Sigma-1 receptor ligands control a switch between passive and active threat responses

AUTHORS

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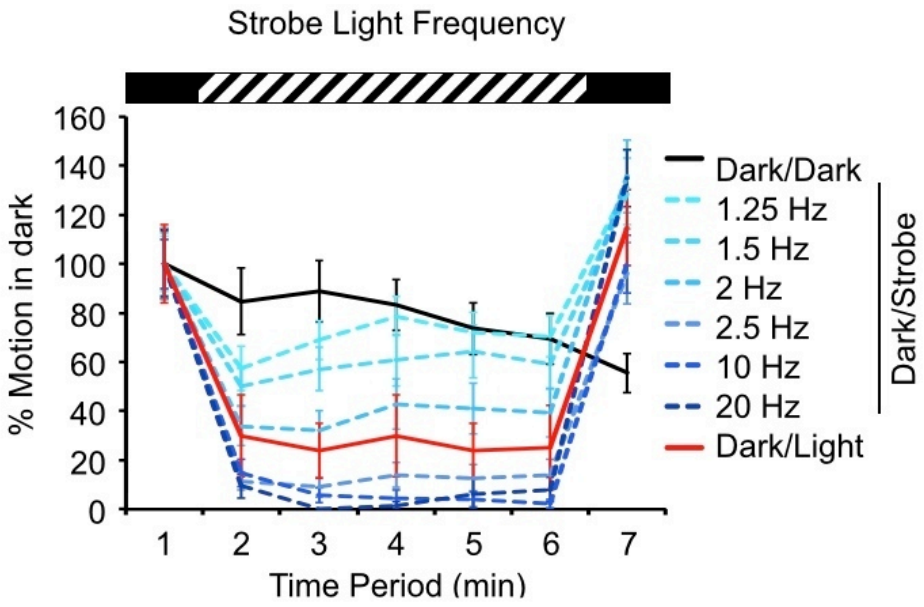
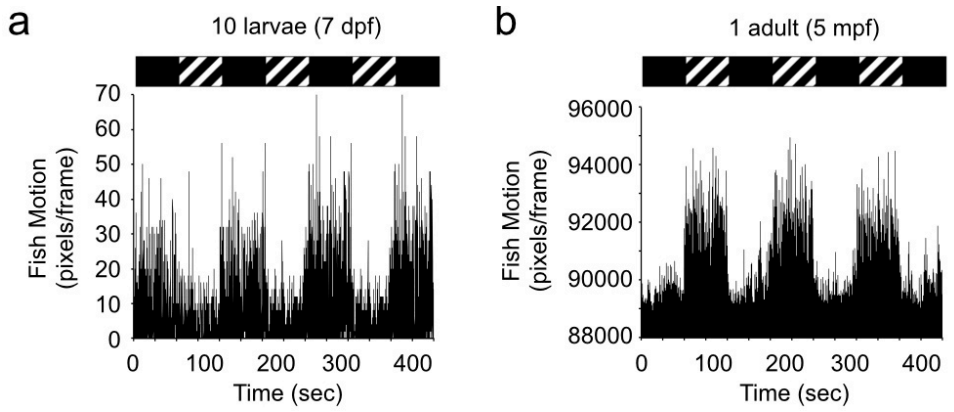
SUPPLEMENTARY RESULTS

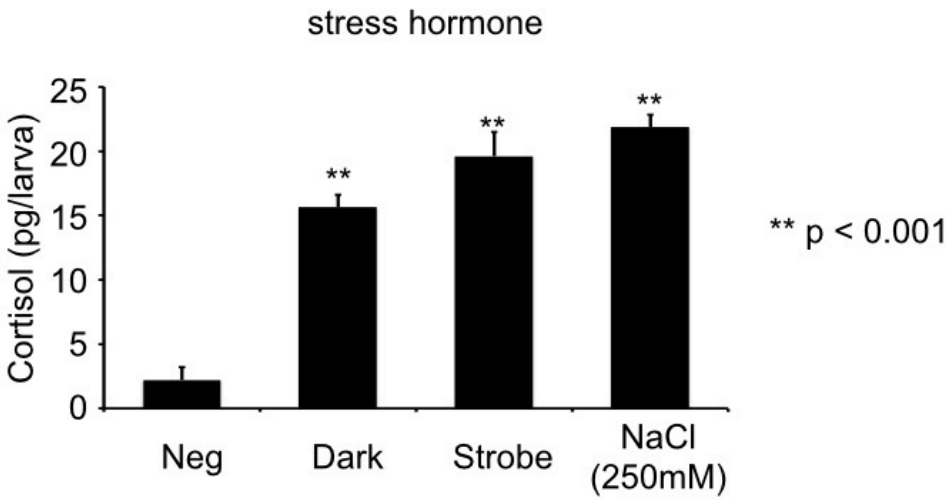
SUPPLEMENTARY FIGURES



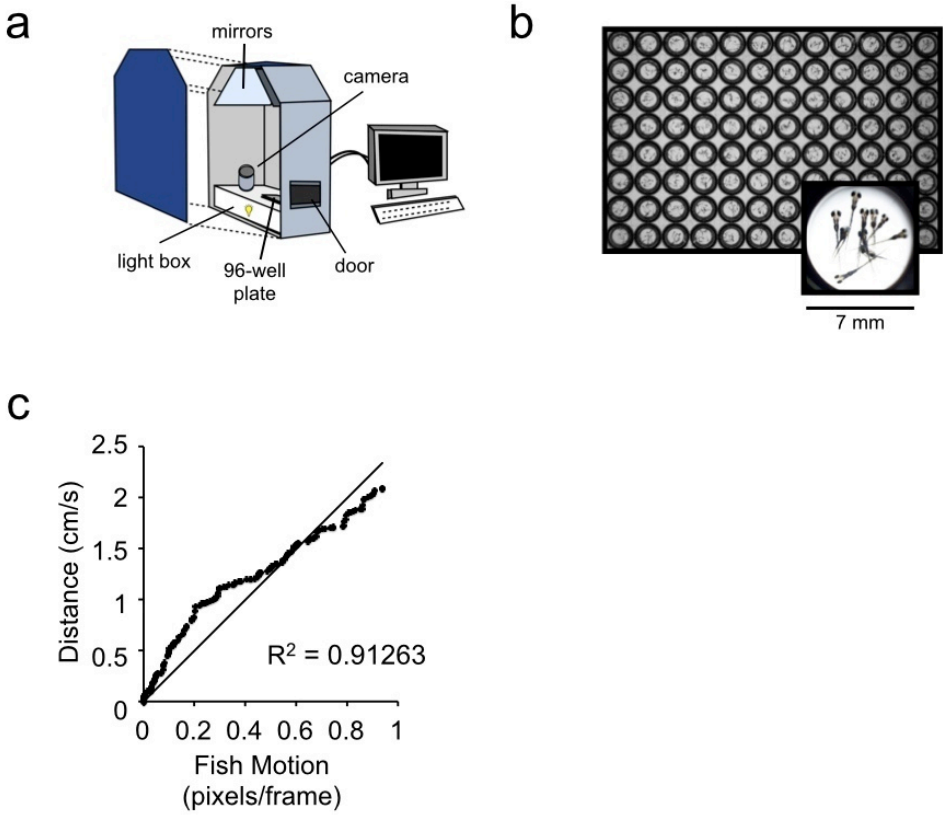
Supplementary Figure 1: Strobe-induced freezing is frequency-dependent. We tested a range of strobe frequencies and found that 10 Hz elicited nearly complete freezing behavior, whereas steady light consistently reduced motion but not to the level of strobe light. 7 dpf zebrafish in 96-well plates were exposed to 1 min of darkness followed by 5 min of additional dark (black line), strobe light (dashed blue lines) or steady light (red line) then returned to dark for the final (7^{th}) minute. Motor activity (in pixels per frame) over 1 min periods was collected (n = 96 for each condition) and graphed as % of initial motion in minute 1. Data represent mean ± s.d.



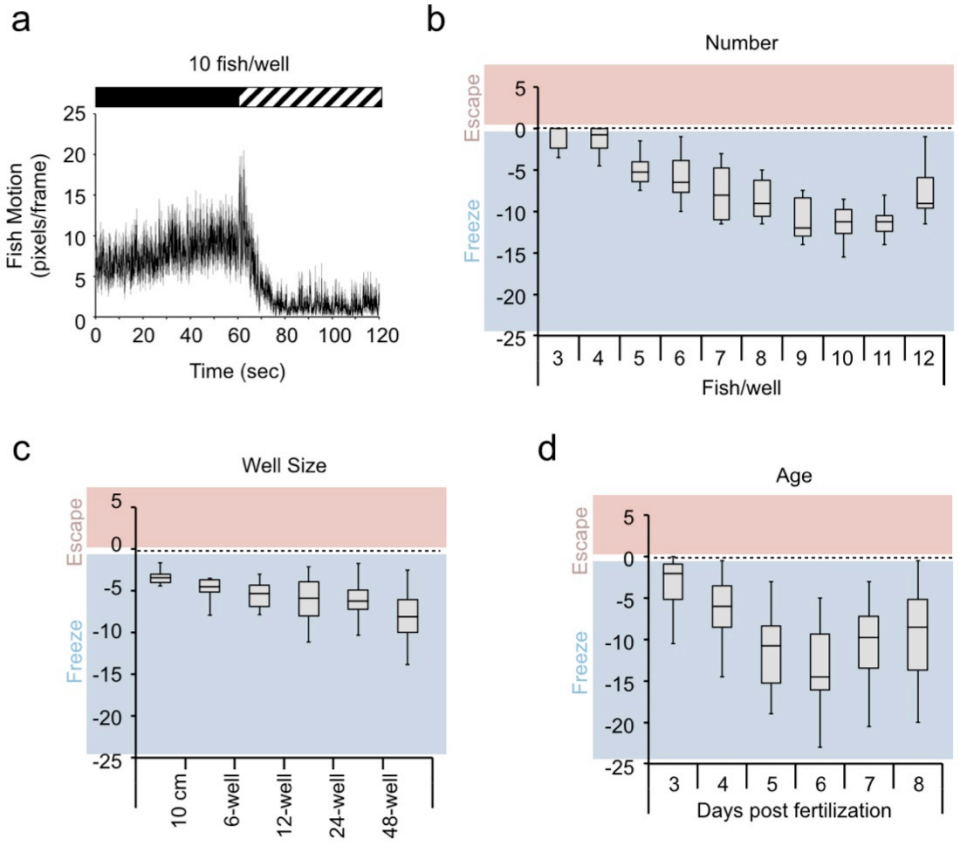
Supplementary Figure 2: Adult zebrafish exhibit the opposite behavior of larvae; they show hyperactive escape behavior in response to strobe light. Representative plot of the aggregate motor activity (in pixels per frame) over time from ten 7 dpf larvae in a single well of a 96-well plate (a) or one 5 month old adult (b) during the 7 minute Strobe Light Response (SLR) assay. Boxes above mark 1 min intervals when the fish are in either darkness (black boxes) or strobe light (hashed boxes).



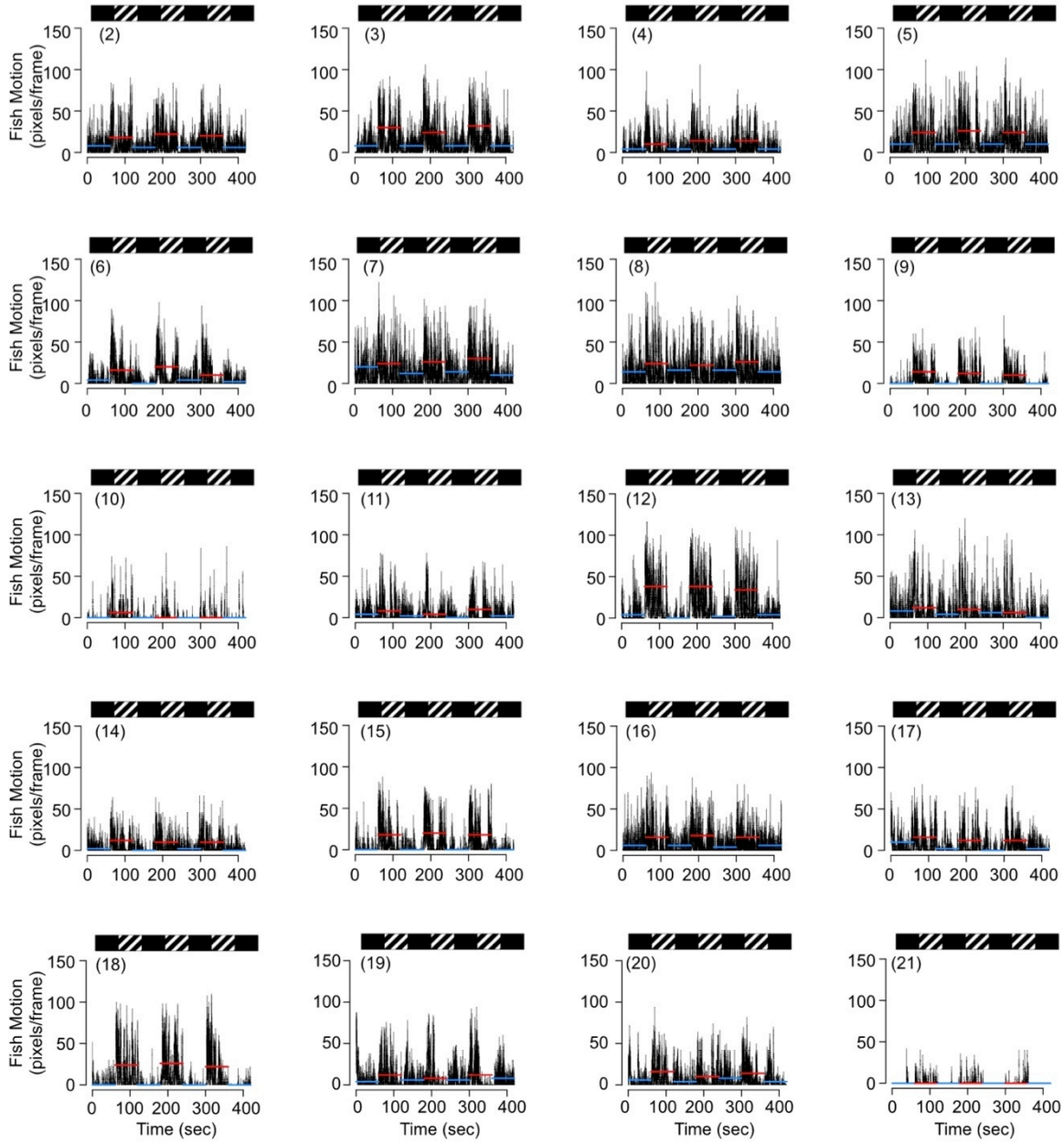
Supplementary Figure 3: Cortisol levels are increased in larvae in response to strobe light. Whole-body cortisol was quantified using a luminescence immunoassay (see Online Methods) in 7 dpf larvae exposed to ambient light (negative control), darkness (positive control), strobe light (10 Hz) or normal E3 medium supplemented with 250 mM NaCl in ambient light (positive control), as indicated. Each bar represents three biological replicates of n = 60 larvae per replicate, normalized by number of larvae. Statistical significance was calculated using a student's *t*-test (2-tailed, unpaired, unequal variance). Data represent mean ± s.d.



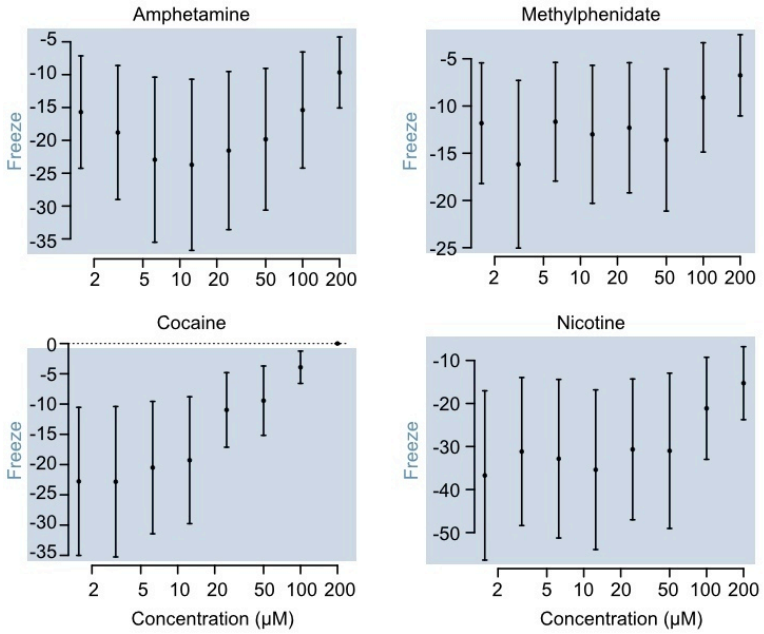
Supplementary Figure 4: Strobe Light Response (SLR) assay set up. (a) Diagram of the ZebraBox platform used to record fish motion over time for the freezing response assay. (b) Example of a standard (127.76 x 85.48 mm) 96 well assay plate containing 10 larvae per well. Diameter of a single well (insert) is 7 mm. (c) Total fish motion was quantified as pixels per video frame recorded at 30 frames per second. ZebraLab software tracks the change in pixel intensity for each well over time producing the 'Fish Motion' (pixels/frame) index, which correlates well with the overall distance traveled by each animal. Fish plated at 1 larvae per well in in two 96-plates (n = 192) were analyzed for 1 min in the dark using the ZebraLab quantification program to obtain Fish Motion (pixel/frame) values. The same fish were then analyzed using the ZebraLab tracking program to obtain distance moved (cm/s). Best-fit linear regression line was plotted and the coefficient of determination (R^2) was calculated using Microsoft Excel.



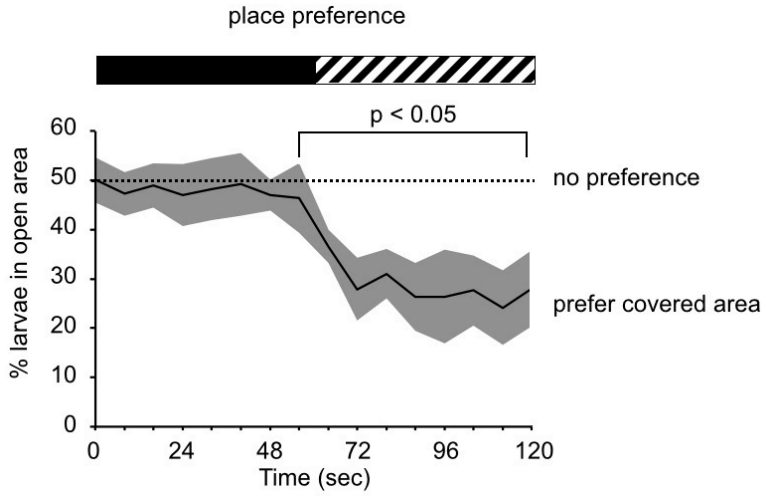
Supplementary Figure 5: Optimization of the Zebrafish Strobe Light Response (SLR) Assay. (a) Aggregate motor activity (in pixels per frame) over time from ten 7 dpf larvae per well of a 96-well plate during a two-minute experiment, n = 48 wells. Boxes above mark 1 min intervals when the fish are in darkness (black box) or strobe light (hashed box). Data are shown as mean (black line) ± s.d. (gray regions) and are representative of 3 independent experiments. (b) Boxplots of the freezing index (see Online Methods) from 7 dpf fish during the 7 min 10 Hz SLR assay, plated in a 96-well at 3–12 fish/well, as indicated. (c) Boxplots from 7 dpf fish during the 7 min, 10 Hz SLR assay, plated 10 fish/well in variously sized wells, as indicated (d) Boxplots from 3–8 dpf fish during the 7 min 10 Hz SLR assay, plated 10 fish/well in 96-well plate format. All boxes represent the interquartile range of the freezing index, whiskers represent the maximum and minimum values observed, n = 12 wells.



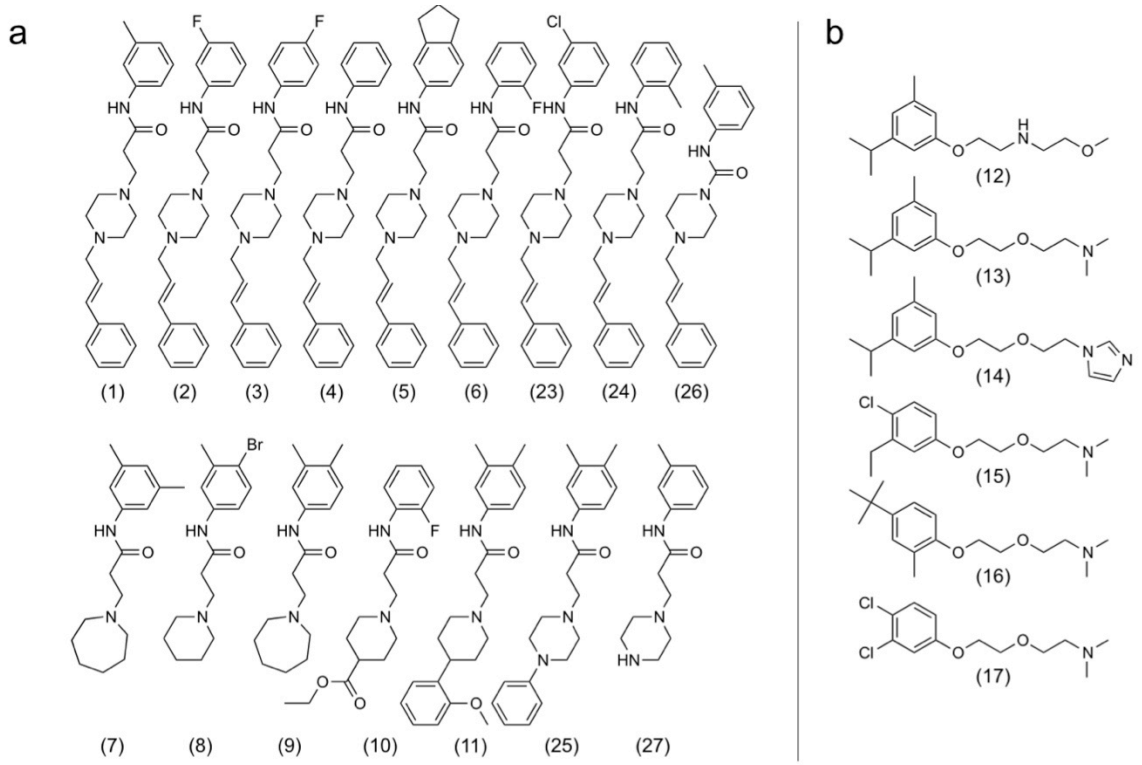
Supplementary Figure 6: Hit compounds that disrupt zebrafish freezing behavior. Fish motion during the strobe assay in wells treated with 3.33 μg/ml (~15 μM) of each compound, as indicated. n = 10 larvae. Data are representative of single well experiments from the initial Strobe Light Response (SLR) chemical screen. Note: not every compound causes hypolocomotion in the dark minutes relative to DMSO (see also Fig 1b for DMSO plot, and Supplementary Table 2 for average fish motion values), e.g. compound 7.



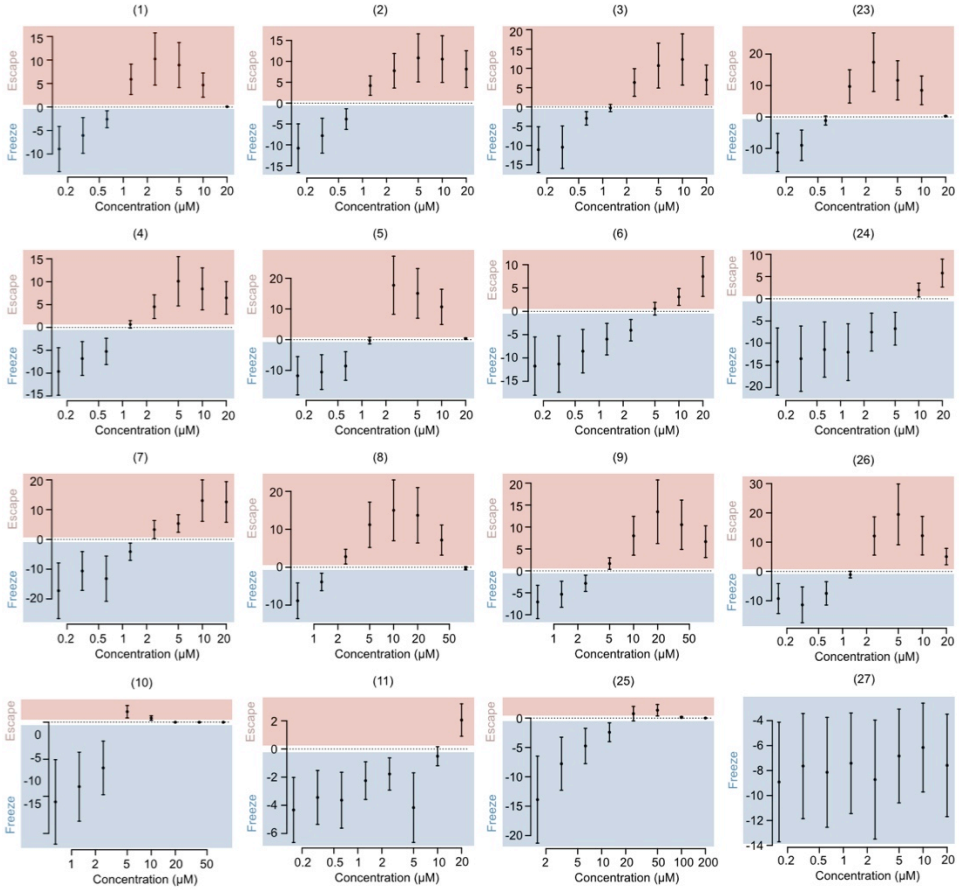
Supplementary Figure 7: Psychostimulants are not disruptors of strobe-induced freezing. General psychostimulants do not cause strobe-induced hyperlocomotion in the SLR assay. Freezing is unaffected in 7 dpf zebrafish larvae treated with various known psychostimulants. By using our 'freezing index' score, which is a measure of the difference between the effects of a compound on general motion in the dark versus motion during strobe light, we were able to exclude any compounds that cause general hyperactivity/hyperlocomotion from our list of hit compounds. Data represent freezing index mean ± s.d. (n = 12 wells per dose).



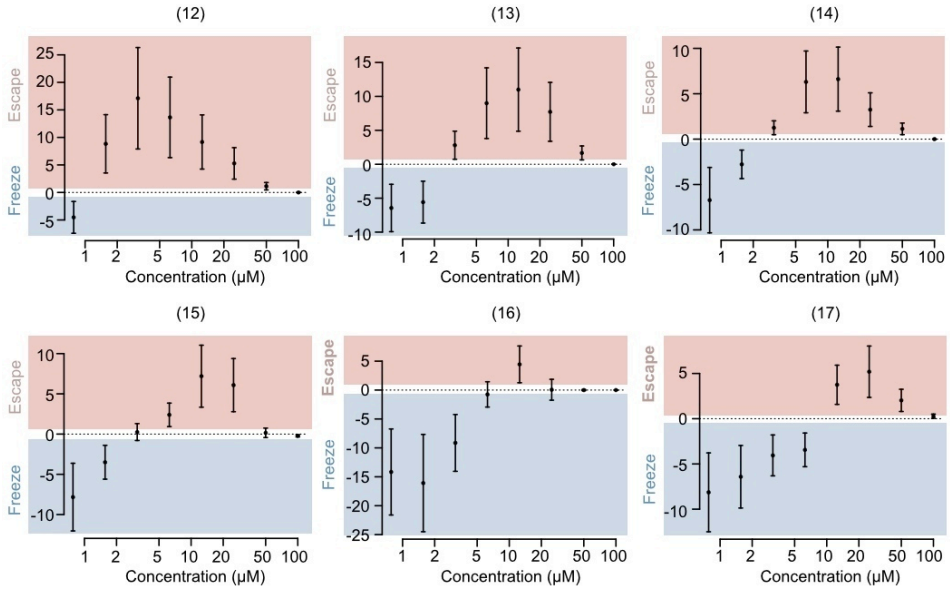
Supplementary Figure 8: Zebrafish larvae exhibit chemically-induced escape behavior when subjected to strobe light. 7 dpf fish were plated in to 10 cm plates at 100 fish/plate and treated for 1 h with compound **1** (5 μM). Half of each plate was covered with opaque black tape. Fish in each plate were placed in darkness and permitted to acclimate for 10 min. After 10 min, 2 min recordings were started. For the first min the fish remained in darkness. For the second min fish in the uncovered half of the plate were exposed strobe light (10 Hz). Numbers of fish in the uncovered region were counted at 8 second intervals and plotted as a function of time. Graph shows mean data from 6 biological replicates (6 different plates of 100 fish each). Data in represents mean (black line) ± s.d. (gray regions). Statistical significance between the final fish counts of each minute (time = 56 and 120 sec) was calculated using a student's *t*-test (2-tailed, unpaired, unequal variance).



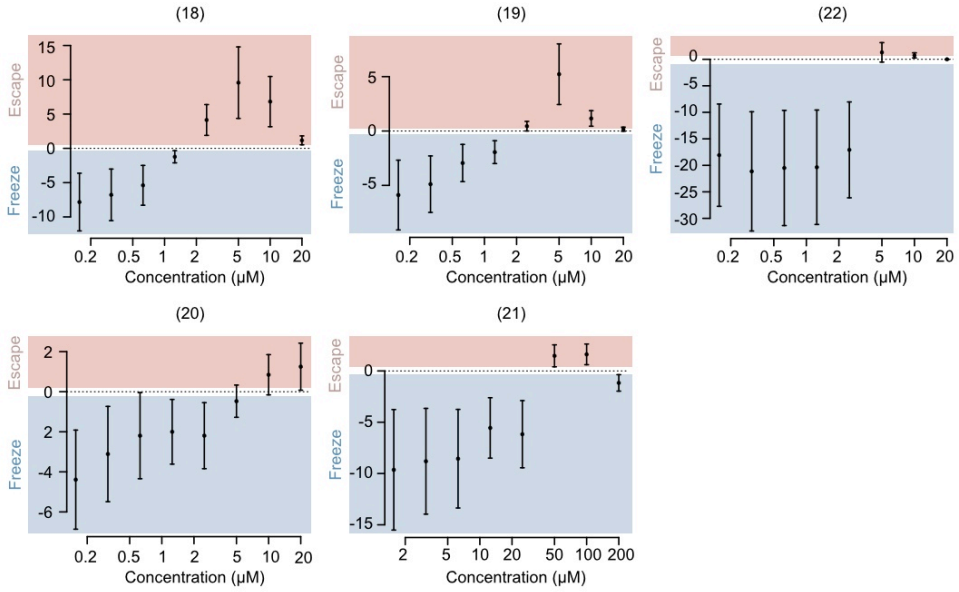
Supplementary Figure 9: Additional chemical structures. (a) Finazine and (b) Finoxetine classes of compounds.



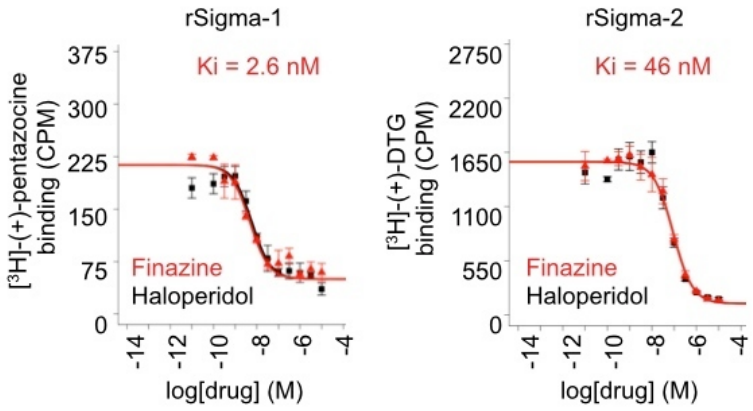
Supplementary Figure 10: Finazine compounds disrupt strobe-induced freezing. Dose curves showing the degree of behavioral switching, measured by the freezing index. Each point represents 12 replicate wells. Data in represents mean ± s.d.



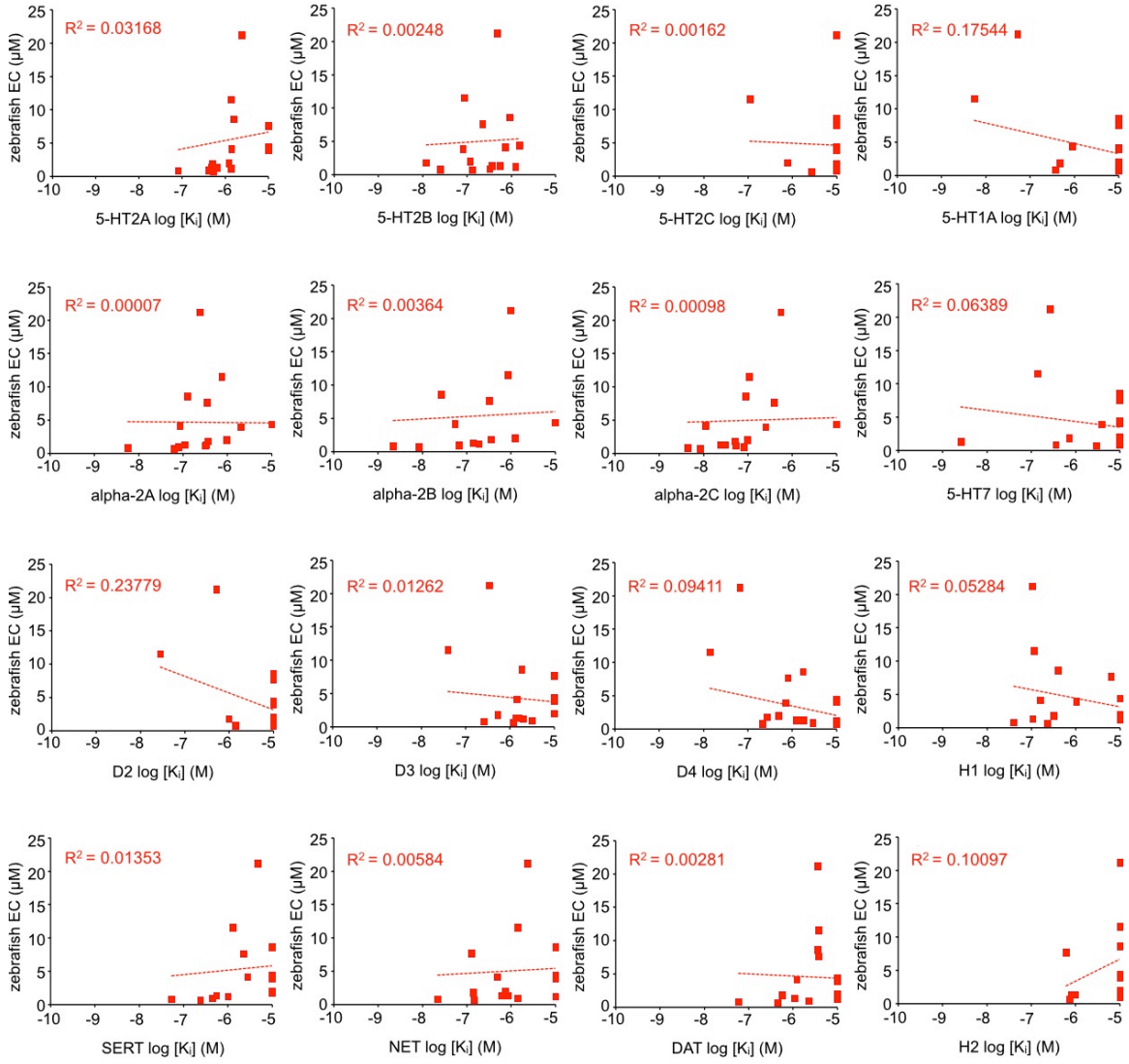
Supplementary Figure 11: Finoxetine compounds disrupt strobe-induced freezing. Dose curves showing the degree of behavioral switching, measured by the freezing index. Each point represents 12 replicate wells. Data in represents mean ± s.d.



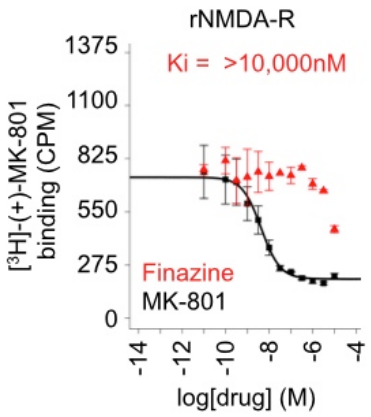
Supplementary Figure 12: Finopidil compounds disrupt strobe-induced freezing. Dose curves showing the degree of behavioral switching, measured by the freezing index. Representative plots for finopidil compounds **18-21** are shown in comparison to the known compound naftopidil **(22)**. Naftopidil, which was not a hit in the screen, was inactive at concentrations less than 20 μM but active when tested at higher concentrations. Each point represents 12 replicate wells. Data in represents mean ± s.d.



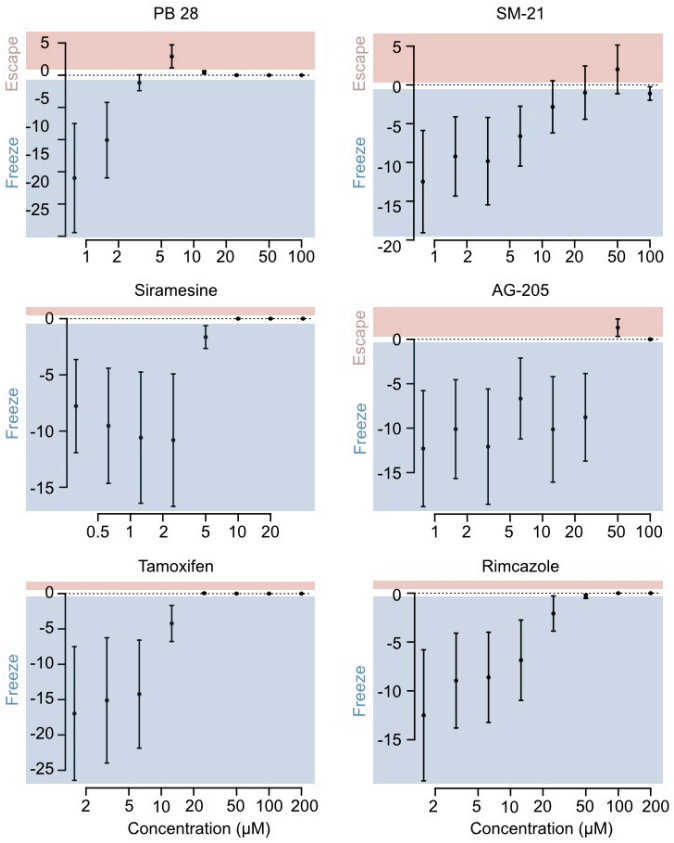
Supplementary Figure 13: Finazine is a high-affinity Sigma-1 ligand. *In vitro* mammalian target binding receptor competition assays using rodent sigma-1 or -2, as indicated. Radioligand binding was measured after co-incubation with an unlabeled competitor, either haloperidol (positive control, black) or finazine (red). Data in represents mean ± s.d.



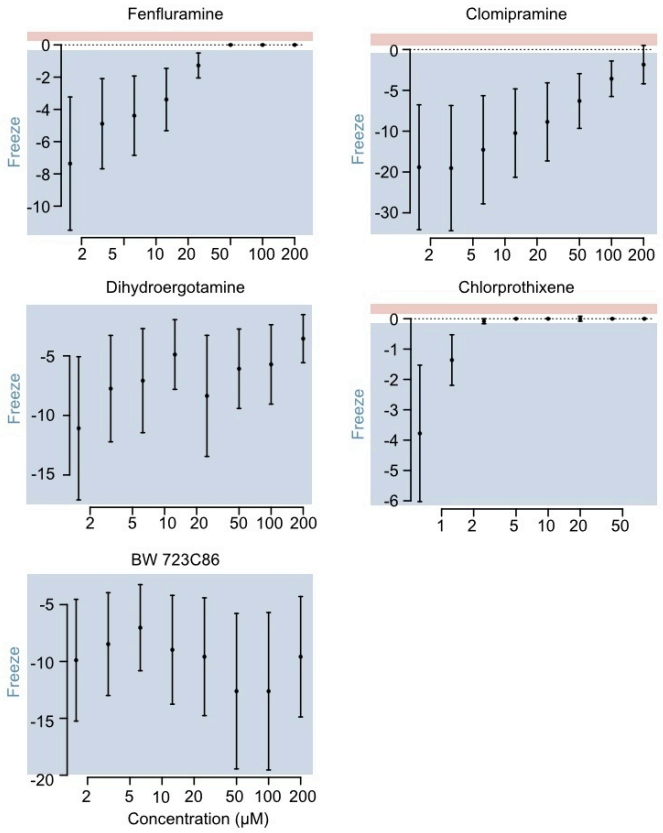
Supplementary Figure 14: No correlation between *in vivo* behavioral activity and *in vitro* binding activity at any of these targets. Each data point represents a different finazine analogue. EC values from the zebrafish strobe light response assay are plotted along with the K_i values obtained from radiologand displacement assays using mammalian proteins. Any K_i value determined to be greater than 10,000 nM (the highest concentration tested) was set at 10,000 nM, for simplicity. This conservative adjustment would make any positively correlated R^2 value estimated here better than reality.



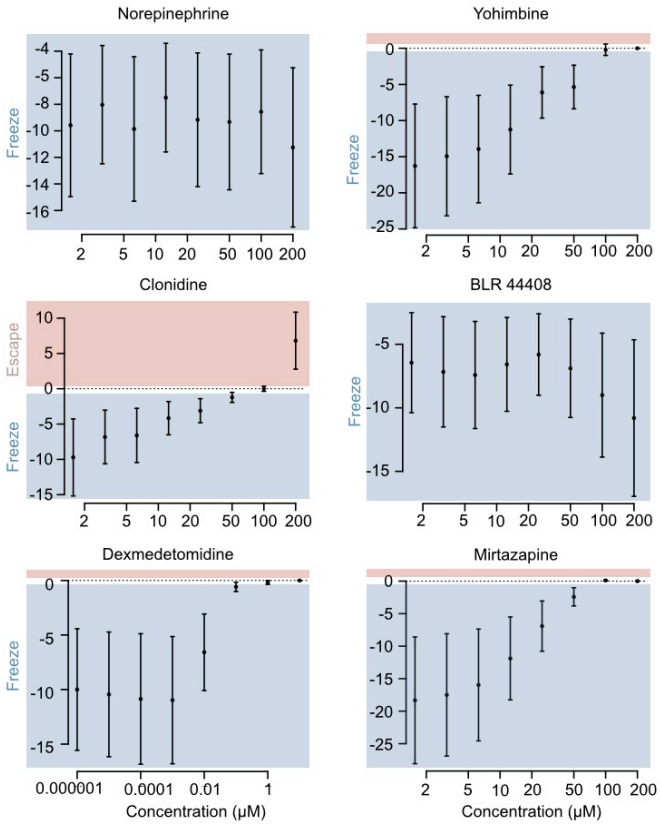
Supplementary Figure 15: Finazine does not bind to the NMDA receptor. *In vitro* mammalian target binding receptor competition assays using cloned NMDA receptor. Radioligand [^{3}H]-(+)-MK-801 binding was measured after co-incubation with an unlabeled competitor, either (+)-MK-801 (positive control, black) or finazine (red).



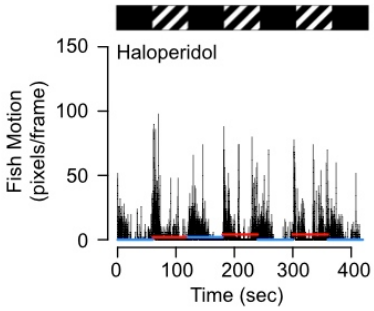
Supplementary Figure 16: Sigma-2 ligands are not potent disruptors of strobe-induced freezing. Dose curves showing the degree of behavioral switching, measured by the freezing index. Each point represents 12 replicate wells. Data in represents mean ± s.d.



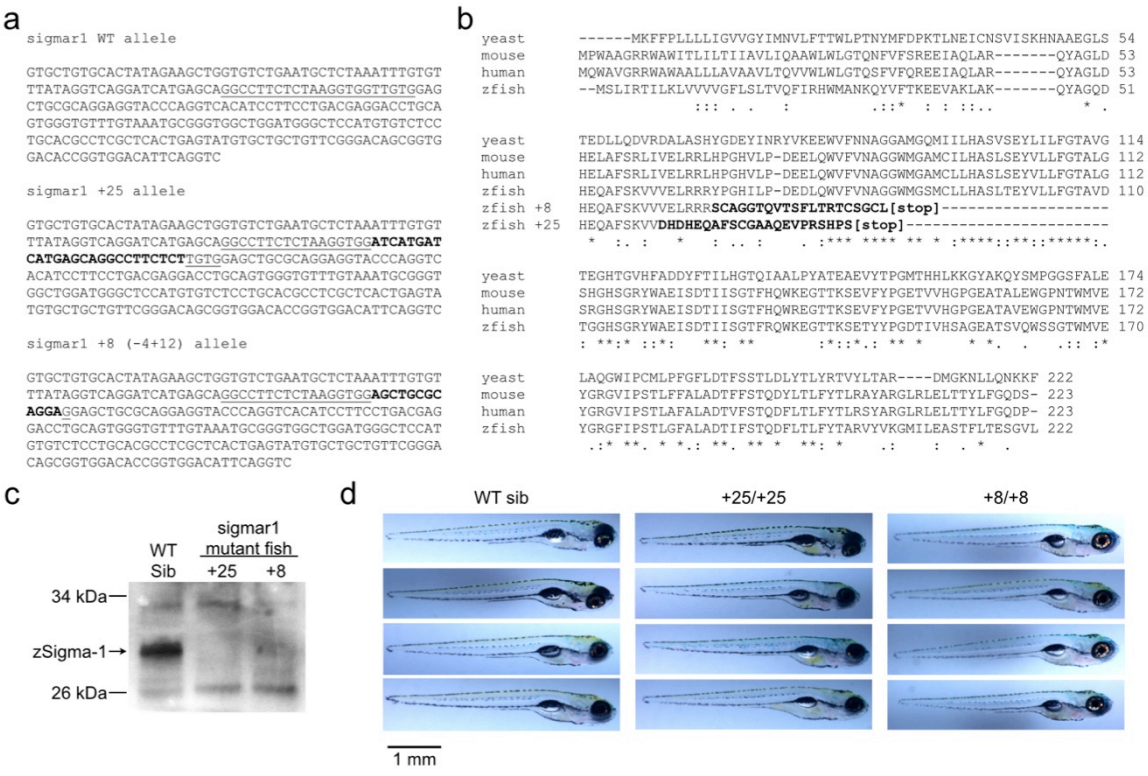
Supplementary Figure 17: Serotonin (5-HT) 2B receptor ligands are not disruptors of strobe-induced freezing. Dose curves showing the degree of behavioral switching, measured by the freezing index. Each point represents 12 replicate wells. Data in represents mean ± s.d.



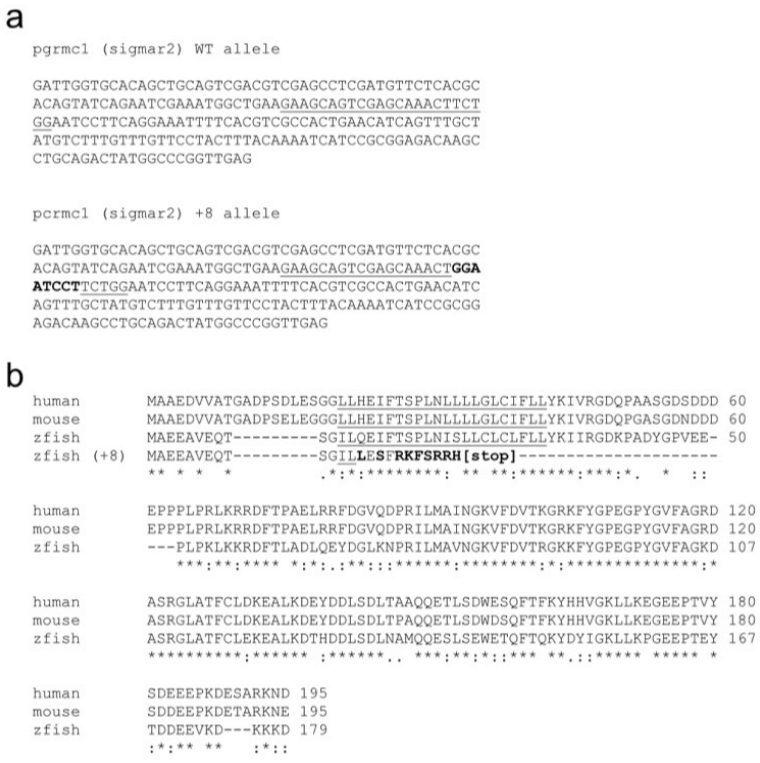
Supplementary Figure 18: Adrenergic alpha-2 receptor ligands are not potent disruptors of strobe-induced freezing. Dose curves showing the degree of behavioral switching, measured by the freezing index. Each point represents 12 replicate wells. Data in represents mean ± s.d. Dexmedetomidine was tested at a lower dose range due to its toxicity at higher doses in larvae.



Supplementary Figure 19: Haloperidol disrupts strobe light induced freezing behavior. Representative plot of the motor activity from all larvae in a single well during the SLR assay. Horizontal lines represent 1 min averages for motion in darkness (blue) and in strobe light (red). Fish were treated with 40 μM haloperidol. Data are representative of 36 wells from 3 independent experiments.



Supplementary Figure 20: Characterization of two zebrafish CRIPR-cas9 induced *sigmar1* frameshift mutant lines. (a) DNA sequencing results from wild-type and mutant zebrafish lines showing exon 2 of the zebrafish *sigmar1* gene. Underlined nucleotides correspond to the variable region of the engineered CRIPR gRNA. Bolded nucleotides represent novel (inserted) genomic material. (b) Predicted protein sequence based on the genetic results. Asterisks (*) denotes amino acids that are highly conserved from yeast to human. Bolded amino acids represent novel (erroneous) residues predicted to occur after frameshift, followed by premature stop codons. (c) Western blot of whole lysates from wild-type or homozygous *sigmar1* mutant 7 dpf zebrafish, probed with anit-Sigma-1 rabbit polyclonal antibody N1C3 (GeneTex). (d) Images taken of wild-type or homozygous *sigmar1* mutant 7 dpf zebrafish. Scale bar is 1 mm.



Supplementary Figure 21: Characterization of zebrafish CRIPR-cas9 induced *pgrmc1 (sigmar2)* frameshift mutant line. (a) DNA sequencing results from wild-type and mutant zebrafish lines showing exon 1 of the zebrafish *pgrmc1 (sigmar2)* gene. Underlined nucleotides correspond to the variable region of the engineered CRIPR gRNA. Bolded nucleotides represent novel (inserted) genomic material. (b) Predicted protein sequence based on the genetic results. Asterisks (*) denotes amino acids that are highly conserved from mouse to human. Bolded amino acids represent novel (erroneous) residues predicted to occurr after frameshift, followed by premature stop codons.

SUPPLEMENTARY TABLES

Category	Parameter	Description
Assay	Type of assay	Whole organism, zebrafish
	Target	Phenotypic, behavioral screen
	Primary measurement	Animal motion
	Key reagents	7 dpf zebrafish larvae (EkkWill strain), in HEPES (10 mM) buffered E3 medium
	Assay protocol	See 'Online Methods' section
	Additional comments	Screen is designed to identify compounds that switch the normal larval strobe light response (SLR) from hypoactivity ('freezing') to hyperactivity ('escape')
Library	Library size	10,000 compounds
	Library composition	'DIVERSetE' library of synthetic, drug-like molecules
	Source	ChemBridge Corporation (San Diego, CA)
	Additional comments	Stored at –80 °C in DMSO
Screen	Format	96-well plates (Fisher Scientific, 12-565-500)
	Concentration(s) tested	3.33 μg/ml (~15 μM), 1% DMSO
	Plate controls	1% DMSO alone negative controls, 16 well per plate, > 2,000 total wells
	Reagent/ compound dispensing system	Manual
	Detection instrument and software	ZebraBox with infrared camera and ZebraLab software (Viewpoint Life Sciences)
	Assay validation/QC	Mean of DMSO controls: –9.22 (Freezing Index) Standard deviation of DMSO controls: 4.86 False positive rate: < 0.05%
	Correction factors	None required
	Normalization	None required
	Additional comments	Screen performed internally within the Peterson Lab
Post-HTS analysis	Hit criteria	Freezing index > 0
	Hit rate	0.98%
	Additional assay(s)	Confirmatory dose curves using the primary assay, Radioligand binding assays with candidate targets
	Confirmation of hit purity and structure	Compounds were repurchased (ChemBridge), Structure and purity were analytically spot-checked
	Additional comments	None

Supplementary Table 1: Small molecule screening data.

Minute	1 dark	2 strobe	3 dark	4 strobe	5 dark	6 strobe	7 dark
DMSO	8.7 ± 0.9	0.3 ± 0.2	11.5 ± 1.1	0.2 ± 0.2	11.8 ± 1.3	0.2 ± 0.2	12.3 ± 1.2
(1)	0.8 ± 0.4	5.0 ± 1.0	1.5 ± 0.4	5.8 ± 1.0	1.0 ± 0.4	6.8 ± 0.8	1.2 ± 0.3
(2)	3.8 ± 0.9	14.7 ± 1.7	3.5 ± 0.9	15.0 ± 1.7	3.8 ± 0.8	15.0 ± 1.6	4.8 ± 0.9
(3)	2.8 ± 0.8	16.7 ± 2.1	2.5 ± 0.9	14.0 ± 1.9	3.3 ± 0.8	16.2 ± 2.1	2.8 ± 0.7
(4)	2.0 ± 0.5	10.0 ± 1.7	3.0 ± 0.5	12.3 ± 1.7	2.5 ± 0.6	11.2 ± 1.6	2.8 ± 0.6
(5)	5.0 ± 0.9	15.8 ± 2.1	4.8 ± 0.9	16.2 ± 2.1	5.8 ± 1.0	16.8 ± 2.1	5.7 ± 0.9
(6)	2.7 ± 1.3	7.8 ± 2.0	2.5 ± 1.1	7.2 ± 1.9	2.0 ± 0.8	8.3 ± 2.1	2.3 ± 0.9
(7)	10.8 ± 2.4	23.8 ± 2.0	8.5 ± 2.3	22.3 ± 2.2	8.3 ± 2.0	21.8 ± 2.6	8.3 ± 1.9
(8)	2.7 ± 0.6	17.3 ± 2.1	2.3 ± 0.6	17.5 ± 1.7	1.8 ± 0.7	16.5 ± 1.6	1.7 ± 0.6
(9)	1.3 ± 0.4	11.2 ± 1.3	0.8 ± 0.6	8.2 ± 1.4	0.7 ± 0.7	7.2 ± 1.2	0.7 ± 0.4
(10)	0.0 ± 0.0	0.7 ± 0.5	0.0 ± 0.0	0.5 ± 0.3	0.0 ± 0.0	0.5 ± 0.4	0.0 ± 0.0
(11)	3.2 ± 0.9	2.5 ± 1.2	2.2 ± 0.6	1.2 ± 0.5	1.7 ± 0.5	1.5 ± 0.9	2.3 ± 0.8
(12)	7.3 ± 0.8	17.5 ± 1.7	6.7 ± 0.7	15.5 ± 1.3	6.8 ± 0.8	15.2 ± 1.5	7.0 ± 0.7
(13)	3.5 ± 1.0	16.2 ± 1.3	1.3 ± 0.3	11.2 ± 1.3	1.3 ± 0.4	10.5 ± 0.4	0.8 ± 0.3
(14)	1.8 ± 0.9	7.8 ± 1.6	0.8 ± 0.5	7.8 ± 1.2	1.3 ± 0.6	7.7 ± 1.2	0.8 ± 0.4
(15)	4.5 ± 1.8	9.7 ± 2.1	2.3 ± 1.2	10.0 ± 1.9	1.7 ± 1.2	9.2 ± 1.4	2.0 ± 1.0
(16)	5.8 ± 0.7	13.8 ± 1.3	9.3 ± 1.1	13.8 ± 1.2	11.0 ± 1.0	15.0 ± 1.4	12.2 ± 0.8
(17)	2.3 ± 1.0	6.7 ± 1.9	0.5 ± 0.3	3.8 ± 1.5	1.2 ± 0.6	4.3 ± 1.3	1.5 ± 0.7
(18)	4.5 ± 0.8	10.8 ± 1.4	4.7 ± 0.8	11.3 ± 1.6	4.2 ± 0.9	12.7 ± 1.9	4.8 ± 1.1
(19)	0.2 ± 0.2	1.8 ± 0.5	0.0 ± 0.0	1.2 ± 0.4	0.2 ± 0.2	0.5 ± 0.3	0.0 ± 0.0
(20)	4.0 ± 0.8	4.2 ± 1.5	2.0 ± 0.7	3.7 ± 1.2	3.7 ± 1.2	3.0 ± 1.2	2.0 ± 0.8
(21)	9.8 ± 1.4	3.8 ± 1.4	10.0 ± 2.0	4.3 ± 1.4	10.0 ± 2.0	4.3 ± 1.8	8.5 ± 1.6

Supplementary Table 2: Actual fish motion values. The motion values (in pixels/frame) used to calculate the freezing index for fish treated with the compounds shown (10 μM) or with DMSO alone (vehicle control). Data represents mean of the third quartile ± s.e.m. (n = 12 wells).

Finazines	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(23)	(24)	(25)	(26)	(27)
Fish EC (μM)	0.8	0.9	1.3	1.2	1.3	4.1	1.8	2.0	3.9	4.4	11.5	0.7	8.6	21.2	7.6	>200
rSigma-1 Ki (nM)	2.6	3.9	9.6	14.0	56.0	16.0	1.4	0.8	1.7	114.0	234.0	4.2	46.0	241.0	9.3	1,350.0
rSigma-2 Ki (nM)	46.0	166.0	115.0	304.0	393.0	272.0	9.4	19.0	30.0	1,338.0	471.0	140.0	426.0	678.0	51.0	>10,000
5-HT1A Ki (nM)	371.0	>10,000	>10,000	>10,000	ND	>10,000	476.0	>10,000	>10,000	894.0	5.5	>10,000	>10,000	52.0	>10,000	>10,000
5-HT1B Ki (nM)	1,799.0	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	4,369.0	>10,000	>10,000	>10,000	>10,000	>10,000
5-HT1D Ki (nM)	496.0	>10,000	3,534.0	3,400.0	2,829.0	>10,000	>10,000	>10,000	>10,000	>10,000	901.0	2,148.0	>10,000	>10,000	>10,000	>10,000
5-HT1E Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
5-HT2A Ki (nM)	82.0	417.0	469.0	1,351.0	649.0	1,414.0	504.0	1,236.0	>10,000	>10,000	1,393.0	533.0	1,572.0	2,396.0	>10,000	>10,000
5-HT2B Ki (nM)	25.0	326.0	367.0	1,245.0	548.0	717.0	12.0	118.0	82.0	1,534.0	89.0	130.0	913.0	485.0	223.0	1,129.0
5-HT2C Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	786.0	>10,000	>10,000	110.0	2,664.0	>10,000	>10,000	>10,000	>10,000
5-HT3 Ki (nM)	1,193.0	>10,000	>10,000	>10,000	>10,000	730.0	256.0	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
5-HT5A Ki (nM)	1,488.0	>10,000	>10,000	>10,000	>10,000	>10,000	1,022.0	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
5-HT6 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	6,971.0	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
5-HT7 Ki (nM)	357.0	>10,000	>10,000	>10,000	2.6	>10,000	720.0	>10,000	3,916.0	>10,000	142.0	2,897.0	>10,000	268.0	>10,000	>10,000
α1A Ki (nM)	771.0	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	8.7	>10,000	>10,000	144.0	>10,000	>10,000
α1B Ki (nM)	947.0	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	403.5	1,446.0	>10,000	>10,000	>10,000	>10,000
α1D Ki (nM)	707.0	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	18.0	>10,000	>10,000	160.0	>10,000	>10,000
α2A Ki (nM)	5.7	79.0	336.0	313.0	110.0	86.0	367.0	950.0	2,014.0	>10,000	753.0	63.0	127.0	241.0	353.0	>10,000
α2B Ki (nM)	2.2	66.0	142.0	191.0	141.0	55.0	363.0	1,249.0	>10,000	>10,000	853.0	8.3	27.0	974.0	329.0	>10,000
α2C Ki (nM)	4.4	81.0	31.0	54.0	24.0	11.0	52.0	98.0	256.0	>10,000	107.0	8.5	90.0	548.0	398.0	>10,000
β1 Ki (nM)	1,254.0	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	3,389.0	>10,000	>10,000	>10,000	>10,000
β3 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
β2 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
D1 Ki (nM)	1,431.0	>10,000	>10,000	>10,000	>10,000	>10,000	10,000.0	>10,000	>10,000	>10,000	1,897.0	>10,000	>10,000	>10,000	>10,000	>10,000
D2 Ki (nM)	1,412.0	>10,000	>10,000	>10,000	>10,000	>10,000	986.0	>10,000	>10,000	>10,000	28.0	>10,000	>10,000	521.0	>10,000	>10,000
D3 Ki (nM)	261.0	3,210.0	1,364.0	1,967.0	1,585.0	1,434.0	528.0	>10,000	>10,000	>10,000	40.0	1,201.0	1,854.0	347.0	>10,000	>10,000
D4 Ki (nM)	216.0	2,963.0	1,242.0	>10,000	1,812.0	>10,000	277.0	502.0	726.0	>10,000	14.0	>10,000	1,760.0	67.0	793.0	>10,000
D5 Ki (nM)	2,828.0	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	1,238.0	>10,000	>10,000	10,000.0	>10,000	>10,000
SERT Ki (nM)	54.0	447.0	ND	1,007.0	557.0	2,794.0	>10,000	>10,000	>10,000	>10,000	1,300.0	238.0	>10,000	4,836.0	2,242.0	>10,000
NET Ki (nM)	22.0	1,419.0	856.0	>10,000	623.5	492.0	138.0	738.0	>10,000	>10,000	1,410.0	145.0	>10,000	2,365.0	128.0	>10,000
DAT Ki (nM)	59.0	2,296.0	1,097.0	>10,000	>10,000	1,232.0	568.0	>10,000	>10,000	>10,000	3,809.0	443.0	3,592.0	3,617.0	3,820.0	>10,000
H1 Ki (nM)	40.0	ND	108.0	>10,000	ND	158.0	312.0	>10,000	1,021.0	>10,000	116.0	230.0	409.0	106.0	6,285.0	>10,000
H2 Ki (nM)	ND	>10,000	990.5	>10,000	816.0	ND	ND	>10,000	>10,000	>10,000	>10,000	744.0	>10,000	>10,000	610.0	>10,000
H3 Ki (nM)	704.0	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	2,499.0	3,122.0	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
H4 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	ND	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
M1 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
M2 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	3,114.0	>10,000	>10,000	>10,000	>10,000
M3 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
M4 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
M5 Ki (nM)	1,064.0	>10,000	>10,000	>10,000	>10,000	3,706.0	2,077.0	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
mu Ki (nM)	867.0	>10,000	>10,000	>10,000	>10,000	>10,000	1,461.0	3,963.0	2,308.0	3,542.0	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
kappa Ki (nM)	422.0	>10,000	>10,000	>10,000	>10,000	>10,000	315.0	489.0	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
delta Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
rPBR Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	4,653.0	>10,000	>10,000	>10,000	778.0	>10,000	>10,000	>10,000	>10,000	>10,000
rGABAA Ki (nM)	10,000.0	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
rBZP Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000

Supplementary Table 3: Summary of *in vitro* mammalian protein binding assays. Several hit compounds from the zebrafish strobe-induced freezing response screen were tested for their ability to inhibit and to displace radiolabeled ligands bound to various neurotransmitter receptors and transporters. All protein targets tested were human except for those indicated "r" for rodent. Results from dose curves are presented as EC values (for the *in vivo* assay strobe light response assay) or K_i values (for ligand binding assays). Detailed descriptions of known radiolabeled and cold competitor ligands used can be found online at https://pdspdb.unc.edu/pdspWeb/content/PDSP%20Protocols%20II%202013-03-28.pdf.

Finoxetines	(12)	(13)	(14)	(15)	(16)	(17)
Fish EC (μM)	1.0	2.5	2.5	3.0	6.9	8.7
rSigma-1 Ki (nM)	5.6	15.0	1,574.0	0.5	0.8	1.0
rSigma-2 Ki (nM)	513.0	49.0	410.0	40.0	47.0	17.0
5-HT1A Ki (nM)	1,189.0	>10,000	>10,000	>10,000	>10,000	>10,000
5-HT1B Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
5-HT1D Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
5-HT1E Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
5-HT2A Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	1,663.0
5-HT2B Ki (nM)	356.0	70.0	969.0	68.0	86.0	90.0
5-HT2C Ki (nM)	>10,000	>10,000	>10,000	820.0	764.0	916.0
5-HT3 Ki (nM)	>10,000	>10,000	671.0	>10,000	>10,000	>10,000
5-HT5A Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
5-HT6 Ki (nM)	1,112.0	498.0	>10,000	>10,000	>10,000	>10,000
5-HT7 Ki (nM)	980.0	>10,000	>10,000	697.0	>10,000	255.0
α1A Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
α1B Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
α1D Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
α2A Ki (nM)	530.0	1,008.0	>10,000	834.0	>10,000	1,077.0
α2B Ki (nM)	>10,000	>10,000	>10,000	510.0	>10,000	1,390.0
α2C Ki (nM)	560.0	>10,000	>10,000	1,379.0	>10,000	1,560.0
β1 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
β3 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
β2 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
D1 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
D2 Ki (nM)	1,939.0	>10,000	>10,000	>10,000	>10,000	>10,000
D3 Ki (nM)	213.0	>10,000	>10,000	>10,000	>10,000	2,591.0
D4 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
D5 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
SERT Ki (nM)	>10,000	1,550.0	>10,000	1,213.0	>10,000	764.0
NET Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	3,094.0
DAT Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
H1 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
H2 Ki (nM)	>10,000	>10,000	>10,000	ND	542.0	729.0
H3 Ki (nM)	>10,000	>10,000	>10,000	1,769.0	>10,000	1,174.0
H4 Ki (nM)	>10,000	>10,000	>10,000	>10,000	ND	1,702.0
M1 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
M2 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
M3 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
M4 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
M5 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
mu Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
kappa Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
delta Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
rPBR Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
rGABAA Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
rBZP Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000

Finipodils	(18)	(19)	(20)	(21)	(22)
Fish EC (μM)	1.5	2.2	6.4	43.8	4.7
rSigma-1 Ki (nM)	67.0	171.0	34.0	620.0	150.0
rSigma-2 Ki (nM)	136.0	19.0	119.0	235.0	37.0
5-HT1A Ki (nM)	>10,000	348.0	58.0	4.7	18.0
5-HT1B Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000
5-HT1D Ki (nM)	>10,000	2,307.0	4,549.0	>10,000	986.0
5-HT1E Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000
5-HT2A Ki (nM)	>10,000	814.0	135.0	>10,000	422.0
5-HT2B Ki (nM)	416.0	412.0	>10,000	248.0	24.0
5-HT2C Ki (nM)	>10,000	113.0	490.0	>10,000	>10,000
5-HT3 Ki (nM)	>10,000	241.0	>10,000	>10,000	>10,000
5-HT5A Ki (nM)	>10,000	>10,000	>10,000	>10,000	989.0
5-HT6 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000
5-HT7 Ki (nM)	>10,000	161.0	82.0	32.0	44.0
α1A Ki (nM)	1,695.0	60.0	336.0	53.0	33.0
α1B Ki (nM)	2,196.0	707.5	501.0	>10,000	117.5
α1D Ki (nM)	>10,000	41.0	1,022.0	50.0	14.0
α2A Ki (nM)	>10,000	792.0	2,902.0	597.0	135.0
α2B Ki (nM)	>10,000	387.0	409.0	1,255.0	97.0
α2C Ki (nM)	1,073.0	868.0	467.0	426.0	90.0
β1 Ki (nM)	>10,000	957.0	>10,000	>10,000	223.0
β3 Ki (nM)	>10,000	1,014.0	>10,000	>10,000	250.0
β2 Ki (nM)	>10,000	752.0	>10,000	>10,000	250.0
D1 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000
D2 Ki (nM)	>10,000	1,359.0	>10,000	216.0	448.0
D3 Ki (nM)	3,372.0	1,157.0	425.0	145.0	184.0
D4 Ki (nM)	416.0	94.0	222.0	7.1	5.2
D5 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000
SERT Ki (nM)	>10,000	1,181.0	2,112.0	>10,000	185.0
NET Ki (nM)	>10,000	2,359.0	1,249.0	>10,000	>10,000
DAT Ki (nM)	>10,000	>10,000	1,762.5	>10,000	>10,000
H1 Ki (nM)	644.0	145.0	304.0	114.0	89.0
H2 Ki (nM)	>10,000	5,058.0	>10,000	>10,000	577.0
H3 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000
H4 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000
M1 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000
M2 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000
M3 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000
M4 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000
M5 Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000
mu Ki (nM)	>10,000	2,351.5	>10,000	>10,000	1,080.0
kappa Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000
delta Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000
rPBR Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000
rGABAA Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000
rBZP Ki (nM)	>10,000	>10,000	>10,000	>10,000	>10,000

Supplementary Table 3 (continued)

SUPPLEMENTARY VIDEOS

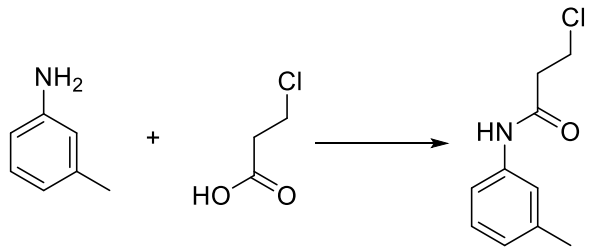
Supplementary Video 1: Movie of the strobe-induced freezing response in zebrafish larvae at low magnification. All wells are negative controls (DMSO alone). Speed of the movie has been increased by 8×.

Supplementary Video 2: Movie of the strobe-induced freezing response in zebrafish larvae treated with ten-fold increasing concentrations of finazine. Each row contains 12 replicate wells. Concentrations are as follows: row A (top), 0.156 μM; row B, 0.312 μM; row C, 0.625 μM; row D, 1.25 μM; row E, 2.5 μM; row F, 5 μM; row G, 10 μM; row H (bottom), 20 μM. Speed of the movie has been increased by 8×.

SUPPLEMENTARY NOTES

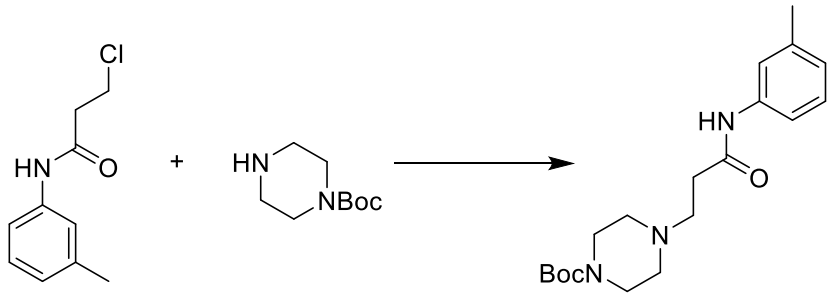
Supplementary Note 1: Synthetic protocol for Compound 27.

Scheme 1



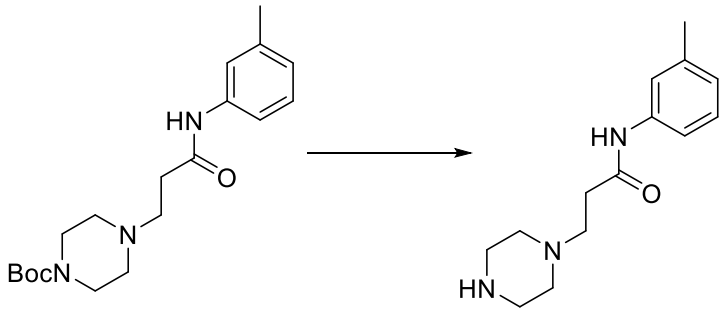
3-chloro-N-(m-tolyl)propanamide：Under a N_2 atmosphere thionyl chloride (15.0 mmol) and DMF (1drop) were added dropwise to a DCM(10ml) solution of 3-chloropropanoic acid (7.5 mmol), after stirring at r.t. for 8 h, solvent and thionyl chloride were removed in vacuo. Disolved the acid chloride in DCM(10ml), *m*-toluidine (5.0 mmol) and triethylamine (7.5 mmol) were added, after stirring at r.t. for 1 h, the reaction was diluted with EtOAc and washed sequentially with saturated aqueous NH_4Cl, saturated aqueous $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4, filtered and condensed. The crude mixture was purified using flash silica gel column chromatography to get the pure product 3-chloro-*N*-(m-tolyl)propanamide (yield 80%). ESI-MS: m/z 198 $(M+H)^+$.

Scheme 2



tert-butyl 4-(3-oxo-3-(m-tolylamino)propyl)piperazine-1-carboxylate：3-chloro-*N*-(m-tolyl)propanamide (1.5 mmol), *tert*-butyl piperazine-1-carboxylate (1.8 mmol), K_2CO_3 (2.25 mmol), KI (0.75 mmol) were combined in a vial, CH_3CN(1.5 mL) was added, and the reaction mixture was stirred at 80 °C overnight, the crude reaction mixture was diluted with EtOAc and washed with H_2O and brine. The organic layer was dried over Na_2SO_4, filtered and condensed. The crude mixture was purified using flash silica gel column chromatography to get the pure product *tert*-butyl 4-(3-oxo-3-(*m*-tolylamino)propyl)piperazine-1-carboxylate (yield 90%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.52 (s, 1H), 7.39 (s, 1H), 7.24 (s, 1H), 7.17 (t, 1H, J = 10.0 Hz), 6.89 (d, 1H, J = 5.0 Hz), 3.58-3.53 (m, 4H), 2.73 (t, 2H, J = 5.0 Hz), 2.58-2.53 (m, 6H), 2.34 (s, 3H), 1.48 (s, 9H); ESI-MS: m/z 348.1 $(M+H)^+$.

Scheme 3



3-(piperazin-1-yl)-N-(m-tolyl)propanamide **(27)**：To a stirring solution of the *tert*-butyl 4-(3-oxo-3-(*m*-tolylamino)propyl)piperazine-1-carboxylate (1 mmol) in dry CH_2Cl_2 (5 mL) at 0 °C, trifluoroacetic acid (0.5 mL) was slowly added and the reaction mixture was stirred at r.t. for 2 h. The mixture was concentrated under vacuum, and suspected in ethyl acetate (10 mL), the saturated $NaHCO_3$ solution was added to adjust pH to 7 at 0 °C. The mixture was extracted with ethyl acetate (3 ×10 mL). The combined organic layer was dried over Na_2SO_4 and concentrated in vacuo. The crude mixture was purified using flash silica gel column chromatography to get the pure product (yield 80%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.60 (br, 1H), 7.39 (s, 1H), 7.30-7.28 (m,

1H), 7.20 (t, 1H, J = 7.5 Hz), 6.90 (d, 1H, J = 7.5 Hz), 3.70 (br s, 1H), 3.07-3.02 (m, 4H), 2.74 (t, 2H, J = 5.5 Hz), 2.64-2.58 (m, 4H), 2.53 (t, 2H, J = 6.0Hz), 2.33 (s, 3H); ESI-MS: m/z 248.0 $(M+H)^+$; HPLC Purity 95.0% (t_R 8.52 min). HPLC condition, instrument, SHIMADZU; column, Pinnacle C18, 3 μm; UV absorption, λ = 254 nm; flow rate, 0.2 mL/min, HPLC gradient went from 5% acetonitrile/95% water to 95% acetonitrile/5% water (both solvents contain 0.1% folic acid) over 30 min.