked to ASDs. Here we investigate the function of Cntnap2 and undertake pharmacological screens to identify phenotypic suppressors. We find that zebrafish cntnap2 mutants display GABAergic deficits, particularly in the forebrain, and sensitivity to drug-induced seizures. High-throughput behavioral profiling identifies nighttime hyperactivity in cntnap2 mutants, while pharmacological testing reveals dysregulation of GABAergic and glutamatergic systems. Finally, we find that estrogen receptor agonists elicit a behavioral fingerprint anti-correlative to that of cntnap2 mutants and show that the phytoestrogen biochanin A specifically reverses the mutant behavioral phenotype. These results identify estrogenic compounds as phenotypic suppressors and illuminate novel pharmacological pathways with relevance to autism.

INTRODUCTION

Autism spectrum disorders (ASDs) are a group of neurodevelopmental syndromes characterized by deficits in social interaction and communication as well as repetitive behaviors and restricted interests (American Psychiatric Association, 2013). Gene discovery in ASDs has accelerated dramatically (De Rubeis et al., 2014; lossifov et al., 2014; Sanders et al., 2015), providing a launching point for the illumination of relevant biological pathways (Parikshak et al., 2013; Willsey et al., 2013). Contactin Associated Protein-like 2 (CNTNAP2) is one of the first genes strongly linked to autism and epilepsy in consanguineous families (Strauss et al., 2006). This gene encodes a cell adhesion molecule of the neurexin family that localizes voltage-gated potassium channels at the juxtaparanodal region of myelinated axons (Poliak et al., 2003). Loss of CNTNAP2 function in mice leads to abnormal neuronal migration, reduced GABAergic neurons, spontaneous seizures, hyperactivity, social deficits, and increased repetitive behaviors (Peñagarikano et al., 2011). However, the function of CNTNAP2 in the CNS and the consequences of its loss for ASD pathology are less well understood.

At present, our ability to advance rapidly from the identification of risk genes to the discovery of biological mechanisms and pharmacological suppressors remains limited. The zebrafish is a model vertebrate system well suited for conducting small molecule screens to uncover modulators of signaling pathways (Ablain and Zon, 2013; Kokel and Peterson, 2011; Rihel et al., 2010). Quantitative behavioral profiling provides a highthroughput approach to characterize psychoactive molecules





Figure 1. cntnap2ab Mutants Display GABAergic Deficits

(A) Cntnap2a and Cntnap2b proteins showing functional domains. DISC, discoidin; EGF, epidermal growth factor; 4.1, 4.1 binding domain; LamG, laminin G; TM, transmembrane.

(B) Western blot analysis of Cntnap2a and Cntnap2b in $cntnap2a^{\Delta 25/\Delta 25}cntnap2b^{\Delta 7/\Delta 7}$ and $cntnap2a^{\Delta 121/\Delta 121}cntnap2b^{31i/31i}$ fish.

(C–F and C'–F') Reporter gene expression in Tg(dlx6a-1.4kbdlx5a/dlx6a:GFP) and Tg(vglut:DsRed) in wild-type (C–F) and $cntnap2a^{\Delta 121/\Delta 121}cntnap2b^{31/31i}$ (cntnap2ab) (C'–F') larvae at 4 dpf. Note the deficit in dlx5a/6a:GFP+ cells in the telencephalon (arrowheads). (C and D, C' and D') Lateral views. (E and F, E' and F') Ventral views. hyp, hypothalamus; OT, optic tectum; tel, telencephalon.

(G) Total number of dix5a/6a:GFP+ cells in the forebrain of wild-type (n = 13) and cntnap2ab mutants (n = 14). *p = 3.08 × 10⁻⁷ (one-way ANOVA).

(H) Average activity in response to PTZ (10 mM) in the progeny of incrossed $cntnap2a^{\Delta 25/+}cntnap2b^{\Delta 7/+}$ fish at 4 dpf (n = 268; cntnap2ab + PTZ, n = 6; wild-type + PTZ, n = 11; cntnap2ab + water, n = 11; wild-type + water, n = 6; p = 0.0013, two-way ANOVA, genotype × dose interaction).

on the basis of the behavioral readout of their effects in zebrafish larvae (Rihel et al., 2010). Here we investigate the consequences of the loss of Cntnap2 in zebrafish and use quantitative behavioral profiling as a platform to conduct rational pharmacological screens to identify phenotypic suppressors and novel pathways with relevance to autism.

RESULTS

cntnap2 Mutants Display GABAergic Deficits

Zebrafish have two *cntnap2* paralogs that are expressed broadly in the developing CNS, with higher expression in the telencephalon (Figures S1A–S1C). Cntnap2a and Cntnap2b proteins show 71% and 65% identity to the human protein, respectively, and contain the same functional domains (Figure 1A). To characterize their function, we generated two loss-of-function mutations in each paralog using zinc finger nucleases and crossed fish carrying these alleles to generate double-mutant fish (*cntna* $p2a^{\Delta 121/\Delta 121}cntnap2b^{31i/31i}$ and *cntnap2a^{\Delta 25/\Delta 25}cntnap2b^{\Delta 7/\Delta 7}*) (Figures S1D–S1F), referred to hereafter as *cntnap2ab* mutants. Each mutation causes a premature stop codon within the discoidin domain and results in loss of protein expression (Figures 1A and 1B), such that double mutants represent a loss of Cntnap2 function.

It has been proposed that an imbalance in excitatory and inhibitory signaling in the CNS is a mechanism underlying ASD and epilepsy (Rubenstein and Merzenich, 2003). To test if this is occurring in *cntnap2ab* mutants, we analyzed inhibitory and excitatory neuronal populations in wild-type and *cntnap2ab* mutants during early brain development, using transgenic lines that label GABAergic neurons and precursors (Tg[*dlx6a-1.4kbdlx5a/ dlx6a:GFP*]) and glutamatergic neurons (Tg[*vglut:DsRed*]) (Ghanem et al., 2003; Kinkhabwala et al., 2011; Zerucha et al., 2000). We observed a significant decrease in GABAergic cells in *cntnap2ab* mutants at 4 days post fertilization (dpf) (Figures 1C–1F, 1C'–1F', S2A–S2H, and S2A'–S2H') in the forebrain and cerebellum (Figures S2I and S2J). In particular, there was an average of 34% fewer GABAergic neurons in the forebrain of *cntnap2ab* mutants compared with wild-type fish at 4 dpf (Figure 1G; $p = 3.08 \times 10^{-7}$, one-way ANOVA). The decreased number of GABAergic cells in the dorsal telencephalon (pallium) at this stage is consistent with a failure in the migration of these cells from the ventral telencephalon (subpallium), similar to findings reported in *Cntnap2* mutant mice (Peñagarikano et al., 2011). In contrast, there were no significant regional deficits in glutamatergic neurons (Figures S2A–S2H, S2A'–S2H', and S2K).

Although there were no gross morphological abnormalities in the structure of the axon scaffold in *cntnap2ab* mutants between 48 hr post fertilization (hpf) and 5 dpf (Figures S1H, S2I, S1H', and S2I'), mutant head size is significantly smaller (Table S1; Figures S1N–S1R and S1N'–S1Q'). In addition, we did not observe gross differences in markers of apoptosis (TUNEL staining) at 28 hpf or proliferation (phospho-histone H3) at 48 hpf (Figures S2R, S2S, S2R', and S2S'). However, deficits in GABAergic neurons and transient delays in commissure formation are evident in the forebrain of mutants at 28 hpf (Figures S1J–S1M, S1J', and S1K'), indicating additional early roles for Cntnap2 in brain development. Together, these data indicate loss of Cntnap2 results in a deficit of inhibitory neurons, particularly in the forebrain.

Increased Seizure Susceptibility in cntnap2 Mutants

Loss of inhibitory neurons can increase susceptibility to seizures (Cobos et al., 2005). To determine the effect of loss of Cntnap2 on seizure susceptibility in zebrafish, we treated wild-type and mutant larvae with pentylenetetrazol (PTZ), a GABA-A receptor antagonist that induces seizures in rodents and zebrafish (Baraban et al., 2005; Watanabe et al., 2010). cntnap2ab mutants display increased sensitivity to PTZ-induced seizures (Figures 1H and S3A-S3F). Drug-induced seizures appear as robust increases in activity and rapid, burst-like and circling movements, followed by periods of inactivity (Baraban et al., 2005). Homozygous double mutants display significantly more activity in response to PTZ (Figure S4B; p = 0.0013, two-way ANOVA, genotype \times dose interaction, n = 268). These results are consistent with increased seizures associated with CNTNAP2 mutations in humans and mice (Peñagarikano et al., 2011; Strauss et al., 2006). Consequently, increased sensitivity to PTZ provides further evidence for GABAergic deficits in cntnap2ab mutants.

cntnap2 Mutants Show Nighttime Hyperactivity

We next adapted a blinded, high-throughput assay to quantify a series of rest-wake cycle behavioral parameters over multiple days (Figure 2A) (Prober et al., 2006; Rihel et al., 2010). Quantitative profiling revealed that *cntnap2ab* mutants display significantly greater nighttime activity compared with wild-type siblings between 4 and 7 dpf (Figures 2B, 2C, and S3G; p =0.00012, one-way ANOVA, n = 838). This phenotype was independently observed in *cntnap2ab* mutants harboring different alleles (Figure S3H; p = 0.0198, n = 187). In contrast, other rest-wake parameters (Table S2), as well as acoustic startle, habituation, and optokinetic response (Table S3), were not significantly affected, further highlighting the specificity of the nighttime hyperactivity phenotype. Combined, these results indicate that loss of Cntnap2 selectively causes nighttime hyperactivity, consistent with GABAergic deficits leading to an imbalance of excitatory and inhibitory signaling in mutant fish.

Differential Responses to Psychoactive Agents in cntnap2 Mutants

Collectively, the day-night, rest-wake cycle parameters of cntnap2ab mutants represent a behavioral fingerprint with a specific nighttime hyperactivity signature. To identify molecular pathways that are dysregulated in the absence of Cntnap2 function, we searched for drugs that phenocopy the mutant behavioral profile. To this end, we compared the cntnap2ab mutant behavioral fingerprint with a data set of the behavioral profiles of wild-type larvae exposed to 550 psychoactive compounds (Rihel et al., 2010). This allowed an unbiased comparison of the different genetic and pharmacological conditions by cluster analysis (Rihel et al., 2010) (Figure 2D) and resulted in the identification of small molecules that strongly correlate with the mutant behavioral profile (Figure 2E). Next, we identified the top 14 drugs that anti-correlate, or generate the opposite phenotype of the mutant behavioral profile (Figure S4H). We reasoned that drugs that induce differential effects in wildtype and mutant larvae might indicate pathways that are dysregulated because of loss of Cntnap2. To test this, we exposed wild-type and $cntnap2a^{\Delta 121/\Delta 121}cntnap2b^{31i/31i}$ larvae to a group of compounds from 4-7 dpf (Figure 3A) that were selected on the basis of the following criteria: (1) correlating; (2) anti-correlating; (3) GABA-A receptor agonists, based on the structural GABAergic deficits; and (4) risperidone, because it is the first U.S. Food and Drug Administration-approved treatment for irritability and aggressive behavior in ASDs (McCracken et al., 2002).

Three lines of evidence indicate that both glutamatergic and GABAergic pathways are dysregulated in *cntnap2ab* mutants. First, we found that both GABA- and NMDA-receptor antagonists are significantly enriched among drugs that strongly correlate with the mutant phenotype (Figures 3D and 3E; p = 0.031 and p = 0.034, respectively, Kolmogorov-Smirnov test). These findings are consistent with the behavioral profile of *cntnap2ab* mutants, given that NMDA receptor antagonists induce night-time hyperactivity in wild-type larvae, and the increased sensitivity of mutants to the GABA-A receptor antagonist, PTZ. Second, *cntnap2ab* mutants are more sensitive to arousal by NMDA receptor antagonists across a range of doses (Figure 3B), suggesting attenuation of glutamatergic signaling.

Third, we found that GABA receptor agonists, such as zolpidem, induce differential behavioral effects in *cntnap2ab* mutants compared to wild-type larvae (Figures 3C and 3F–3H). For this analysis, we performed hierarchical clustering and principal-component analysis (PCA) to quantify differential mutant responses compared with wild-type across 18 rest-wake activity parameters (Rihel et al., 2010). Specifically, we calculated the Euclidean distance between the mutant + drug and wild-type + drug behavioral profiles, representing the difference between the mutant and wild-type responses to each drug (Figures 3H and S4A–S4F; see Supplemental Experimental Procedures).



Figure 2. cntnap2ab Mutants Display Nighttime Hyperactivity

(A) Experimental setup (Prober et al., 2006; Rihel et al., 2010).

(B) Locomotor activity of *cntnap2a*^{Δ25/Δ25}*cntnap2b*^{Δ7/Δ7} (*cntnap2ab*, red) and wild-type (WT; blue) sibling-matched larvae over 72 hr. Hyperactivity in mutants worsens on successive nights (arrows). The magnified activity profile on night 6 is shown.

(C) Average locomotor activity of *cntnap2ab* versus wild-type. *p = 0.00012 (one-way ANOVA, comparing all genotypes on all nights); p = 0.0193, 0.0236, and 0.0073, nights 4, 5, and 6, respectively.

(D) Hierarchical clustering of the *cntnap2ab* behavioral fingerprint (red arrow) compared with the fingerprints of wild-type larvae exposed to a panel of 550 psychoactive agents from 4–6 dpf (Rihel et al., 2010). Each rectangle in the clustergram represents the *Z* score, or the average value in SDs relative to the behavioral profiles of wild-type exposed to DMSO alone (magenta, higher than DMSO; cyan, lower than DMSO). The *cntnap2ab* profile correlates with agents that induce nighttime arousal ("Correlating Drugs").

(E) A magnified section of the clustergram, highlighting compounds that correlate with the cntnap2ab mutant behavioral fingerprint.



Figure 3. Differential Behavioral Responses of cntnap2ab Mutants to Psychoactive Agents

(A) The 14 psychoactive drugs tested in *cntnap2a*^{$1121/\Delta121}$ *cntnap2b*^{<math>311/311} and wild-type larvae, their biological targets, and the rationale for their selection. The following classes of drugs are shown: correlating (blue), anti-correlating (purple), drugs interacting with the GABA-A receptor (orange), and risperidone (green). (B and C) Dose-response effects of the NMDA receptor antagonist, (-)-MK-801, on night waking activity at 5 dpf (B) and the non-benzodiazepine GABA-A receptor agonist zolpidem on average night waking activity at 4–6 dpf (C) in wild-type (WT; blue) and *cntnap2ab* (red) larvae (p = 0.002, (-)-MK-801; p = 0.0003, zolpidem; two-way ANOVA, genotype × drug interaction).</sup>

(D and E) Significant enrichment of NMDA receptor antagonists (D) and GABA receptor antagonists (E) in the top ranks of correlating drugs (p = 0.031, NMDA-R antagonists; p = 0.034, GABA-R antagonists; Kolmogorov-Smirnov test).

(F) Hierarchical clustering of the behavioral profiles of wild-type or *cntnap2ab* larvae exposed to 14 psychoactive agents at three doses each. Each rectangle in the clustergram represents the *Z* score relative to the behavior of wild-type or mutant larvae exposed to DMSO alone (magenta, higher than DMSO; cyan, lower than DMSO).

(G) Magnified sections highlight the behavioral fingerprints of wild-type and cntnap2ab larvae in response to zolpidem (20 μM).

(H) Pairwise Euclidean distances between wild-type and *cntnap2ab* responses to psychoactive agents in the PCA. Note that zolpidem and (±)-baclofen produce the strongest differential responses.

Using this approach, we found that the GABA receptor agonists zolpidem and (±)-baclofen produce the strongest differential responses of the drugs tested (Figure 3H), suggesting perturbation of GABAergic signaling pathways in mutants. Taken together, our quantitative behavioral profiling reveals dysfunction in glutamatergic as well as GABAergic pathways due to loss of Cntnap2.

Estrogens Reverse the Behavioral Phenotype of *cntnap2ab* Mutants

Next, we searched for small molecules that suppress the mutant phenotype of nighttime hyperactivity. We reasoned that such compounds might elicit a behavioral fingerprint in wild-type opposite to that of mutant larvae. We found that estrogenic compounds are significantly enriched in the top ranks of small molecules that anti-correlate with the cntnap2ab mutant behavioral fingerprint (Figure 4A; p = 0.0003 by random permutation). Specifically, four of the top ten anti-correlating drugs have known estrogenic activity. On the basis of these results, we hypothesized that such drugs might be able to rescue the mutant phenotype. To test this, we analyzed the effects of estrogenic compounds and other selected molecules in wild-type and mutant larvae using quantitative behavioral profiling and PCA (Figure 3A; see Supplemental Experimental Procedures). Behavioral rescue was defined as the shortest Euclidean distance between the mutant + drug and wild-type + no drug behavioral profiles (Figure 4D).

We found that biochanin A, a plant-derived estrogen, and β-estradiol 17-cypionate most strongly reverse the mutant behavioral phenotype (Figures 4B-4D and S4G). Indeed, the behavioral response of cntnap2ab larvae treated with biochanin A (0.1 μ M) from 4–7 dpf most strongly correlates with the wildtype phenotype, decreasing nighttime activity with little effect on other measures of rest and activity (Figures 4B and 4C). In contrast, risperidone (0.001 µM) reverses nighttime hyperactivity but alters other rest-wake cycle parameters, indicating that it elicits a less specific phenotypic rescue than biochanin A (Figures 4C, 4E, and 4F). In addition, we found that early exposure to biochanin A does not reverse the GABAergic deficits (Figure S4M) or drug-induced seizures (Figure S4K) and that chronic exposure followed by washout does not rescue nighttime hyperactivity (Figure S4L), providing evidence for an acute mechanism of action. Consistent with estrogenic activity, we found that biochanin A (10 µM) significantly activates the expression of estrogen response genes in zebrafish larvae (Figure S4N). However, the rescue dose (1 μ M) shows only a weak effect on target genes, suggesting that the behavioral rescue might occur independently of the robust transcriptional activation of estrogen target genes. Moreover, we found that biochanin A (0.1-1 µM) leads to selective suppression of nighttime activity at 5 dpf and no change in daytime activity in background-matched larvae (Figures 4G and 4H), indicating its effects are not due to background effects or generalized sedation. Furthermore, β -estradiol (1 μ M) is also able to rescue nighttime hyperactivity in cntnap2ab mutants (Figure S4J). Together, these results provide evidence that biochanin A and β-estradiol acutely suppress a specific behavioral phenotype in a genetic loss-of-function model of an ASD risk gene.

DISCUSSION

This study represents the first characterization of zebrafish mutants of an ASD risk gene using quantitative behavioral profiling to identify biological pathways with relevance to ASDs. We demonstrate that zebrafish cntnap2ab mutants display reductions in GABAergic neurons, increased sensitivity to drug-induced seizures, and nighttime hyperactivity. Further, our pharmacological data support dysregulation of GABAergic and glutamatergic signaling in mutants. Altered NMDA signaling has been shown to cause GABAergic deficits, particularly in parvalbumin-positive (PV+) interneurons, and has been proposed as a mechanism underlying neuropsychiatric disorders (Keilhoff et al., 2004; Saunders et al., 2013). Moreover, mouse Cntnap2 knockouts display GABAergic deficits, most prominently in PV+ interneurons (Peñagarikano et al., 2011). Further studies are required to determine if PV+ subpopulations are altered in cntnap2ab mutants, but our study highlights that GABAergic deficits are likely to occur in concert with alterations in NMDA circuits, not previously associated with Cntnap2.

Next, our results uncover the ability of estrogens to rescue the cntnap2 mutant behavioral phenotype, suggesting that these compounds serve as modifiers of neural circuits disrupted in mutants. Indeed, estrogens can signal to multiple downstream pathways, including transcriptional activation of estrogen response genes, regulation of other transcription factors, and rapid activation of intracellular signaling pathways (Marino et al., 2006). The extent to which estrogens alter one or more downstream intracellular signaling pathways remains to be determined, but our findings suggest that the rescue activity likely involves an acute mechanism other than robust activation of estrogen target genes. Moreover, estrogens have been shown to affect glutamatergic signaling. Estradiol increases dendritic spine density and long-term potentiation via an NR2B-dependent mechanism that can be blocked by exposure to NMDA antagonists, such as MK-801 (Smith et al., 2009), Therefore, estrogens may be acting upstream of the identified NMDA and GABA deficits. Although there is growing interest in the effects of sex hormones on brain development and ASD risk, given the 4:1 male/female ratio of classical autism (Baron-Cohen et al., 2011; Schaafsma and Pfaff, 2014), further investigations are required to determine whether such a mechanism might contribute to the observed female protective effect suggested by recent human genetics studies of ASDs (Jacquemont et al., 2014).

As the list of reliable ASD risk genes continues to expand, there is a growing need for biologically relevant systems to advance from gene discovery to pharmacological screens. We demonstrate that quantitative behavioral profiling of zebrafish *cntnap2ab* mutants can be used as a novel platform for the prediction and in vivo screening of compounds to identify suppressors of a behavioral phenotype resulting from loss of an ASD risk gene. Future testing in a mammalian system is a critical next step. Moreover, the study of zebrafish mutants of the ASD risk gene, *CNTNAP2*, and its differential responses to psychoactive agents reveals the strength of this approach to identify molecular mechanisms and potential pharmacological candidates for further evaluation.



Figure 4. Biochanin A Reverses Nighttime Hyperactivity in cntnap2ab Mutants

(A) Rank-sorting of the anti-correlating data set with respect to estrogenic compounds shows significant enrichment of estrogenic agents in the top ranks (p = 0.0003 by random permutation). Black lines indicate drugs defined as having estrogenic activity (25 compounds in total).

(B) Hierarchical clustering of the behavioral fingerprints of $cntnap2a^{\Delta 121/\Delta 121}cntnap2b^{31i/31i}$ larvae exposed to 14 psychoactive agents at three doses each relative to the wild-type + no drug fingerprint. Each rectangle in the clustergram represents the Z score of drug-exposed mutants relative to untreated wild-type (magenta, higher than wild-type; cyan, lower than wild-type).

(C) Magnified sections of the clustergram show relative suppression of the mutant fingerprint by biochanin A (0.1 µM) compared with risperidone (0.001 µM). The red box highlights parameters that measure nighttime activity.

(D) Pairwise Euclidean distances ("Differential Drug Effects") between the mutant responses to psychoactive agents compared with untreated wild-type in the PCA (Figure S4G). Biochanin A (0.1 μ M) produces the strongest suppression of the mutant phenotype in this assay, with the fewest effects on other behavioral parameters.

(E and F) Dose-response effects of biochanin A (E) and risperidone (F) on nighttime activity at 5 dpf (p = 0.0001, biochanin A; p = 0.0034, risperidone; two-way ANOVA, gene \times drug interaction). Although there is some experimental variability in the baseline activity of both wild-type and mutant larvae, nighttime hyperactivity is consistently observed.

(G and H) Effect of the blind addition of biochanin A (0.1–1 μ M) or DMSO on activity at night (G) and day (H) in the progeny of incrosses of *cntna*- $p2a^{\Delta25/*}cntnap2b^{\Delta7/*}$ fish at 5 dpf. *p = 0.045 (two-way ANOVA, gene × dose interaction).

EXPERIMENTAL PROCEDURES

Zebrafish

Mutations in *cntnap2a* and *cntnap2b* were generated using zinc finger nucleases. The *cntnap2a*^{$\Delta 121/\Delta 121} cntnap2b$ ^{31/31i} and *cntnap2a*^{$\Delta 25/\Delta 25$} cntnap2b^{$\Delta 7/\Delta 7$} lines were generated by incrossing double homozygotes, which are viable and fertile. Tg(*dlx6a-1.4kbdlx5a/dlx6a:GFP*) and Tg(*vglut:DsRed*) were obtained from the laboratories of M. Ekker and J. Fetcho, respectively. *cntnap2a*^{$\Delta 121/\Delta 121} cntnap2b$ ^{31/31i} mutants were crossed to these transgenic lines. Animal experiments were conducted in accordance with Institutional Animal Care and Use Committee regulatory standards (Yale University) and the UK Animals (Scientific Procedures) Act 1986.</sup></sup>

Pharmacological Screen

Larval activity was monitored from 4–7 dpf using a custom-modified Zebrabox and automated video tracking system (Viewpoint; LifeSciences) (Rihel et al., 2010). Correlation analysis was done in MATLAB (R2014a; The MathWorks) to identify compounds that correlate or anti-correlate with the *cntnap2a*^{Δ25/Δ25}*cntnap2b*^{Δ7/Δ7} phenotype. Three doses of each compound (Figure 3A; Table S4) were tested in 10–12 *cntnap2a*^{Δ121/Δ121}*cntnap2b*^{31/31i} or wild-type replicate larvae with 10–12 control larvae per 96-well plate (Rihel et al., 2010). Hierarchical clustering was conducted in MATLAB with the statistics and bioinformatics toolboxes.

ACCESSION NUMBERS

The accession numbers for the sequences reported in this paper are GenBank KU376408 and KU376409.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2015.12.039.

AUTHOR CONTRIBUTIONS

E.J.H., M.W.S., and A.J.G. designed the project. E.J.H. generated and characterized *cntnap2ab* mutants. E.J.H. and K.J.T. performed immunohistochemistry. J.M.F. assisted in genotyping, zebrafish husbandry, and in situ hybridizations. D.C. adapted methods of mutagenesis and screening. R.A.J. and M. Granato analyzed startle and habituation. F.K. and H.B. analyzed the optokinetic response. B.R.B. provided in situ probes and discussions. E.J.H. and M.J.F.B. analyzed forebrain commissures. E.J.H., S.I., K.J.T., and S.W.W. analyzed CNS structure and GABAergic deficits. E.J.H., M. Ghosh, and J.R. performed behavioral tracking and pharmacological screens. J.R. performed correlation analyses and PCA. E.J.H. wrote the manuscript with input from S.W.W., J.R., M.W.S., and A.J.G.

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REFERENCES

Ablain, J., and Zon, L.I. (2013). Of fish and men: using zebrafish to fight human diseases. Trends Cell Biol. *23*, 584–586.

American Psychiatric Association (2013). Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (Arlington: American Psychiatric Publishing).

Baraban, S.C., Taylor, M.R., Castro, P.A., and Baier, H. (2005). Pentylenetetrazole induced changes in zebrafish behavior, neural activity and c-fos expression. Neuroscience *131*, 759–768.

Baron-Cohen, S., Lombardo, M.V., Auyeung, B., Ashwin, E., Chakrabarti, B., and Knickmeyer, R. (2011). Why are autism spectrum conditions more prevalent in males? PLoS Biol. *9*, e1001081.

Cobos, I., Calcagnotto, M.E., Vilaythong, A.J., Thwin, M.T., Noebels, J.L., Baraban, S.C., and Rubenstein, J.L. (2005). Mice lacking DIx1 show subtype-specific loss of interneurons, reduced inhibition and epilepsy. Nat. Neurosci. 8, 1059–1068.

De Rubeis, S., He, X., Goldberg, A.P., Poultney, C.S., Samocha, K., Cicek, A.E., Kou, Y., Liu, L., Fromer, M., Walker, S., et al.; DDD Study; Homozygosity Mapping Collaborative for Autism; UK10K Consortium (2014). Synaptic, transcriptional and chromatin genes disrupted in autism. Nature *515*, 209–215.

Ghanem, N., Jarinova, O., Amores, A., Long, Q., Hatch, G., Park, B.K., Rubenstein, J.L., and Ekker, M. (2003). Regulatory roles of conserved intergenic domains in vertebrate DIx bigene clusters. Genome Res. *13*, 533–543.

Iossifov, I., O'Roak, B.J., Sanders, S.J., Ronemus, M., Krumm, N., Levy, D., Stessman, H.A., Witherspoon, K.T., Vives, L., Patterson, K.E., et al. (2014). The contribution of de novo coding mutations to autism spectrum disorder. Nature 515, 216–221.

Jacquemont, S., Coe, B.P., Hersch, M., Duyzend, M.H., Krumm, N., Bergmann, S., Beckmann, J.S., Rosenfeld, J.A., and Eichler, E.E. (2014). A higher mutational burden in females supports a "female protective model" in neurodevelopmental disorders. Am. J. Hum. Genet. *94*, 415–425.

Keilhoff, G., Becker, A., Grecksch, G., Wolf, G., and Bernstein, H.G. (2004). Repeated application of ketamine to rats induces changes in the hippocampal expression of parvalburnin, neuronal nitric oxide synthase and cFOS similar to those found in human schizophrenia. Neuroscience *126*, 591–598.

Kinkhabwala, A., Riley, M., Koyama, M., Monen, J., Satou, C., Kimura, Y., Higashijima, S., and Fetcho, J. (2011). A structural and functional ground plan for neurons in the hindbrain of zebrafish. Proc. Natl. Acad. Sci. U S A *108*, 1164–1169.

Kokel, D., and Peterson, R.T. (2011). Using the zebrafish photomotor response for psychotropic drug screening. Methods Cell Biol. *105*, 517–524.

Marino, M., Galluzzo, P., and Ascenzi, P. (2006). Estrogen signaling multiple pathways to impact gene transcription. Curr. Genomics 7, 497–508.

McCracken, J.T., McGough, J., Shah, B., Cronin, P., Hong, D., Aman, M.G., Arnold, L.E., Lindsay, R., Nash, P., Hollway, J., et al.; Research Units on Pediatric Psychopharmacology Autism Network (2002). Risperidone in children with autism and serious behavioral problems. N. Engl. J. Med. *347*, 314–321.

Parikshak, N.N., Luo, R., Zhang, A., Won, H., Lowe, J.K., Chandran, V., Horvath, S., and Geschwind, D.H. (2013). Integrative functional genomic analyses implicate specific molecular pathways and circuits in autism. Cell *155*, 1008–1021.

Peñagarikano, O., Abrahams, B.S., Herman, E.I., Winden, K.D., Gdalyahu, A., Dong, H., Sonnenblick, L.I., Gruver, R., Almajano, J., Bragin, A., et al. (2011). Absence of CNTNAP2 leads to epilepsy, neuronal migration abnormalities, and core autism-related deficits. Cell *147*, 235–246. Poliak, S., Salomon, D., Elhanany, H., Sabanay, H., Kiernan, B., Pevny, L., Stewart, C.L., Xu, X., Chiu, S.Y., Shrager, P., et al. (2003). Juxtaparanodal clustering of Shaker-like K+ channels in myelinated axons depends on Caspr2 and TAG-1. J. Cell Biol. *162*, 1149–1160.

Prober, D.A., Rihel, J., Onah, A.A., Sung, R.J., and Schier, A.F. (2006). Hypocretin/orexin overexpression induces an insomnia-like phenotype in zebrafish. J. Neurosci. *26*, 13400–13410.

Rihel, J., Prober, D.A., Arvanites, A., Lam, K., Zimmerman, S., Jang, S., Haggarty, S.J., Kokel, D., Rubin, L.L., Peterson, R.T., and Schier, A.F. (2010). Zebrafish behavioral profiling links drugs to biological targets and rest/wake regulation. Science *327*, 348–351.

Rubenstein, J.L., and Merzenich, M.M. (2003). Model of autism: increased ratio of excitation/inhibition in key neural systems. Genes Brain Behav. *2*, 255–267.

Sanders, S.J., He, X., Willsey, A.J., Ercan-Sencicek, A.G., Samocha, K.E., Cicek, A.E., Murtha, M.T., Bal, V.H., Bishop, S.L., Dong, S., et al.; Autism Sequencing Consortium (2015). Insights into Autism Spectrum Disorder Genomic Architecture and Biology from 71 Risk Loci. Neuron *87*, 1215–1233.

Saunders, J.A., Tatard-Leitman, V.M., Suh, J., Billingslea, E.N., Roberts, T.P., and Siegel, S.J. (2013). Knockout of NMDA receptors in parvalbumin interneurons recreates autism-like phenotypes. Autism Res. 6, 69–77.

Schaafsma, S.M., and Pfaff, D.W. (2014). Etiologies underlying sex differences in Autism Spectrum Disorders. Front. Neuroendocrinol. *35*, 255–271.

Smith, C.C., Vedder, L.C., and McMahon, L.L. (2009). Estradiol and the relationship between dendritic spines, NR2B containing NMDA receptors, and the magnitude of long-term potentiation at hippocampal CA3-CA1 synapses. Psychoneuroendocrinology *34* (*Suppl 1*), S130–S142.

Strauss, K.A., Puffenberger, E.G., Huentelman, M.J., Gottlieb, S., Dobrin, S.E., Parod, J.M., Stephan, D.A., and Morton, D.H. (2006). Recessive symptomatic focal epilepsy and mutant contactin-associated protein-like 2. N. Engl. J. Med. *354*, 1370–1377.

Watanabe, Y., Takechi, K., Fujiwara, A., and Kamei, C. (2010). Effects of antiepileptics on behavioral and electroencephalographic seizure induced by pentetrazol in mice. J. Pharmacol. Sci. *112*, 282–289.

Willsey, A.J., Sanders, S.J., Li, M., Dong, S., Tebbenkamp, A.T., Muhle, R.A., Reilly, S.K., Lin, L., Fertuzinhos, S., Miller, J.A., et al. (2013). Coexpression networks implicate human midfetal deep cortical projection neurons in the pathogenesis of autism. Cell *155*, 997–1007.

Zerucha, T., Stühmer, T., Hatch, G., Park, B.K., Long, Q., Yu, G., Gambarotta, A., Schultz, J.R., Rubenstein, J.L., and Ekker, M. (2000). A highly conserved enhancer in the DIx5/DIx6 intergenic region is the site of cross-regulatory interactions between DIx genes in the embryonic forebrain. J. Neurosci. *20*, 709–721. Neuron, Volume 89

Supplemental Information

Estrogens Suppress a Behavioral

Phenotype in Zebrafish Mutants

of the Autism Risk Gene, CNTNAP2

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Figure S1, Related to Figure 1. Generation of cntnap2ab mutants and characterization of brain structure.

Figure S1, Related to Figure 1. Generation of cntnap2ab mutants and characterization of brain structure.

A, **B**. Fluorescent *in situ* hybridization of *cntnap2a* and *cntnap2b* expression and acetylated tubulin in wild-type embryos at 30 and 48 hpf.

C. Whole mount *in situ* hybridization of *cntnap2a* and *cntnap2b* in wild-type embryos at 24 and 48 hpf. Both paralogs are expressed in the CNS, though *cntnap2b* shows a more specific expression pattern in the telencephalon and diencephalon (24 hpf), the optic tectum, midbrain-hindbrain boundary, and hindbrain (48 hpf).

D. Genomic sequences of insertion-deletion mutations generated by zinc finger nucleases in each *cntnap2* paralog. These include $\Delta 121$ and $\Delta 25$ in exon 3 of *cntnap2a* (top) and $\Delta 7$ in exon 2 and a 31-nt insertion in exon 4 of *cntnap2b* (bottom), all of which produce a frameshift and premature stop codon.

E. Alternatively spliced transcripts resulting from the 31-nt allele in exon 4 of *cntnap2b* identified by RT-PCR of exons 1 to 6 at 5 dpf. These transcripts include: (i) the 31-nt sequence itself, as in the genomic sequence; (ii) $\Delta 10$ in exon 4: because the 31-nt insertion occurs close to the intron-exon 4 boundary, an alternate splice site is used (the next "TCAG" in the genomic sequence after the actual intronic splice site), which is 10 nt after the beginning of exon 4; (iii) $\Delta 148$ due to splicing of the entire exon 4. All 3 transcripts produce a frameshift and a premature stop codon. We did not identify alternatively spliced transcripts resulting from the other mutations in either gene.

F. Sequence alignment of wild type Cntnap2a (top) and Cntnap2b (bottom) proteins and the translation of mutant transcripts. All mutant transcripts are predicted to cause premature stop codons in the discoidin domains of both proteins (Figure 1A).

G-I. Acetylated tubulin (AcTub) and synaptic vesicle protein 2 (SV2) labeling in wild-type (**G-I**) and $cntnap2a^{A121/A121}cntnap2b^{31i/31i}$ (**G'-I'**) fish at 24, 48, and 120 hpf. While there is a transient delay in the formation of the anterior and post-optic commissures in *cntnap2ab* mutants at 28 hpf (see **K**, **K'**, **M**), these commissures form by later stages. Brain structure is not otherwise grossly disrupted in *cntnap2ab* mutants up to 120 hpf.

J-K. Acetylated tubulin and GABA (**J**, **J**') or GFAP (glial cells) (**K**, **K**') in wild-type and $cntnap2a^{4121/4121}cntnap2b^{31i/31i}$ (*cntnap2ab*) mutants at 28 hpf.

L. There are significantly fewer GABA+ cells in both the telencephalon (arrowheads) and diencephalon (double arrowheads) of *cntnap2ab* (**J**') versus wild-type (**J**) larvae at 28 hpf. GABA+ cells were counted in z-stack images by three blinded raters. (*p=0.00782, telencephalon; **p=0.0181, diencephalon, two-way ANOVA, genotype factor).

M. The formation of the anterior commissure (AC) and post-optic commissure (POC) is delayed in $cntnap2a^{A121/A121}cntnap2b^{31i/31i}$ (cntnap2ab) (**K'**) compared to wild-type (**K**) larvae (*p=0.00428, AC; **p=0.0159, POC, two-way ANOVA, genotype factor.) Three blinded raters quantified the degree of commissure formation based on a qualitative rating scale ranging from no axons crossing (1) to complete formation (7), as previously described (Barresi et al., 2005). The POC appears to be less fasciculated in mutants. Glial bridge formation is largely intact.

N-R Head and brain size in wild-type (**N-Q**) and $cntnap2a^{A121/A121}cntnap2b^{31i/31i}$ (*cntnap2ab*) (**N'-Q'**). Representative tracings done in Fiji are shown in yellow. The posterior boundaries of the head and brain were defined by the otic vesicle and the midbrain-hindbrain boundary, respectively.

R. *cntnap2ab* mutant heads are 6.5% and 9.7% smaller than wild-type at 30 hpf and 96 hpf, respectively, and mutant brains are 18% smaller than wild-type at 96 hpf. (* $p=9.71x10^{-7}$, 30 hpf head size; **p=0.00513, 96 hpf head size; *** $p=3.89x10^{-6}$, 96 hpf brain size; one-way ANOVA). In addition, eye and yolk size measurements indicate that mutant fish are smaller overall (Table S1).

(A, B): frontal views; (C, N-O, N'-O'): lateral views; (G-J, G'-J', P-P'): lateral views of dissected embryos with the eye removed; (K-K'): frontal views of the forebrain; (Q-Q'): ventral views; tel, telencephalon; di, diencephalon; OT, optic tectum; PC, posterior commissure; AC, anterior commissure; POC, post-optic commissure; E, eye; MHB, midbrain-hindbrain boundary; HB, hindbrain.



Figure S2, Related to Figure 1. Inhibitory and excitatory neurons in *cntnap2ab* mutants.

Figure S2, Related to Figure 1. Inhibitory and excitatory neurons in *cntnap2ab* mutants.

Immunostainings of reporter gene expression in Tg(dlx6a-1.4kbdlx5a/dlx6a:GFP) and Tg(vglut:DsRed) in wild-type (A-H) and $cntnap2a^{Al21/Al2l}cntnap2b^{3li/3li}$ (A'-H') larvae at 4 dpf. (A-A', E-E'): The deficit in GABAergic neurons and precursors (dlx5a6a:GFP+ cells) is evident in the ventral and dorsal telencephalon (tel), hypothalamus (hyp), and cerebellum (CB) (arrowheads in A-A', E-E'). The reduction in GABAergic neurons by surface area of acetylated tubulin is significant in the forebrain and cerebellum (J).

I-K. *dlx5a6a*:GFP+ cells, *vglut*:DsRed and acetylated tubulin stainings were quantified by brain region at 4 dpf: forebrain (small brackets in **D-D'**, **H-H'**); hypothalamus (large brackets in **D-D'**, **H-H'**); cerebellum (arrowheads in **E-E'**, **H-H'**); and optic tectum (arrowheads in **E-E'**, **H-H'**). (forebrain and hypothalamus: wild-type, n=9; *cntnap2ab*, n=9; optic tectum and cerebellum: wild-type, n=7; *cntnap2ab*, n=8)

I. *dlx5a6a*:GFP+ cell number normalized to wild-type. (*p<0.001; **p<0.01; ***p<0.02; one-way ANOVA, Bonferroni corrected).

J. dlx5a6a:GFP+ cell number relative to acetylated tubulin surface area normalized to wild-type. Because there are significant reductions in GABAergic neurons relative to area in the forebrain and cerebellum, we concluded that the GABAergic deficits in these regions are not primarily attributable to the reduction in brain size. (*p<0.02; one-way ANOVA, Bonferroni corrected).

K. *vglut*:DsRed surface area relative to acetylated tubulin surface area normalized to wild-type. Unlike GABAergic neurons, there are no significant reductions in glutamatergic surface area relative to acetylated tubulin surface area across regions, suggesting these differences are due to the overall reduction in brain size as opposed to regional deficits (p=0.225, forebrain; p=0.444, hypothalamus; p=0.408, optic tectum; p=0.490, cerebellum; one-way ANOVA).

L-S. Analysis of the CNS of wild-type (**L-S**) and $cntnap2a^{A121/A121}cntnap2b^{31i/31i}$ (cntnap2ab) (**L'-S'**) by in situ hybridization at 48 hpf: (**L, L'**): glutamate decarboxylase 1b (gad1b); (**M, M'**): vesicular glutamate transporter or solute carrier family 17, member 6b (vglut2a or slc17a6b); (**N, N'**): solute carrier family 6 (neurotransmitter transporter, glycine), member 5 (glyt2 or slc6a5); (**O, O'**): tyrosine hydroxylase (th1 or th); (**P, P'**): neuronal differentiation 1 (neurod or neurod1); (**Q, Q'**); transiently expressed axonal glycoprotein (tag1) or contactin 2 (cntn2); (**R, R'**): TUNEL staining at 28 hpf; (**S, S'**): phospho-histone H3 (H3P) (green) and acetylated tubulin (blue) immunostaining at 48 hpf. We observed that expression of gad1b was variable. No gross differences in the other neurotransmitter systems, markers of neuronal differentiation, apoptosis, or cell proliferation were observed.

(A-D, A'-D', E-H, E'-H') show all channels for the images in Figure 1C-F and 1C'-F', respectively. (A-D, A'-D'): ventral views; (E-H, E'-H', L-N, L'-N'; P-P', R-R', S-S'): lateral views; (O-O', Q-Q'): dorsal views. (tel, telencephalon; hyp, hypothalamus; OT, optic tectum; CB: cerebellum; HB, hindbrain; E, eye.)



Figure S3, Related to Figures 1 and 2. Drug induced seizure sensitivity and nighttime hyperactivity in *cntnap2ab* mutants.

Figure S3, Related to Figures 1 and 2. Drug-induced seizure sensitivity and nighttime hyperactivity in *cntnap2ab* mutants.

A. Activity tracings of wild-type (WT) and $cntnap2a^{\Delta l2l/\Delta l2l}cntnap2b^{3li/3li}$ (*cntnap2ab*) larvae at 6 dpf before and after exposure to 10 mM PTZ or water (ribbon shows +/- SEM).

B. Average activity 1 h before and after the blind addition of 10 mM PTZ or water to the progeny of incrosses of $cntnap2a^{A25/+}cntnap2b^{A7/+}$ fish, as in Figure 1H, with intermediate genotypes shown. Note that $cntnap2a^{A25/A25}cntnap2b^{A7/A7}$ larvae show the greatest PTZ response (p=0.0013, two-way ANOVA, genotype x dose interaction).

C. Dose-response curve showing the percent increase in activity over baseline per fish of wild-type or $cntnap2a^{A121/A121}cntnap2b^{31i/31i}$ larvae at 6 dpf exposed to water or increasing concentrations of PTZ for 1 h (p=0.0004, two-way ANOVA, genotype x dose interaction). Inset shows that the average baseline activity for 1 h prior to the addition of water or PTZ is not significantly different in wild-type and cntnap2ab larvae.

D. Dose-response curve of the log of the percent increase in activity over baseline per fish for wild-type and $cntnap2a^{A121/A121}cntnap2b^{31i/31i}$ larvae at 4 dpf exposed to water or increasing concentrations of PTZ for 1 h. Compared to larvae at 6 dpf (**C**), baseline activity at 4 dpf is relatively low, such that the PTZ response (normalized to baseline) appears greater. Increased PTZ sensitivity in mutants is evident at 4 dpf as well (p<10⁻⁶, two-way ANOVA, genotype x dose interaction). *cntnap2ab* mutants decrease their activity in response to water, in contrast to wild-type fish, in which water is mildly activating. We hypothesize this difference may reflect deficits in the ability of *cntnap2ab* mutants to respond to mildly stressful stimuli. Given this observation, normalization in the dose-response experiments was done to baseline, pre-PTZ activity per fish.

E. Cumulative histogram showing the percent of all activity in the blinded experiment that occurred 1 h after the addition of PTZ or water (ribbon shows +/- SEM). Most (>90%) baseline activity of 4 dpf larvae is ≤ 0.3 /sec, though PTZ induces robust increases in activity. *cntnap2ab* mutants are more likely than background-matched wild-type larvae to have activity bouts 0.5/sec in response to PTZ. Inset shows the average activity (± SEM) centered on all locomotor events that occurred within 1 h of the addition of PTZ or water. Note that PTZ-induced activity bouts occur more sparsely in mutants compared to wild-type, as evidenced by the decreased likelihood of a bout to be immediately preceded or followed by another bout (arrowheads).

F. Plot of average activity by *cntnap2* allele before and after the blind addition of 10 mM PTZ or water at 6 dpf. This graph highlights that increased sensitivity to PTZ is primarily driven by the *cntnap2b* allele (p=0.5703, a allele x drug interaction; p=0.0046, b allele x drug interaction; p=0.10, a allele versus b allele interaction, two-way ANOVA).

G. Rest-wake locomotor activity of $cntnap2a^{A25/A25}cntnap2b^{A7/A7}$ (*cntnap2ab*, red) and wild-type (blue) siblingmatched larvae over 72 h, as in Figure 2B. The bar graph shows the average locomotor activity of wild-type, $cntnap2a^{A25/A25}cntnap2b^{A7/A7}$ and all intermediate genotypes on all nights. We observed a general trend in strengthening of the phenotype with a reduction in *cntnap2ab* gene dosage (see also Figure S3H). (*p=1.2x10⁻⁴, oneway ANOVA followed by Tukey's post-hoc testing shows the differences lie between the double homozygous larvae and the genotypes marked with the black bar.)

H. Locomotor activity of $cntnap2a^{4l2l/4l2l}cntnap2b^{3li/3li}$ (cntnap2ab, red) and wild-type (blue) background-matched larvae over 72 h. In total, 187 larvae were tracked blind to genotype. Magnified graphs of activity on each night are shown. The bar graph shows average locomotor activity of wild-type, $cntnap2a^{4l2l/4l2l}cntnap2b^{3li/3li}$ and all intermediate genotypes on all nights. Note that double homozygous mutants demonstrate the greatest level of activity, though single homozygotes for the cntnap2b mutation ($cntnap2a^{+/+}cntnap2b^{3li/3li}$ or $cntnap2a^{+/-}cntnap2b^{3li/3li}$ larvae) are hyperactive as well, indicating the cntnap2b allele may be driving the hyperactivity phenotype in this line (*p=0.0198, all nights, one-way ANOVA followed by Tukey's post-hoc testing shows the differences lie between the double homozygous larvae and the genotypes marked with the black bar.) The difference in the relative allelic effects in the two mutant lines may be due to background variation that influences allelic expression, the smaller dataset for the $cntnap2a^{4l2l/4l2l}cntnap2b^{3li/3li}$ line, or the nature of the mutations themselves, i.e. alternatively spliced transcripts. However, the effect of total disruption of Cntnap2 on nighttime activity in double homozygotes is robust and consistent in both mutant lines.



Figure S4, Related to Figures 3 and 4. Differential pharmacological responses of *cntnap2ab* mutants and suppression of nighttime hyperactivity by estrogenic compounds.

Figure S4, Related to Figures 3 and 4. Differential pharmacological responses of *cntnap2ab* mutants and suppression of nighttime hyperactivity by estrogenic compounds.

A. Magnified sections of the clustergram of the behavioral profiles of wild-type (WT) or $cntnap2a^{A121/A121}cntnap2b^{31i/31i}$ (MUT) larvae exposed to 14 psychoactive agents (as in Figure 3F) show differential responses to zolpidem (10 μ M, 20 μ M). Each rectangle in the clustergram represents the Z-score relative to the behavior of wild-type or $cntnap2a^{A121/A121}cntnap2b^{31i/31i}$ larvae exposed to DMSO alone (magenta, higher than DMSO; cyan, lower than DMSO). The eigenvectors for the first four PCA components are plotted below the clustergram to show the relative contribution of the 18 rest-wake cycle parameters (above) to each PCA component. Compared to the 26 rest-wake parameters, as shown in Figure 2E, in subsequent hierarchical clustering and PCA analyses (Figures 3F and 4B), the length and latency parameters were removed, because these parameters are non-normally distributed across drug-exposed samples, thereby skewing the PCA analysis.

B. Scree plot showing the percentage of the variance that is explained by each principal component in the PCA. Note that four components explain \sim 50% of the variance. The first four components are shown in magenta, green, blue, and yellow, respectively, corresponding to the components plotted in (A).

C-E. To confirm the stability of the PCA, we asked how the distribution of eigenvectors would change if each drugdose combination was removed sequentially from the dataset, following the method of (Woods et al., 2014). We applied this "left-out" modeling approach to our dataset (black) and to a randomized permutation of our dataset (orange).

C. Plot of the cumulative probability versus the similarity of eigenvectors for the "left-out" models of our dataset (black) and the randomized dataset (orange). The eigenvectors remain more similar in our model compared to the randomized dataset.

D. Plot of cumulative probability versus length in the "left-out" models shows that the distance from the origin of eigenvectors in our dataset (black) is greater compared to the randomized dataset (orange).

E. Comparison of the distances between vectors for each drug-dose combination in the "left-out" model of our dataset (black dots) versus the randomized set (orange dots). Observe that in our model, the spatial distance between eigenvectors is relatively maintained, unlike in the randomized dataset.

F. Wild-type (blue) and $cntnap2a^{A121/A121}cntnap2b^{31i/31i}$ (*cntnap2ab*, red) behavioral responses to the three doses of zolpidem (solid lines) and (\pm)-baclofen (dashed lines) can be represented by plotting the first two components of the PCA. Note the distinct location and shape of the wild-type and mutant plots, indicating that these drugs induce differential responses, as shown quantitatively for zolpidem in Figure 3C. In contrast, plots of the first two components of the PCA showing the behavioral responses to three doses of clonidine (gray) in wild-type (thick line, blue dots) and *cntnap2ab* (thin line, red dots) are shown. Note that the location and shape of the mutant and wild-type curves is similar for clonidine, which indicates that this drug has a similar behavioral effect in wild-type (blue) and *cntnap2ab* (red) larvae to other drugs in PCA space. A representative plot of average night activity at 4 dpf for clonidine (inset) shows that clonidine induces similar dose-dependent behavioral effects on nighttime activity at 4 dpf in wild-type (blue) and *cntnap2ab* (red) larvae (p=0.744, two-way ANOVA, genotype x drug interaction).

G. Magnified section of the clustergram in Figure 4B. Observe that biochanin A (0.1 μ M, 1 μ M) and three doses of β -estradiol 17-cypionate (0.1, 1, and 10 μ M) are in this cluster, which highlights the drugs that induce the strongest phenotypic suppression. Each rectangle in the clustergram represents the Z-score of mutants relative to wild-type + no drug (cream color = wild-type level; magenta, higher than wild-type; cyan, lower than wild-type). The eigenvectors for the first three PCA components are plotted below the clustergram to show the relative contribution of the rest-wake cycle parameters (above) to each PCA component, as in (A). The red box highlights the parameters that measure nighttime activity. Scree plot (lower left inset) shows that three components explain ~50% of the variance. These components are shown in magenta, green, and blue, respectively, corresponding to the components plotted on the graph. The stability of the PCA was confirmed using the "left-out" model (Woods et al., 2014), as in (**B-E**) (data not shown). Of note, we found that the lowest dose of risperidone (0.001 μ M) reverses nighttime hyperactivity in *cntnap2ab* mutants, yet based on the PCA, it appears to rescue with less phenotypic selectivity than biochanin A (Figure 4C, D, F), suggesting that its effect on nighttime hyperactivity is less phenotypically specific.

At the same time, the effects of risperidone appear to be highly dose-dependent, suggesting that it may affect various pharmacological pathways at different dose ranges. Further experiments are needed to investigate the differential responses of wild-type and mutant larvae to risperidone across a range of doses.

H. The top anti-correlating psychoactive agents and their degree of anti-correlation relative to the $cntnap2a^{A25/A25}cntnap2b^{A7/A7}$ mutant behavioral profile. Drugs shown in bold purple letters were selected for further testing in wild-type and mutant fish (Figure 3A). It is possible that other agents on this list in addition to biochanin A may have the ability to suppress the mutant behavioral phenotype. It will be interesting in future studies not only to test all of the anti-correlating small molecules, but to test all 550 psychoactive agents in *cntnap2ab* and other zebrafish mutants of ASD genes, which will provide substantial leverage to investigate convergent biological mechanisms among ASD risk genes.

I. Locomotor activity of $cntnap2a^{A121/A121}cntnap2b^{31i/31i}$ (red) and wild-type (blue) non-background-matched larvae over 72 hours (n=40 per group). cntnap2ab mutants display nighttime hyperactivity relative to unrelated wild-type (*night 5, p=0.0079; **night 6, p=0.022; one-way ANOVA), consistent with our findings in background-matched larvae. Mutants also show significantly less daytime activity relative to unrelated wild-type controls (p=0.005, one-way ANOVA). Because this effect did not reach statistical significance in sibling-matched control experiments (Table S2), we concluded that this difference is likely due to background or environmental variation.

J. Effect of β -estradiol (1 μ M) on average night waking activity at 5 dpf in wild-type and *cntnap2d*^{A121/A121}*cntnap2b*^{31i/31i} larvae (*p=0.017, two-way ANOVA, genotype x drug interaction).

K. To assess the extent to which biochanin A reverses PTZ-induced seizures, wild-type and $cntnap2a^{4121/4121}cntnap2b^{31i/31i}$ mutants were exposed daily and chronically to biochanin A (1 µM) or DMSO from 24 hpf to 4 dpf, and then exposed to PTZ in the presence of biochanin A or DMSO. Exposure to biochanin A did not rescue the increased sensitivity of *cntnap2ab* mutants to PTZ (p=0.237, three-way ANOVA, genotype x drug exposure x PTZ interaction). *cntnap2ab* mutants demonstrated increased sensitivity to PTZ irrespective of biochanin A exposure (p=3.57x10⁻⁴, genotype x PTZ exposure).

L. To assess the extent to which long-term exposure to biochanin A reverses nighttime hyperactivity, wild-type and $cntnap2a^{A121/A121}cntnap2b^{31i/31i}$ (*cntnap2ab*) embryos were exposed daily to biochanin A (1 µM) or DMSO alone beginning at 24 hpf. After removal of the drug solution at 4 dpf, the locomotor activity of wild-type and *cntnap2ab* larvae was quantified for 48 hours using the automated tracking assay. Exposure to biochanin A followed by washout did not rescue nighttime hyperactivity (*p=5.51x10⁻⁵, two-way ANOVA, genotype factor; p=0.454, two-way ANOVA, genotype x drug interaction).

M. To determine whether early exposure to biochanin A reverses the GABAergic deficits in *cntnap2ab* mutants, wild-type and *cntnap2a^{A121/A121}cntnap2b^{31i/31i}* (*cntnap2ab*) embryos were raised in the presence of biochanin A (0.1 μ M) or DMSO, which was added at 30 hpf. Immunostainings of reporter gene expression using the transgenic line, Tg(*dlx6a-1.4kbdlx5a/dlx6a:GFP*), were performed and *dlx5a/6a:GFP*+ cells in the forebrain of wild-type and *cntnap2ab* mutants at 4 dpf were counted manually. There are significantly fewer GABAergic neurons in the forebrains of *cntnap2ab* mutants compared to wild-type larvae at 4 dpf irrespective of exposure status (*p=6.03x 10⁻¹³, two-way ANOVA, genotype factor). Early exposure to biochanin A did not alter the number of GABAergic neurons in *cntnap2ab* or wild-type fish (p=0.190, two-way ANOVA, genotype x drug interaction; wild-type + DMSO, n=13; wild-type + biochanin A, n=17; *cntnap2ab* + DMSO, n=14; *cntnap2ab* + biochanin A, n=15).

N. To ascertain the extent to which biochanin A (BchA) upregulates estrogen response genes, expression of three known estrogen target genes (*cytochrome P450, family 19, subfamily A, polypeptide 1 (aromatase B, cyp19a1b*); *vitellogenin 1 (vtg1*); and *estrogen receptor 1 (esr1*)) (Hao et al., 2013) was measured by qPCR in total RNA extracts from wild-type larvae exposed overnight to biochanin A (1 μ M, 10 μ M) or DMSO from 4 to 5 dpf. Fold change in gene expression is shown. Note that biochanin A (10 μ M) significantly activates estrogen response genes, while the rescue dose (1 μ M) has only a weak effect (*p=0.001; **p=0.002; [#]p=0.1; Dunnett's test).

Table S1, Related to Figure 1. Head and Brain Size Measurements in Wild-type and
 $cntnap2a^{A121/A121}cntnap2b^{31i/31i}$ Mutants at 30 and 96 hpf.

Age	Head Size (Rel. to WT) (± SEM)	р	Eye Size (Rel. to WT) (± SEM)	р	Yolk Size (Rel. to WT) (± SEM)	р
30 hpf	0.935 ± 0.00783	9.71x10 ⁻⁷	0.851 ± 0.0190	1.50x10 ⁻⁶	0.902 ± 0.00613	0.00132
96 hpf	0.903 ± 0.0236	0.00513	0.873 ± 0.0198	0.00106	N/A	N/A
96 hpf brain	0.820 ± 0.0226	3.89x10 ⁻⁶	N/A	N/A	N/A	N/A

Age	Head:E	Cye Ratio	Head:Yolk Ratio					
	WT	Mutant	р	WT	Mutant	р		
30 hpf	4.80 ± 0.0679	5.33 ± 0.112	0.00082	0.574 ± 0.00672	0.595 ± 0.0068	0.11		
96 hpf	2.14 ± 0.0549	2.21 ± 0.0795	0.458	N/A	N/A	N/A		

At 30 hpf: WT, n=22; Mutant, n=24; 96 hpf: WT, n=9; Mutant, n=7; 96 hpf brains WT, n=11, Mutant, n=14. For yolk size measurements at 30 hpf: WT, n=21; Mutant n=23.

 Table S2, Related to Figure 2. Rest-wake Cycle Parameters in Background-Matched Wild-type and

 $cntnap2a^{A25/A25}cntnap2b^{A7/A7}$ Larvae at 4-6 dpf.

Time	Wild-type	cntnap2ab	р	Wild-type	cntnap2ab	р	Wild-type	cntnap2ab	р
Total Sleep (minutes)			Numbe	Number of Sleep Bouts			Sleep Bout Length (minutes)		
Night 4	453 ± 11.1	414 ± 16.7	0.0451	59.8 ± 3.57	64.0 ± 3.76	0.4143	11.0 ± 1.07	8.47 ± 0.853	0.0793
Night 5	362 ± 16.0	324 ± 21.0	0.1459	76.9 ± 3.44	75.4 ± 4.08	0.7826	6.28 ± 0.709	5.94 ± 0.945	0.7714
Night 6	342 ± 15.9	309 ± 19.6	0.194	81.1 ± 3.45	81.8 ± 4.29	0.8973	6.09 ± 0.939	5.53 ± 1.04	0.6904
Day 5	351 ± 29.3	417 ± 34.8	0.1448	66.3 ± 5.44	59.5 ± 4.09	0.3132	6.85 ± 0.759	7.12 ± 0.779	0.8068
Day 6	234 ± 26.8	283 ± 32.2	0.2459	59.9 ± 4.70	73.0 ± 7.15	0.1159	4.01 ± 0.697	4.51 ± 0.714	0.6186
	Sleep Latency (minutes)		Average Activity (seconds/minute)			Waking Ac	ctivity (seconds/mi	inute)	
Night 4	17.0 ± 0.493	21.1 ± 3.73	0.2264	0.281 ± 0.0315	0.440 ± 0.0783	0.0478	0.916 ± 0.0588	1.00 ± 0.0880	0.4103
Night 5	12.0 ± 1.61	10.3 ± 0.902	0.372	0.363 ± 0.0342	0.596 ± 0.103	0.0234	0.767 ± 0.059	0.969 ± 0.104	0.0825
Night 6	7.46 ± 0.820	9.81 ± 1.22	0.1025	0.349 ± 0.0257	0.649 ± 0.139	0.0222	0.659 ± 0.0310	0.940 ± 0.144	0.0394
Day 5	3.60 ± 1.14	4.69 ± 1.58	0.5695	1.88 ± 0.177	1.57 ± 0.212	0.2615	2.89 ± 0.189	2.47 ± 0.212	0.137
Day 6	13.2 ± 7.86	7.07 ± 2.13	0.4878	2.42 ± 0.190	2.037 ± 0.215	0.1817	3.19 ± 0.209	2.72 ± 0.253	0.1462

Table S3, Related to Figure 2. Acoustic Startle, Habituation, and Optokinetic Response Assays in Wild-type and *cntnap2a*^{A121/A121}*cntnap2b*^{31i/31i} Larvae at 5-7 dpf.*

	Short Latency Sensitivity (9	C-bend (SLC) ^a % Response)	Long Latency (% Re	C-bend (LLC) ^a sponse)	SLC Habituation	Prepulse Inhibition
Genotype	13 dB	26 dB	13 dB	19.8 dB	Average (%)	Average (%)
Wild-type	7.5 ± 2.45	83.7 ± 4.27	22.9 ± 3.36	37.2 ± 4.1^{b}	96.2 ± 1.07	64.0 ± 3.35
$cntnap2a^{\Delta 121/+}2b^{31i/+}$	10.6 ± 1.22	86.9 ± 1.87	23.6 ± 2.16	$34.1 \pm 2.23^{\circ}$	95.5 ± 1.12	60.0 ± 3.09
$cntnap2a^{\Delta 121/\Delta 121} 2b^{31i/31i}$	8.89 ± 2.41	89.4 ± 3.92	15.6 ± 5.00	$22.6\pm4.62^{\text{b,c}}$	97.4 ± 0.97	62.3 ± 3.66

Acoustic Startle and Habituation

^aTested in background-matched controls. ${}^{b}p = 0.025$, ${}^{c}p = 0.039$, *t-test*

Optokinetic Response								
Genotype % contrast (# saccades/min ± SEM)						Spontaneous		
	100	70	40	20	10	5	Saccades	
Wild-type	10.3 ± 0.52^{d}	10.3 ± 0.67	8.04 ±0.51	6.99 ± 0.45	6.34 ± 0.52	5.9 ± 0.52	5.59 ± 0.45	
$cntnap2a^{\Delta 121/+}2b^{31i/+}$	10.4 ± 0.49^{e}	9.59 ± 0.61	8.07 ± 0.46	6.84 ± 0.55	5.87 ± 0.43	5.69 ± 0.36	4.95 ± 0.50	
$cntnap2a^{\Delta 121/\Delta 121} 2b^{31i/31i}$	$8.74 \pm 0.47^{d, e}$	8.72 ± 0.43	6.49 ± 0.53	5.60 ± 0.53	5.15 ± 0.49	4.84 ± 0.44	5.67 ± 0.42	

 $^{d}p = 0.04, ^{e}p = 0.036, t$ -test

*While there were no significant differences in acoustic startle, habituation, or pre-pulse inhibition (PPI) in *cntnap2a*^{A121/A121}*cntnap2b*^{31i/31i} fish at 5 to 7 dpf, mutants were significantly less likely to perform long-latency "Cbends" (LLC) in response to a 19.8 dB acoustic stimulus. LLC responses are kinematically distinct from shortlatency C-bend startle responses (SLC) and have different genetic and neural circuit requirements than SLC responses. While the specific neurons required for LLC behavior are not well understood, LLC-like responses may be involved in coordinated escape movements in schooling fish (Burgess and Granato, 2007). In addition, we did not detect differences in spontaneous saccadic eye movements or OKR performance at most contrast sensitivities in *cntnap2ab* mutants at 5 to 7 dpf, though at the highest contrast level (100%), mutants displayed significantly lower OKR performance compared to wild-type fish. The absence of robust phenotypes in these larval behavioral assays further supports the specificity of the nighttime hyperactivity phenotype detected in our rest-wake assay. Further studies are indicated to dissect the deficits in circuitry underlying these subtle behavioral differences.

Compound	Catalog #	Company	Dose Range (µM)
Amantadine hydrochloride	A1260	Sigma	3, 10, 30
(±)-Baclofen	B5399	Sigma	0.1, 1, 10
β-estradiol 17-cypionate	E8004	Sigma	0.1, 1, 10, 30
Biochanin A	D2016	Sigma	0.1, 1, 10
Chlorzoxazone	C4397	Sigma	1, 10, 30
Clonidine hydrochloride	C7897	Sigma	1, 10, 30
CSG-12066 maleate salt	C106	Sigma	0.1, 1, 10
Diazepam C-IV	D0899	Sigma	0.1, 1, 10
β-Estradiol	E1024	Sigma	1, 10, 30
L-701,324	L0258	Sigma	3, 10, 30
(-)-MK-801 hydrogen maleate	M108	Sigma	3, 10, 30
Risperidone	R3475	LKT Laboratories	0.001, 0.01, 1, 10, 30
Ticlopidine hydrochloride	T6654	Sigma	1, 10, 30
Zolpidem	N/A	Gift from	1, 10, 20
-		David Sugden	

Table S4, Related to Figures 3 and 4. Final concentrations of each psychoactive compound tested in wild-type and $cntnap2a^{4121/4121}cntnap2b^{31i/31i}$ larvae.

Supplemental Experimental Procedures

Zinc finger nuclease design

CompoZr custom zinc finger nucleases (ZFN) targeting zebrafish *cntnap2a* and *cntnap2b* were designed by Sigma-Aldrich. CompoZr ZFN contain a DNA-binding domain and an obligate-heterodimer *FokI* nuclease domain, engineered for improved specificity (Miller et al., 2007). Activity of ZFN pairs as determined by the yeast MEL-1 reporter assay (Doyon et al., 2008) was >50% for all pairs (*cntnap2a*, 115.1%; *cntnap2b*, Exon 2, 141.0%; *cntnap2b*, Exon 4: 86.3%). Target sequences for each ZFN pair are shown below. ZFN left and right array binding sites are in capital letters and the cut sites are shown in bold lower case letters.

cntnap2a, Exon 3: AACTGGAAACCATaccatcAAGATGGCAACATCTGGG

cntnap2b, Exon 2: AGCGCCTTCACCAGCTCCtctgtgCTGGCAGCGGAT

cntnap2b, Exon 4: CTCTTCTTCTGCCTCAGAcattttCAGGTAACTGGAACTCAG

Generation and screening of *cntnap2ab* double mutants

To generate mutants, 25, 50, or 75 pg of each left and right ZFN mRNA directed against either *cntnap2a* or *cntnap2b* was injected into wild-type embryos (F_0) at the one-cell stage. Plasmids encoding ZFN (pZFN) were linearized by XhoI digestion. Capped mRNA was synthesized by in vitro transcription and the polyA tail was added using the mMessage mMachine T7 Ultra transcription kit (Ambion) following the manufacturer's protocol. mRNA was purified using the RNeasy Mini Kit (Qiagen). Each ZFN set was screened to determine the optimal concentration that induced somatic mutations at the target site while minimizing toxicity, as previously described (Foley et al., 2009; Sander et al., 2011). In brief, genomic DNA was isolated from 10 injected 24 hpf embryos that were viable and lacked developmental defects, and the ZFN target region was amplified by limited-cycle PCR and cloned using the TOPO-TA kit (Invitrogen). The somatic mutation frequency was determined based on the number of insertion-deletion mutations observed by high-resolution fragment analysis of PCR-amplified plasmid DNA from 100 transformed colonies. The following somatic mutation frequencies were ascertained for each ZFN set: cntnap2a: 36%, cntnap2b, Exon 2: 70%, cntnap2b, Exon 4: 12%. Morphologically normal embryos were raised to adulthood and screened for germline mutations, according to the method described in (Cifuentes et al., 2010), with some modifications. Briefly, to genotype the progeny of intercrossed founders, 32 F₁ embryos were screened in groups of two, which were sorted into the wells of a 96-well plate. 50 µl of 100 mM NaOH was added to each well and the embryos were boiled for 20 minutes at 95°C, producing a crude DNA extract, which was neutralized by the addition of 15 µl of 1 M Tris-HCl, pH 7.4 (Sigma-Aldrich). 2 µl of this DNA extract was used per PCR reaction, and 1 µl of PCR product was diluted in 9 µl of formamide for high-resolution fragment analysis (DNA Analysis Facility at Yale University). Chromatograms were analyzed by GeneMapper software (Applied Biosystems) to identify insertion-deletion mutations. F_0 founders are mosaic and some were found to have multiple germline mutations. F_0 were backcrossed to wild-type fish and F_1 heterozygotes carrying mutations of interest in *cntnap2a* and *cntnap2b* were crossed to generate double heterozygotes (F₂). Germline frequencies for each mutation were the following: *cntnap2a*: Δ121, Δ25: 17%; *cntnap2b*, Exon 2: Δ7, 63%; Exon 4: 31ins, 29%.

Genotyping was performed using the following sets of PCR primers: *cntnap2a*, Exon 3, 485 bp: 5' ACCCTTAAAATTGATAAAAGAACACG 3' / 5' GCAGAAAAGGGGCTAAATTAAAA 3' *cntnap2b*, Exon 2, 388 bp: 5' TGCGATGTGTATCATATGTTCTTTT 3' / 5' AAAAAGGTAGCTCAAACTGTAATTG 3' *cntnap2b*, Exon 4, 404 bp: 5' CCTTGCATAATTAAACTGAAATGCT 3' / 5' TGGTTCTCTTGTTTCATTTGGTC 3'

Sequences of full-length cntnap2a and cntnap2b

Because annotations of *cntnap2a* and *cntnap2b* were incomplete at the 5' end in earlier assemblies of the zebrafish genome, Rapid Amplification of cDNA Ends (5' RACE) was performed using the GeneRacer kit (Life Technologies) following the manufacturer's protocol. First, total RNA was isolated from 24, 48, 72 and 120 hpf

embryos using TRIzol reagent (Life Technologies) and purified using the RNeasy Mini Kit (Qiagen). mRNA was isolated from pooled RNA from these four stages using oligo(dT) primer (Invitrogen) fixed to streptavidin magnetic beads (New England Biolabs). The following gene-specific 5' RACE reverse primers were used:

cntnap2a: 5' CCCAGATGTTGCCATCTTGATGGTATGG 3'

cntnap2b: 5' CAGTCCAGTGGGATGAAACGCACATA 3'

After determining the sequence of the first exon of each gene by 5'RACE, full-length *cntnap2a* (BamHI-XhoI) and *cntnap2b* (BstBI-XbaI) were cloned into pCS2+ (+/- 3'HA tags) by RT-PCR using pooled cDNA from 24, 48, 72, and 120 hpf embryos (or 72 and 120 hpf only). The current annotation of *cntnap2a* in GRCz10 does not include the sequence of the first exon as determined by 5'RACE in our experiments. The accession numbers for the full-length sequences of *cntnap2a* and *cntnap2b* reported in this paper are GenBank: KU376408 and KU376409.

To assess for alternate start sites of either transcript, 5'RACE was performed using the following genespecific reverse primers:

cntnap2a: 5' GTGGAGGTGCTGAAGCTGAAGGTCA 3' cntnap2a, Nested: 5' CTGCTTCAAAGAAACCACCAACATC 3' cntnap2a, 2nd Nested: 5' CATTTGCCACCATTACGACAATACA 3' cntnap2b #1: 5' GGTGTGTTGAAAGAAGCAGCGTTCC 3' cntnap2b #1, Nested: 5' CAACCACAACCTGACTCACAGGTAA 3' cntnap2b #1, 2nd Nested: 5' GTGCAGTTGCGTTCAATACCACA 3' cntnap2b #2: 5' CACCGCAGACTCGCCTTCCTTTAGT 3' cntnap2b #2, Nested: 5' TGCAGTCGCATGAGTATCCATTGTA 3' cntnap2b #2, 2nd Nested: 5' ATAACTGCTACAGTGGCCCGAACAT 3'

For *cntnap2a*, one specific 5'RACE product of \sim 3kb was identified corresponding to the full-length transcript with the same start site (data not shown). No other specific 5'RACE products were identified. For *cntnap2b*, mispriming of the GeneRacer 5' Nested primer within *cntnap2b* yielded nonspecific products. These results indicated that there were no shorter transcripts or alternate start sites identified.

Regarding current annotations of zebrafish *cntnap2*, the full-length sequence of *cntnap2a* that we cloned by RT-PCR is not annotated completely in GRCz10, in that it does not include the sequence of the first exon as determined by 5'RACE in our experiments. In the Ensembl genome browser (GRCz10), there is one gene on chromosome 24 corresponding to *cntnap2a* (ENSDARG00000058969) that is predicted to have 2 transcripts: ENSDART00000154039 and ENSDART00000155843, both of which have incomplete 5' coding sequences. The function of ENSDART00000155843, which is predicted to yield a product of 190 aa, is unknown and requires further investigation. In Ensembl (GRCz10), there is one gene on chromosome 2 corresponding to *cntnap2b* (ENSDARG00000074558) that is predicted to have 2 transcripts: ENSDART00000155125, which is predicted to encode a protein of 566 aa. Our 5'RACE and western blotting experiments would be expected to detect the longer transcript of *cntnap2a* (ENSDART00000154039) and both transcripts of *cntnap2b* (ENSDART00000108900; ENSDART00000155125). No other shorter proteins were detected by western blot using our custom antibody (data not shown), indicating our ZFN-induced mutants containing indels in exons 2 to 4 would be expected to disrupt Cntnap2a and Cntnap2b function.

In situ hybridization

In situ hybridization was performed according to the method previously described (Thisse et al., 1993). After staining, embryos were fixed in 4% PFA, washed in PBST and sequentially dehydrated in methanol. Embryos were cleared in benzyl benzoate/benzyl alcohol for imaging. Fluorescent *in situ* hybridization was adapted from the protocol described in (Julich et al., 2005). Probes were detected with anti-Digoxigenin-POD, Fab fragments (Roche, 1:1000) and TSA Plus Cyanine 3 System (Perkin Elmer). Primary antibody to acetylated tubulin (mouse monoclonal IgG2b, #T7451, Sigma-Aldrich, RRID:AB_609894) was used at 1:500. Following staining, embryos were mounted for confocal imaging in low melting point agarose (0.8-1%).

Probes for *cntnap2a* and *cntnap2b* were amplified from a pooled sample of cDNA from 0, 6, 24, 48, and 120 hpf embryos and cloned into pBluescript (BamHI-XhoI). Sequences of PCR primer pairs for the *cntnap2a* and

cntnap2b probes are:

cntnap2a, **520 bp:** 5' GCATAATCCACAACCTTCCAGTC 3' / 5' TTCCCCTGGTGCGAGACGGACAC 3' *cntnap2b*, **1108 bp:** 5' GTGCTGGCAGCGGATTTTGGTG 3' / 5' CCTCCACCCCTCCAGGCATGAGC 3'

In addition, a second probe (517 bp) located closer to the 3' end of *cntnap2b* was found to have the same expression pattern as the longer probe (data not shown). Primers for this probe are:

5' CGAGTCTGCGGTGCTGGGCATTGTG 3' /

5' GACTCCCTGTATGGAGGCAGTGGAC 3'

Plasmids for transcribing the *in situ* probes for *gad1b*, *vglut2a*, *glyt2*, and *neurod1* were gifts from Brent Bill from the laboratory of Daniel Geschwind. The primer pairs for generating these probes are:

gad1b: 5' CGTCTTCTGCACCTTCTTCCT 3' / 5' AGATGTGAACAGCACGAGCC 3' vglut2a: 5' AGGGAGCCTGCTGGTTTTAG 3' / 5' CTGCAGGTCCTAGCAGCTTAG 3' glyt2: 5' TTGTGACGTGTACGAACAGC 3' / 5' CAAGTGGGTCGATCATGTTCT 3' neurod1: 5' GCAGGATGCCTCCAACTGA 3' / 5' GTGACCGCAACGTAGAAGC 3'

The plasmid for transcribing *tag1* was a gift from the laboratory of Gavin Wright (Thisse et al., 2008), and *th1* was a gift from the laboratory of Pertti Panula (Chen et al., 2009).

Antisense digoxigenin (DIG)-labeled RNA probes were synthesized for 3 hours at 37°C in 20 μ l reactions containing the following: 2 μ l 10X transcription buffer (Roche), 2 μ l 10X DIG RNA labeling mix (Roche), 1 μ l 100 mM DTT (Roche), 1 μ l RNaseOUT recombinant ribonuclease inhibitor (Invitrogen), 2 μ l RNA polymerase (T7, SP6, or T3) (Roche), and 12 μ l of linearized purified plasmid. Following probe synthesis, 2 μ l of DNase I (amplification grade) (Invitrogen) was added to each sample for 30 minutes at 37°C. Probes were purified using the RNeasy Mini Kit (Qiagen). 24-48 hpf embryos were fixed in 4% paraformaldehyde (PFA) overnight at 4°C, washed in PBST (PBS + 0.1% Tween 20), dehydrated sequentially in methanol/PBST, and stored at -20°C at least overnight.

Immunohistochemistry

Whole mount antibody staining of dissected embryos was performed as previously described, with some modifications (Wilson et al., 1990). In brief, embryos were fixed in 4% sucrose/4% PFA at room temperature for 1 h per 24 h of development. Following fixation, embryos were washed in PBS and dissected according to the method previously described (Wilson et al., 1990), then dehydrated sequentially in methanol-PBT (PBS + 0.5% Triton X-100) and stored in methanol at -20°C at least overnight. Embryos were rehydrated sequentially, washed in PBT, digested with proteinase K (10 µg/ml for 10 minutes (28 hpf) or 20 minutes (48 hpf); 20-40 µg/ml for 72, 96, and 120 hpf, respectively for 20 minutes), and post-fixed in 4% sucrose/4% PFA for 20 minutes at room temperature. Embryos were blocked for at least 1 h at room temperature in 10% normal goat serum/1% DMSO/0.5% Triton X-100 in PBS, and incubated in primary antibodies overnight at 4°C. Embryos were washed 4-6 times for at least 30 minutes in PBT at room temperature and incubated in secondary antibodies for 2 h at room temperature or overnight at 4°C. Embryos were washed 4-6 times for at least 30 minutes at room temperature or overnight at 4°C in PBT and mounted for imaging in low melt agarose (0.8-1%). Tubes containing embryos were rotated at each step. Immunostaining to visualize forebrain commissure formation (Figure S1K-K') was performed in undissected embryos according to the method previously described (Barresi et al., 2005). The following primary antibodies were used: acetylated-Tubulin (mouse monoclonal IgG2b, #T7451, Sigma-Aldrich, RRID:AB 609894, 1:500 or 1:800); SV2 (mouse monoclonal IgG1, DSHB, 1:500); GABA (rabbit polyclonal, #A2052, Sigma-Aldrich,

RRID:AB_477652, 1:500); GFP (rat monoclonal IgG2a, #04404-84, Nacalai Tesque, Inc., RRID:AB_10013361, 1:1000); RFP (rabbit polyclonal, #600-401-379, Rockland, RRID:AB_2209751, 1:1000); GFAP (rabbit polyclonal, #Z0334, DAKO, RRID:AB_10013382, 1:400); Phospho-Histone H3 (Ser10) (rabbit polyclonal, #9701, Cell Signaling, RRID:AB_331535, 1:200). The following Alexa Flour secondary antibodies were used (Life Technologies, 1:200): 488 or 633 goat anti-mouse IgG2b; 568 goat anti-mouse IgG1; 488 or 546 goat anti-rabbit IgG (H+L); 488 goat anti-rat IgG (H+L).

TUNEL

TUNEL labeling to detect apoptotic cells was performed using the ApopTag kit (Chemicon International) with modifications to the standard protocol. 28 hpf embryos (undissected) were fixed in 4% PFA overnight at 4°C, washed in PBST (PBS + 0.1% Tween 20), sequentially dehydrated in methanol-PBST, and stored in methanol at -20°C at least overnight. Embryos were sequentially rehydrated and washed in PBST, then digested with proteinase K (10 μ g/ml) for 18 minutes at room temperature and re-fixed in 4% PFA for 20 minutes at room temperature. Next, embryos were washed in PBST and incubated for 10 minutes in 2:1 ethanol:acetone at -20°C, washed again and incubated in equilibration buffer for 1 hour at room temperature. Embryos were rotated during all room temperature incubations. The following ratio of ApopTag enzyme and buffer was used: 12 μ l TdT enzyme, 24 μ l reaction buffer, and 1 μ l of 10% Triton X-100. 18 μ l of the reaction mix was added per tube containing 5-10 embryos overnight at 37°C. After the labeling reaction, embryos were washed in PBST, blocked, and developed as in the *in situ* hybridization protocol (Xu et al., 1994).

Imaging and data processing

Whole-mount immunostained embryos were imaged by confocal microscopy (Leica and Zeiss Systems, Yale Center for Cellular and Molecular Imaging; Leica Systems, UCL Research Department of Cell and Developmental Biology). Maximum intensity projections are shown for all confocal images, which were processed using Fiji (Schindelin et al., 2012), Imaris (Bitplane), or Volocity (Improvision). Dissected 24 hpf embryos were mounted laterally and imaged at 25X (0.8W) (Z-distance \approx 74 µm). Dissected 48-120 hpf embryos were mounted at various orientations and imaged at 25X (0.95W) using Z-stacks ranging from ~150-250 µm. Embryos stained by fluorescent *in situ* hybridization were analyzed at 20X (0.8W) or 25X (0.95W). Figures were assembled using Illustrator (CC, Adobe).

To quantify GABA+ neurons at 28 hpf, dissected embryos were mounted laterally and imaged at 25X (0.8W). Z-stacks (80-110 μ m, average=93 μ m) started at the first appearance of the telencephalic neuropil and ended immediately past the midline at the point where GABA+ cells could no longer be visualized. Three blinded raters performed GABA cell counts on Z-stack images using the Cell Counter plug-in for Fiji (http://rsb.info.nih.gov/ij/plugins/cell-counter.html). This plug-in allows raters to manually mark individual cells in a z-stack and counts the cells as they marked. To quantify dlx5a/6a:GFP+ neurons in the forebrains of Tg(dlx6a-1.4kbdlx5a/dlx6a:GFP) larvae at 96 hpf, with or without early exposure to biochanin A (0.1 μ M), dissected embryos were mounted laterally or ventrally and imaged at 25X (0.95W). Z-stacks (WT: lateral=300-340 μ m, average=323 μ m; ventral=250-290 μ m, average=273 μ m; *cntnap2ab*: lateral=260-330 μ m, average=302 μ m; ventral=240-300 μ m, average=267 μ m) were obtained of the whole brain, beginning and ending with the first and last appearance of the axon scaffold, respectively. dlx5a/6a:GFP+ neurons were counted in the forebrains of wild-type and mutant larvae in lateral and ventral images manually and blindly by a single rater using the Cell Counter plug-in for Fiji.

To measure head size, lateral images of live 30 hpf embryos or 96 hpf larvae anesthetized with MS-222 were obtained using a dissecting microscope. A single blinded rater traced the perimeter of the head using the otic vesicle as a posterior boundary, eye, and yolk using Fiji (Figures S1N-O and S1N'-O'). To measure brain size, maximum intensity projections of lateral and ventral confocal Z-stacks (WT: lateral=300-340µm, average=318µm; ventral=250-280µm, average=271µm; *cntnap2ab*: lateral=270-330µm, average=297µm; ventral=240-300µm, average=265 µm) of 96 hpf dissected whole brains stained with anti-acetylated tubulin were imaged at 25X (0.95W). Using Fiji, a single blinded rater traced the perimeter of the brain with the midbrain-hindbrain boundary as

the posterior boundary (Figures S1P-Q and S1P'-Q').

To analyze forebrain commissure formation, embryos were cleared in 75% glycerol, mounted between two cover slips, and imaged at 40X (1.2W) in Z-stacks of ~14 μ m. Three blinded raters assessed the degree of forebrain commissure formation in maximum intensity projection images based on a previously defined scale (Barresi et al., 2005). Whole mount *in situ* images were analyzed using Zeiss Axioimager M1 and Discovery microscopes and photographed with a Zeiss Axiocam digital camera. Images were processed with Zeiss AxioVision 3.0.6.

Imaris 7.7.2 software (Bitplane) was used to quantify dlx5a/6a:GFP+ neurons and vglut:DsRed and acetylated tubulin surface area by brain region in Tg(dlx6a-1.4kbdlx5a/dlx6a:GFP::vglut:DsRed) larvae at 4 dpf. First, a surface was generated to demarcate each region. To quantify markers in lateral images, the surface was traced from the lateral edge to the mid-line of the brain, which demarcated the region where the staining was strongest. The following mounting orientations were used to quantify the respective regions: forebrain and hypothalamus: ventral and lateral; optic tectum and cerebellum: dorsal and lateral. dlx5a/6a:GFP+ cells were counted using an estimated XY diameter = 6 µm and filtered by quality with the following cutoffs: forebrain and hypothalamus, 23; cerebellum, 15; optic tectum, 10. vglut:DsRed and acetylated tubulin surface area were measured using a surface area detail level = 1.000 µm and the smooth function with the following absolute intensity cutoffs for glutamate (vglut) and tubulin (AcTub): forebrain: vglut, 40 and AcTub, 25; hypothalamus: vglut, 40 (ventral images)/30 (lateral images) and AcTub, 25; cerebellum: vglut, 30 (dorsal images)/25 (lateral images) and AcTub, 25; optic tectum, vglut, 25 and AcTub, 15. Cell number and surface area values were normalized to the wild-type average for each orientation. Images were analyzed blindly by a single rater. Statistical analyses were conducted using StatPlus (AnalystSoft).

RT-PCR

Total RNA was extracted from 25 wild-type, $cntnap2a^{A121/A121}cntnap2b^{31i/31i}$ or $cntnap2a^{A121/A25}cntnap2b^{31i/A7}$ larvae at 5 dpf using TRIzol reagent (Life Technologies) and purified using the RNeasy Mini Kit (Qiagen). First strand cDNA synthesis was performed according to the manufacturer's recommendations using SuperScript II or III First-Strand Synthesis System (Invitrogen). Limited-cycle PCR to amplify exons 1 to 6 of *cntnap2a* and *cntnap2b*, which is the region containing ZFN-induced indel mutations, was performed using the following primer pairs:

cntnap2a, 808 bp: 5' ATTTTAAGCCACATCAGCATCTCTC 3' /

5' TTCTCCTGTAGCGCTCAATTACTAC 3'

cntnap2b, 718 bp: 5' TATGGTGTGTGTTTATGTGAAGCTCAG 3' /

5' TGAATGGAGCCGTACTGATTACT 3'

Genomic DNA lengths for these primer pairs are: cntnap2a: 356,671 bp; cntnap2b: 40,857 bp. Sangersequencing of cntnap2a and cntnap2b RT-PCR products from wild-type cDNA produced clear sequences with no evidence of exon-skipping involving exons 1-6. Sanger sequencing of RT-PCR products from cDNA from $cntnap2a^{4121/425}cntnap2b^{31i/47}$ transheterozygotes indicated that both mutant alleles were being transcribed. To resolve the sequences of these transcripts and to assess for alternatively spliced transcripts in this region in $cntnap2a^{4121/4121}cntnap2b^{31i/31i}$ and $cntnap2a^{4121/425}cntnap2b^{31i/47}$ larvae, RT-PCR products from these mutants were cloned using either TOPO-TA or Zero Blunt PCR TOPO Cloning Kit (Invitrogen) and plasmid DNA was isolated (QIAprep Miniprep kit, Qiagen) from ~10 colonies per PCR product and Sanger-sequenced (Keck Biotechnology Resource Laboratory, Yale University).

Generation of antibodies against zebrafish Cntnap2a and Cntnap2b

Custom antibody recognizing zebrafish Cntnap2a and Cntnap2b (YZ3970) was generated by YenZym Antibodies, LLC, using a peptide antigen corresponding to amino acids 867 to 886 of Cntnap2a: TPLNDDQWHRVSAERNTKEAC-amide. This region is 90% identical between Cntnap2a and Cntnap2b and occurs within the third Laminin-G domain of both proteins. To verify that this antibody recognized both Cntnap2 proteins, HA-tagged full-length Cntnap2a and Cntnap2b were cloned into pCS2+ and transfected into HEK cells using lipofectamine 2000 reagent (Invitrogen). Total protein was isolated from transfected HEK cells and analyzed

by SDS-PAGE and western blotting. YZ3970 was found to recognize both overexpressed proteins by western blot (data not shown).

Western blot

To analyze Cntnap2a and Cntnap2b expression, total protein was isolated from the heads of 72 hpf larvae with the following genotypes: wild-type, *cntnap2ab* double homozygotes, single homozygotes, and double heterozygotes from both mutant lines $(cntnap2a^{A121/A121}cntnap2b^{31i/31i})$ and $cntnap2a^{A25/A25}cntnap2b^{47/A7})$. 10 ml of lysis buffer was prepared fresh containing 1 ml of 10X Cell Lysis Buffer (#9803, Cell Signaling), 100 µl of 0.1 M PMSF, 1 complete protease inhibitor cocktail tablet (Roche), and 9 ml distilled water. Protein was isolated using 150 µl of lysis buffer per 62 decapitated 72 hpf heads. Samples were homogenized on ice by mechanical grinding, then rotated at 4°C for 20 minutes and immediately stored at -80°C at least overnight. Upon thawing, samples were centrifuged at 13,000 rpm for 3 minutes to pellet insoluble material. 20 µl of the supernatant from each sample was diluted 1:1 in 2X loading buffer (prepared from 4X NuPAGE LDS Sample Buffer, Invitrogen) with 20% βmercaptoethanol, and boiled for 2 minutes at 95°C. 30-34 µl of each sample was loaded per lane and analyzed by SDS-PAGE using 7.5% mini-PROTEAN TGX precast gels (Bio-Rad). The polyacrylamide gel was transferred to a PVDF membrane using a wet transfer system (Bio-Rad). Primary antibodies used for western blotting included: Affinity-purified custom antibody directed against Cntnap2A and Cntnap2B (YenZym, YZ3970, 1:500); β-actin (mouse monoclonal, #A1978, Sigma-Aldrich, RRID:AB 476692, 1:5000). After probing with primary antibody, blots were stripped with Restore Western Blot Stripping Buffer (Thermo Scientific) and reprobed with antibody to β-actin. Secondary antibodies included peroxidase-conjugated AffiniPure donkey anti-rabbit IgG (H+L) and peroxidase-conjugated AffiniPure donkey anti-mouse IgG (H+L) (Jackson ImmuoResearch Laboratories, Inc., 1:10:000).

Locomotor activity assay

The behavioral assay was conducted as described previously (Prober et al., 2006; Rihel et al., 2010). In brief, individual 4 dpf larvae were placed in a 96-well plate (7701-1651; Whatman, Clifton, NJ) containing 650 μ l of standard embryo water (0.3 g/L Instant Ocean, 1 mg/L methylene blue, pH 7.0). The 96-well plate was maintained at a constant temperature (28.5°C) and exposed to a 14h:10h white light:dark schedule with constant infrared illumination within a custom-modified Zebrabox (Viewpoint, LifeSciences) (Figure 2A). Larval activity was monitored for 60-72 hours using an automated video tracking system (Zebrabox and Videotrack software; Viewpoint Life Sciences, Montreal, Quebec, Canada). Larval movement was recorded using Videotrack quantization mode. The Videotrack detection parameters were empirically defined for clean detection of larval movement with minimal noise. To control for differences in genetic background, all tracking experiments were performed blindly on the progeny of double heterozygous *cntnap2a*^{+/-}2b^{+/-} fish. After tracking, genomic DNA was isolated from individual larvae and genotyping was done by PCR and high-resolution fragment analysis (DNA Analysis Facility at Yale University).

Pentylenetetrazol activity assay

For the dose-response experiments, individual $cntnap2a^{A121/A121}cntnap2b^{31i/31i}$ or wild-type larvae at 4 or 6 dpf were pipetted into the wells of a 96-well plate containing 650 µl of standard embryo water. As above, the plate was placed in a custom-modified Zebrabox. Larval movement was recorded by the Videotrack system for 1 h before and after the addition of PTZ or water. Videotrack detection parameters were determined as above. PTZ was diluted in sterile distilled water and either PTZ or water alone was pipetted directly into each well to yield the following PTZ final concentrations: 2.5, 5, 7.5, 10, and 15 mM. At 6 dpf, the total number of larvae tested per concentration of PTZ was: 2.5, 5, 7.5, 10 mM, n=42-48; 15 mM, n=24; water only, n=68. At 4 dpf: 2.5, 7.5, 15 mM, n=10-12; 5, 10 mM, water only, n=34. Data were analyzed by normalizing the post-PTZ activity of each fish to itself prior to the addition of PTZ or water. For blinded experiments, the progeny of incrosses of $cntnap2a^{A25/+}cntnap2b^{A7/+}$ fish at 4 dpf (n=268) were pipetted individually into the wells of a 96-well plate. Sterile water or PTZ (final concentration=10 mM) was pipetted directly into the wells of one-half of the same 96-well plate. 10 mM PTZ was

selected because this dose was found to illicit the largest differential response in wild-type versus mutant fish at 6 dpf (Figure S3C). Genomic DNA isolation and genotyping were performed after each experiment.

Chemical screens

Selection of psychoactive agents was based on the following criteria (Figure 3A): i) behavioral phenotype of wild-type fish plus drug strongly correlates with the phenotype of $cntnap2a^{A25/A25}cntnap2b^{A7/A7}$ mutants (L-701,324 and (-)-MK-801); ii) behavioral phenotype of wild-type fish plus drug strongly anti-correlates with the mutant phenotype (9 drugs); iii) GABAergic mechanism (zolpidem, diazepam); iv) clinical use in treating aggression or irritability in ASD (risperidone). Three doses of each drug were selected based on previous studies of the dose range that induces behavioral effects and minimizes toxicity in larval zebrafish (Rihel et al., 2010). All agents were dissolved in DMSO with the exception of baclofen, which was dissolved in water. DMSO alone at <0.2% has been shown not to affect behavior (Rihel et al., 2010). Each drug concentration was tested on 10-12 $cntnap2a^{A121/A121}cntnap2b^{31i/31i}$ or wild-type larvae, with 10-12 control larvae per 96-well plate. Stock solutions of each compound dissolved in DMSO or DMSO alone were pipetted directly into the wells of a 96-well plate containing 650 µL of standard embryo water.

Final concentrations of each drug tested in wild-type and $cntnap2a^{A121/A121}cntnap2b^{31i/31i}$ larvae are shown in Table S4. Amantadine was identified as anti-correlating by using a more extensive dataset of 6,000 compounds, which was previously filtered to yield the list of 550 compounds (Rihel et al., 2010). For blinded experiments, the progeny of incrosses of $cntnap2a^{A25/+}cntnap2b^{A7/+}$ fish (n=560, 2 independent experiments) were pipetted individually into the wells of a 96-well plate. DMSO or biochanin A (final concentration=0.1-1µM) was pipetted directly into the wells of one-half of the same 96-well plate. Genomic DNA was isolated and fish were genotyped after each experiment.

Hierarchical clustering

Behavioral phenotypes of wild-type fish exposed to a panel of 550 psychoactive agents from 4 to 7 dpf were ascertained as previously described (Rihel et al., 2010). To compare the behavioral fingerprints of wild-type larvae exposed to each drug and the $cntnap2a^{A25/A25}cntnap2b^{A7/A7}$ mutant behavioral fingerprint, hierarchical clustering analysis was performed as in (Rihel et al., 2010).

Correlation analysis

Correlation analysis was performed in Matlab (R2014a; The Mathworks, Inc.) as previously described (Rihel et al., 2010).

Rank correlation analysis

To conduct rank correlation analysis of NMDA antagonists and estrogenic analogs, compounds were assigned to pharmacological classes as described (Rihel et al., 2010). Estrogenic agents were additionally annotated by hand blind to rank using PubChem (pubchem.ncbi.nlm.nih.gov/). Assays of bioactivity in a set of *in vitro* estrogen receptor- α tests deposited at PubChem were included. Insecticides were the only class of compounds that were excluded due to their strong effects on sodium channels. Rank correlation analysis was performed as described using the Kolmogorov-Smirnov statistic to assess significance (Rihel et al., 2010). Rank-sorting of anti-correlating drugs revealed that estrogenic compounds are significantly enriched in this dataset. Specifically, 25 compounds in our dataset of 550 were identified as estrogenic. Four of the top 10 anti-correlating agents are estrogenic. We created 10,000 randomly permuted datasets and found similar or better enrichment in only three permuted datasets, giving a p-value estimate of 3/10,000 or 0.0003. Using both strict and relaxed criteria for defining estrogenic agents demonstrated enrichment of these agents in the top ranks.

Principal components analysis

Data were aggregated for 18 rest-wake parameters (sleep, rest bouts, average total activity, average waking activity; each of these parameters was determined for nights 5 and 6 and days 5 and 6; and night and day average

waking activity) and each drug-dose combination. For the differential response PCA, each data point was defined as the Z-score of the response of wild-type or $cntnap2a^{A121/A121}cntnap2b^{31i/31i}$ mutant larvae to each drug compared to their baseline (DMSO alone). For the PCA to identify drugs that reverse the behavioral fingerprint of mutants, each data point was defined as the Z-score of the response of $cntnap2a^{A121/A121}cntnap2b^{31i/31i}$ mutant larvae exposed to each drug compared to the behavioral fingerprint of untreated wild-type larvae. Hierarchical clustering was conducted in Matlab (R2014a), with the statistics and bioinformatics toolboxes. PCA was performed according the method described in (Woods et al., 2014).

Long-term Exposure to Biochanin A

To assess the extent to which exposure to biochanin A affects GABAergic cell numbers, biochanin A dissolved in DMSO or DMSO alone was added to petri dishes containing Tg(dlx6a-1.4kbdlx5a/dlx6a:GFP) wild-type or *cntnap2d*^{d121/d121} *cntnap2b*^{31i/31i} embryos at 30 hpf (n=6-11/dish) and embryos were raised in the presence of either biochanin A (final concentration=0.1 µM) or DMSO. At 4 dpf, larvae were fixed, dissected, immunostained and imaged by confocal microscopy, as described above. dlx5a/6a:GFP+ cells in the forebrain were counted manually as described.

To determine the extent to which chronic exposure to biochanin A affects nighttime hyperactivity, wildtype or $cntnap2a^{A121/A121}cntnap2b^{31i/31i}$ embryos were exposed to biochanin A (1 µM) dissolved in DMSO or DMSO alone at 24 hpf (n=50/dish). Biochanin A (1 µM) or DMSO was added daily. At 4 dpf, each larva was pipetted into fresh embryo water, allowed to swim for 30 seconds, and placed in a 96-well plate with fresh embryo water. The locomotor activity of wild-type or mutant embryos was tracked for 48 hours using the automated tracking assay described above.

To test the effect of biochanin A exposure on PTZ-induced seizures, wild-type or $cntnap2a^{A121/A121}cntnap2b^{31i/31i}$ embryos were exposed to biochanin A (1 μ M) dissolved in DMSO or DMSO alone at 24 hpf (n=50/dish) and biochanin A (1 μ M) or DMSO was added daily. At 4 dpf, larvae were plated in a 96-well plate containing either biochanin A or DMSO. Larvae were allowed to habituate for 15 minutes and baseline locomotor activity was tracked for two hours, after which 10 mM PTZ was added to each well and locomotor activity was tracked for two hours using the automated assay described above.

qPCR

To assess the effect of biochanin A exposure on estrogen target genes, groups of ~35 wild-type larvae were exposed to biochanin A (1 μ M, 10 μ M) or DMSO overnight from 4 to 5 dpf. Biochanin A (1 μ M, 10 μ M) and DMSO exposure were conducted in duplicate samples. Total RNA was isolated using TRIzol reagent (Life Technologies) and purified using the RNeasy Mini Kit (Qiagen). cDNA was synthesized using 1000 ng of total RNA and the SuperScript III First-Strand Synthesis System (Invitrogen). Expression of the following three genes was analyzed by qPCR: *vitellogenin 1 (vtg1)*; *estrogen receptor 1 (esr1)*; *cytochrome P450, family 19, subfamily A, polypeptide 1 (aromatase B, cyp19a1b)*. These genes were selected because they were found to be upregulated in response to 17 β -estradiol in zebrafish embryos (Hao et al., 2013). We selected *cyp19a1b* because it is expressed in the brain during early development (Hao et al., 2013). *cyp19a1b* primer sequences were obtained from (Hao et al., 2013). Primer sequences for *vtg1* and *esr1* are shown below:

vtg1, 106 bp: 5' GAGATGCAAGAGGCTGGAG 3' /

5' GGCTCAGATCTTTAGACTTTGTCAC 3'

esr1, 122 bp: 5' TTACGGAGTCTGGTCGTGTG 3' /

5' AGCTCTTTCGACGGTTTCTG 3'

Other larval behavioral assays

Acoustic startle, habituation, and PPI were analyzed in $cntnap2a^{A121/A121}cntnap2b^{31i/31i}$ (n \approx 70), $cntnap2a^{A121/+}cntnap2b^{31/+}$ (n \approx 70), and wild-type (n \approx 50) larvae at 5 to 7 dpf by raters blind to genotype, according to the method previously described (Wolman et al., 2011). After the initial analysis indicated that there may be a difference in the LLC response rate of mutants, the progeny of incrosses of $cntnap2a^{A121/+}cntnap2b^{31i/+}$ fish were

blindly tested for LLC responsiveness and genotyped after the experiment (total n=318; $cntnap2a^{A121/A121}cntnap2b^{31i/31i}$, n=14; $cntnap2a^{A121/+}cntnap2b^{31i/4}$, n=92; wild-type, n=24). The optokinetic response assay was performed on $cntnap2a^{A121/A121}cntnap2b^{31i/31i}$ (n=18), $cntnap2a^{A121/+}cntnap2b^{31/4}$ (n=11), and wild-type (n=14) larvae at 5 to 7 dpf with different contrast levels of the visual stimulus (5-100%). Two independent trials were conducted by raters blind to genotype, according to the previously described method (Schoonheim et al., 2010).

Supplemental References

Barresi, M.J., Hutson, L.D., Chien, C.B., and Karlstrom, R.O. (2005). Hedgehog regulated Slit expression determines commissure and glial cell position in the zebrafish forebrain. Development *132*, 3643-3656.

Burgess, H.A., and Granato, M. (2007). Sensorimotor gating in larval zebrafish. The Journal of neuroscience 27, 4984-4994.

Chen, Y.C., Priyadarshini, M., and Panula, P. (2009). Complementary developmental expression of the two tyrosine hydroxylase transcripts in zebrafish. Histochemistry and cell biology *132*, 375-381.

Cifuentes, D., Xue, H., Taylor, D.W., Patnode, H., Mishima, Y., Cheloufi, S., Ma, E., Mane, S., Hannon, G.J., Lawson, N.D., *et al.* (2010). A novel miRNA processing pathway independent of Dicer requires Argonaute2 catalytic activity. Science *328*, 1694-1698.

Doyon, Y., McCammon, J.M., Miller, J.C., Faraji, F., Ngo, C., Katibah, G.E., Amora, R., Hocking, T.D., Zhang, L., Rebar, E.J., *et al.* (2008). Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. Nature biotechnology *26*, 702-708.

Foley, J.E., Maeder, M.L., Pearlberg, J., Joung, J.K., Peterson, R.T., and Yeh, J.R. (2009). Targeted mutagenesis in zebrafish using customized zinc-finger nucleases. Nature protocols *4*, 1855-1867.

Hao, R., Bondesson, M., Singh, A.V., Riu, A., McCollum, C.W., Knudsen, T.B., Gorelick, D.A., and Gustafsson, J.A. (2013). Identification of estrogen target genes during zebrafish embryonic development through transcriptomic analysis. PloS one *8*, e79020.

Julich, D., Hwee Lim, C., Round, J., Nicolaije, C., Schroeder, J., Davies, A., Geisler, R., Lewis, J., Jiang, Y.J., Holley, S.A., *et al.* (2005). beamter/deltaC and the role of Notch ligands in the zebrafish somite segmentation, hindbrain neurogenesis and hypochord differentiation. Developmental biology *286*, 391-404.

Miller, J.C., Holmes, M.C., Wang, J., Guschin, D.Y., Lee, Y.L., Rupniewski, I., Beausejour, C.M., Waite, A.J., Wang, N.S., Kim, K.A., *et al.* (2007). An improved zinc-finger nuclease architecture for highly specific genome editing. Nature biotechnology *25*, 778-785.

Sander, J.D., Dahlborg, E.J., Goodwin, M.J., Cade, L., Zhang, F., Cifuentes, D., Curtin, S.J., Blackburn, J.S., Thibodeau-Beganny, S., Qi, Y., *et al.* (2011). Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA). Nature methods *8*, 67-69.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., *et al.* (2012). Fiji: an open-source platform for biological-image analysis. Nature methods *9*, 676-682.

Schoonheim, P.J., Arrenberg, A.B., Del Bene, F., and Baier, H. (2010). Optogenetic localization and genetic perturbation of saccade-generating neurons in zebrafish. The Journal of neuroscience *30*, 7111-7120.

Thisse, B., Wright, G.J., and Thisse, C. (2008). Embryonic and Larval Expression Patterns from a Large Scale Screening for Novel Low Affinity Extracellular Protein Interactions.

Thisse, C., Thisse, B., Schilling, T.F., and Postlethwait, J.H. (1993). Structure of the zebrafish snail1 gene and its expression in wild-type, spadetail and no tail mutant embryos. Development *119*, 1203-1215.

Wilson, S.W., Ross, L.S., Parrett, T., and Easter, S.S., Jr. (1990). The development of a simple scaffold of axon tracts in the brain of the embryonic zebrafish, Brachydanio rerio. Development *108*, 121-145.

Wolman, M.A., Jain, R.A., Liss, L., and Granato, M. (2011). Chemical modulation of memory formation in larval zebrafish. Proceedings of the National Academy of Sciences of the United States of America *108*, 15468-15473.

Woods, I.G., Schoppik, D., Shi, V.J., Zimmerman, S., Coleman, H.A., Greenwood, J., Soucy, E.R., and Schier, A.F. (2014). Neuropeptidergic signaling partitions arousal behaviors in zebrafish. The Journal of neuroscience *34*, 3142-3160.

Xu, Q., Holder, N., Patient, R., and Wilson, S.W. (1994). Spatially regulated expression of three receptor tyrosine kinase genes during gastrulation in the zebrafish. Development *120*, 287-299.