Supporting Information

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SI Materials and Methods

Detailed Experimental Procedures for Data Presented in the Main Text. ***Experimental animals.*** Adult zebrafish (the laboratory-bred AB strain from the University of Oregon), aged 6–9 mo, were used for all experiments except where noted. Adult zebrafish were maintained and bred following standard procedures (1). Other zebrafish strains used in the study include EK, a commercially available strain from Ekkwill, Florida, and Wik, a laboratory-bred strain from Tubingen, Germany. They were kept on a 14/10-h L/D cycle and at a temperature of 27–28 °C. The average body weight for the animals used in our experiments was ~0.25 g. All experiments were carried out in accordance with the guidelines stipulated by the National Institutes of Health regarding the care and use of animals for experimental procedures.
Behavioral assays. Animals tested in a given experiment were siblings group-housed (8–12 per housing unit) in identical tanks and tested in identical experimental settings. Behavior room lighting and temperature were the same as in the animal housing rooms. Continuous background noise was provided to minimize startling of the animals during behavioral testing. All behavioral chambers were placed in a large brown box to isolate zebrafish from experimenters.

L/D choice assay. A chamber (23.5 × 13.5 × 13 cm, length × width × depth) was decorated half with black tape and half with white tape (covering both the bottom and the side walls) and filled with 1 L of zebrafish system water [5 g of instant ocean salt (Aquatic Ecosystem) + 3 g of sodium bicarbonate per 20 L of deionized water). Individual animals were introduced into the chamber by netting and allowed to habituate for 2 min, followed by a 5-min video recording. A Dynamic Image Analysis System (DIAS; Soltec) or Ethovision (Noldus) and Excel (Microsoft) were used for data analysis. Data were represented as mean ± SEM of choice index (% time in dark area − % time in white area)/100. A choice index of 1 indicates 100% time in the dark area, whereas a choice index of −1 represents 100% time in the light area.

For the computer-projected L/D visual image, a chamber with the same dimensions (23.5 × 13.5 × 13 cm, length × width × depth) was placed atop a computer monitor that could be remotely controlled. The initial projection from the monitor was of a brown color matching the color of the boxed environment. Each animal was introduced into the chamber and allowed to habituate overnight individually. The next morning, the projected image was changed from the initial brown color to a half light and half dark color. At the same time, a 5-min recording was collected for subsequent behavioral analysis. For L/D + H, before changing the projected image, individual fish were netted out of the chamber; after changing the projected image to half light and half dark, individual animals were netted back into the cage.

Locomotor activity assay. To measure zebrafish locomotor activity, individual zebrafish were introduced into a transparent tank (20.5 × 25 × 4 cm, length × width × depth). The distance traveled was recorded for 10 min and analyzed by DIAS and Excel. Data were represented as mean ± SEM in units of millimeters per second.

Visual acuity assay. We adopted a protocol as previously described (2). Briefly, individual zebrafish were netted into a transparent circular water container containing 1 L of system water. A rod was secured in the middle of the container to prevent fish from swimming diagonally. Just outside the container, a rotating drum (dark vertical bar of 2.5 cm in width against a light background or gray vertical bar against a dark background, matching those used in the choice chambers) was visible to the fish. The fish had 30 s to habituate in the environment without the drum rotating. After 30 s, the drum started to rotate at 25 rounds per minute for 2 min. The behavior of the fish was recorded with a camera placed 0.6 m above the container. Data were generated by dividing the number of times the zebrafish reacted to the rotating bar by the total number of times the zebrafish encountered the rotating bar. Data were represented as mean ± SEM.
Pharmacological study. Buspirone hydrochloride and chlordiazepoxide hydrochloride were purchased from Sigma–Aldrich. Each drug was dissolved in a fish tank containing 1 L of system water to the desired concentrations. Three zebrafish were treated at the same time for 1 h, followed by behavioral analyses as described above.
Statistical analysis. To address whether experimental groups were significantly different from the control group, we used the Student's *t* test or, whenever necessary, one-way ANOVA, followed by the post hoc Tukey's test or Bonferroni test. Data are considered significantly different when $P < 0.05$.
Analysis of c-fos. The *c-fos* gene sequence information (National Center for Biotechnology Information Gene ID code 394198) was used to prepare the in situ probe. For *c-fos* induction, 30 min after initial exposure to either L/D or L/D + H, zebrafish were killed and brains were perfused with PBS, followed by 4% (wt/vol) paraformaldehyde (PFA). Whole zebrafish were further fixed with 4% (wt/vol) PFA at 4 °C for 2 d. Afterward, brains were extracted and washed with PBS and cryoprotected in 30% (wt/vol) sucrose/PBS overnight at 4 °C. Next, brains were embedded in Tissue-Tek medium (Electron Microscopy Sciences) and cryosectioned to 30 μm. For *c-fos* detection by in situ hybridization, brain sections were prefixed in 4% PFA at room temperature (RT) for 10 min. Following PBS washes, sections were treated with 1 μg/mL Proteinase K (Roche Diagnostics) at 37 °C for 10 min, immediately followed by PBS washes. After postfixation in 4% (wt/vol) PFA at RT for 5 min, sections were treated for 10 min with 0.1 M triethanolamine (TEA) and acetic anhydride (250 μL per 100 mL of TEA) to reduce nonspecific RNA probe binding. The reaction was stopped with PBS washes, and the sections were allowed to dry for 1 h at RT. Sections were then incubated with digoxigenin-labeled *c-fos* antisense RNA (1 μg/mL), which was diluted in hybridization buffer [50% (vol/vol) formamide, 5× SSC, 50 μg/mL heparin, 0.1% Tween-20, 5 mg/mL Torula (yeast) RNA], at 65 °C overnight. Sections were postwashed in 4× SSC, 2× SSC, 1× SSC, 0.5× SSC, and 0.1× SSC at 55 °C, followed by 0.1× SSC and 1× MAB (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) washes at RT. Sections were then blocked with blocking reagent buffer [1× MAB, 2% (wt/vol) Blocking Reagent (Roche Diagnostics), 5% (vol/vol) goat serum] for 30 min at RT, followed by anti-Digoxigenin-Alkaline Phosphatase (Roche Diagnostics, 1:1,000 dilution) incubation at 4 °C overnight. After further 1× MAB washes, sections were treated with NTMT [0.1 M Tris-Cl (pH 9.5), 50 mM $MgCl_2$, 0.1 M NaCl, 0.1% Tween-20) for 10 min at RT. A colormetric reaction was achieved by incubation with 175 μg/mL 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 337.5 μg/mL nitro blue tetrazolium chloride (NBT). The reaction was stopped by brief washes with PBS. Sections were then fixed with 4% (wt/vol) PFA at RT, washed with PBS, air-dried, and coverslipped with xylene and Entellan (Electron Microscopy Sciences). Images were taken with a Zeiss compound microscope. For the quantitative analysis of *c-fos*–positive cells, total numbers of *c-fos*–positive cells were

counted in all sections containing the brain regions of interest. The interpretation of neuroanatomy follows the adult zebrafish brain atlas (3). Data were presented as the average number of *c-fos*–positive cells per 30-μm section.

Assessment of chlordiazepoxide binding in zebrafish brain tissues. Saturation analysis indicated that [^{3}H]flunitrazepam binds with high affinity to zebrafish whole-brain homogenates in a saturable manner. Equilibrium K_d and B_{max} values for [^{3}H]flunitrazepam in zebrafish whole brain are 1.5 ± 0.4 nM and 125 ± 50 fmol/mg protein (n = 3), respectively, as determined by nonlinear regression (Fig. S2*A*). Through a series of competition assays, we displaced [^{3}H]flunitrazepam from zebrafish brain membrane homogenates by the anxiolytic benzodiazepine receptor ligands diazepam (IC_{50} = 60.5 ± 8 nM, K_i = 23 ± 4 nM) and chlordiazepoxide (IC_{50} = 374 ± 138 nM, K_i =143 ± 55 nM) (n = 4–5, Hill coefficient = 1). The anxiolytic tricyclic antidepressant desipramine, which has no substantial affinity for benzodiazepine receptors in mammals, did not displace [^{3}H]flunitrazepam binding from zebrafish brain membranes at physiologically relevant concentrations (IC_{50} = 24,555 ± 5,597 nM, K_i =10,096 ± 2,534 nM) (Fig. S2*B*).

In summary, the zebrafish brain benzodiazepine binding site, as defined by [^{3}H]flunitrazepam, exhibits a pharmacological profile that is very similar, if not identical, to those found in human brain (4, 5). These data, together with our previous experience with pharmacological compound actions in zebrafish (6–8), allow us to extrapolate that most pharmacological compounds developed in mammalian systems will likely produce similar actions in zebrafish.

Detailed Experimental Procedures for Data Presented in *SI Text*. [^{3}H] flunitrazepam saturation and displacement binding to zebrafish $GABA_A$ sites was conducted for the data presented in *SI Text*. Radioligand binding to $GABA_A$ benzodiazepine sites was performed in membrane homogenates from adult zebrafish whole brains using [^{3}H]flunitrazepam (Perkin–Elmer), following previously established procedures (9). Whole brains (∼250 mg of wet-weight tissue) from 12 to 16 zebrafish adults of mixed gender were pooled together and homogenized in 25 mL of 4 °C 170 mM Tris-HCl buffer, pH 7.4, at 4 °C. Homogenates were centrifuged for 10 min at 30,600 × g at 4 °C, and pellets were resuspended in buffer and recentrifuged. Final pellets were resuspended, and protein concentration was determined spectrophotometrically using Bradford reagent (Sigma). Binding assays were carried out for 1 h at 4 °C in pH 7.4 buffer. The [^{3}H]flunitrazepam concentration ranged from 0.05 to 40 nM for saturation assays or was 2.5 nM for displacement assays. Diazepam, chlordiazepoxide, and desipramine (all from Sigma) at concentrations ranging from 0.5 pM to 5 μM were used to displace [^{3}H] flunitrazepam in competition assays. Nonspecific binding for the saturation assays was defined by 5 μM diazepam and was 8–20% of total binding.

The incubation was terminated with 4 mL of buffer, pH 7.4, at 4 °C. Labeled homogenates were captured by rapid filtration under vacuum using a Brandel tissue harvester onto number 25 glass fiber filters (Schleicher and Schuell) presoaked in 0.5% polyethylenimine (Sigma) and washed twice with 4 mL of buffer. ^{3}H-radioactivity trapped on filters was counted using a Packard liquid scintillation counter with 40% efficiency. Data were analyzed by nonlinear regression using DeltaGraph (Red Rock), and Cheng–Prusoff correction (10) was used to determine K_i values for competition curves.

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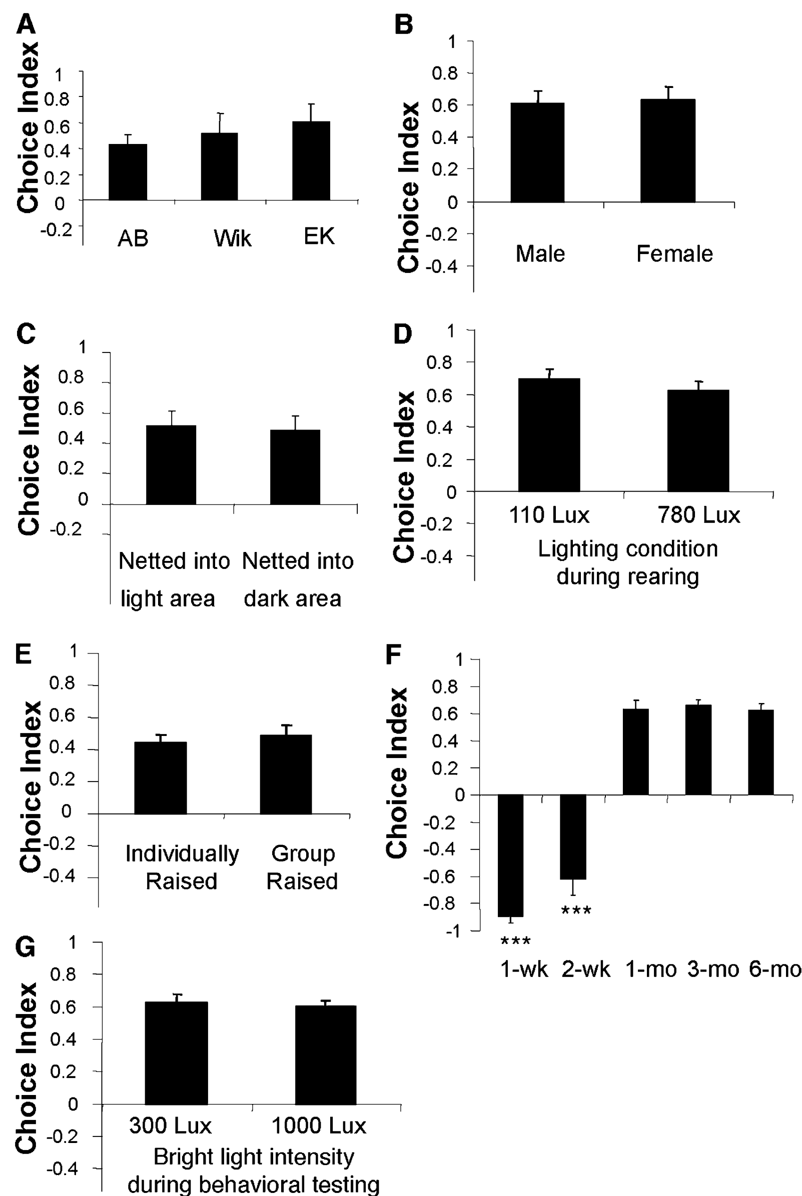


Fig. S1. Characterization of an innate light avoidance behavior in adult zebrafish. (*A*) Three different WT strains of adult zebrafish exhibit light avoidance behavior (*n* = 11 for all groups). (*B*) Male and female adult zebrafish exhibit similar light avoidance behavior (*n* = 13 for all groups). (*C*) Initial placement of adult zebrafish on either the light or dark side does not affect the light avoidance behavior (light, *n* = 11; dark, *n* = 10). (*D*) Use of two different lighting conditions during the raising of zebrafish does not affect light avoidance behavior (*n* = 12 for each group). (*E*) Raising in isolation or in groups does not affect light avoidance behavior (*n* = 12 for all groups). (*F*) Larval and adult zebrafish display a choice reversal under the same experimental conditions and using the L/D choice chamber designed proportionally to their body size (1-wk, 2-wk, 1-mo, *n* = 12; 3-mo, *n* = 24; 6-mo, *n* = 26; ANOVA, ***$P < 0.001$). (*G*) Bright light intensity of 1,000 Lux during behavioral testing does not further enhance the light avoidance behavior (300 Lux, *n* = 26; 1,000 Lux, *n* = 24). All data represent mean ± SEM.

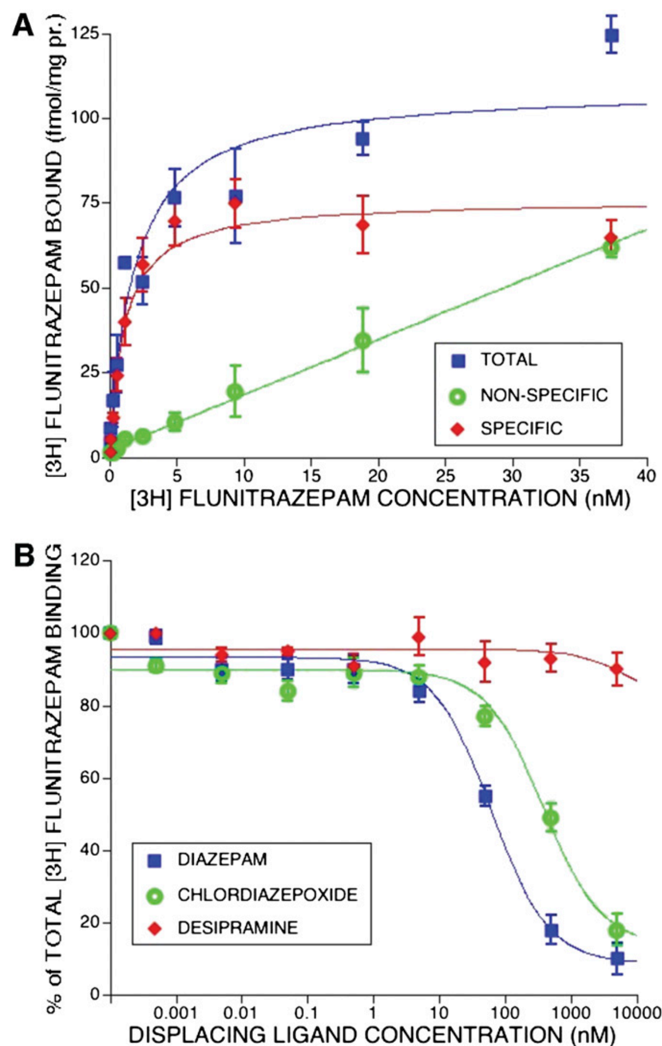


Fig. S2. [^{3}H]flunitrazepam saturation and displacement binding to zebrafish $GABA_A$ sites. (*A*) Saturation binding analysis of [^{3}H]flunitrazepam binding to benzodiazepine binding sites in zebrafish brain membranes by nonlinear regression (n = 3). Membranes pooled from adult mixed-gender zebrafish were incubated with concentrations of [^{3}H]flunitrazepam ranging from 0.05 to 40 nM. Nonspecific binding was defined by 5 μM diazepam and was subtracted from the total binding curve in *A* to obtain specific binding values. The K_d of the zebrafish benzodiazepine binding site is 1.5 ± 0.4 nM, and the B_{max} is 125 ± 50 fmol/mg protein. (*B*) Displacement of 2.5 nM [^{3}H]flunitrazepam from zebrafish whole-brain membranes by diazepam and chlordiazepoxide (n = 4–5 displacement assays). Diazepam has a greater affinity (K_i = 23 ± 4 nM) for the zebrafish benzodiazepine binding site on $GABA_A$ receptors than chlordiazepoxide (K_i = 143 ± 55 nM), as indicated by its displacement of [^{3}H]flunitrazepam at a lower concentration. Desipramine exhibits negligible affinity for the zebrafish benzodiazepine binding site (K_i = 10,096 ± 2,534 nM).

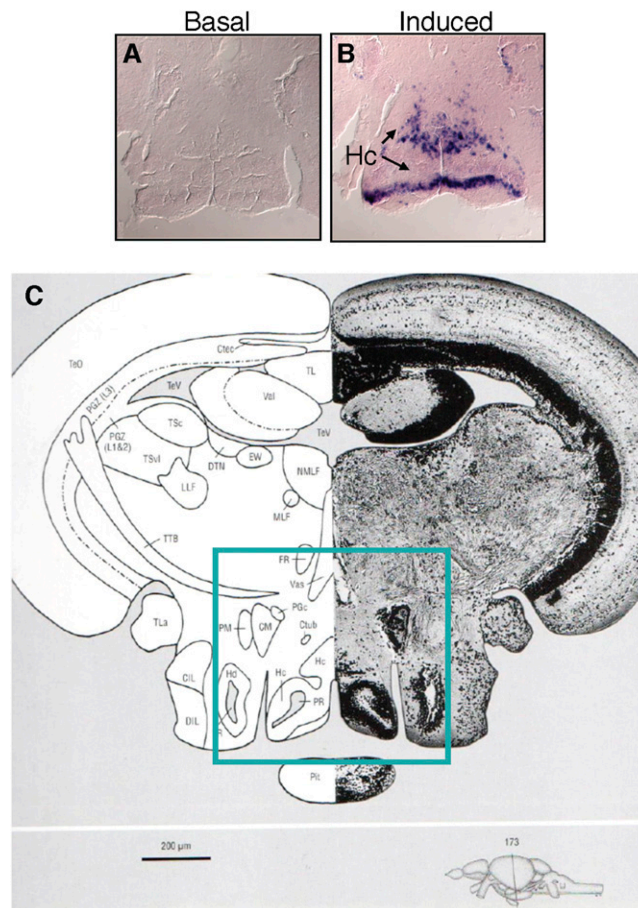


Fig. S3. In situ hybridization of *c-fos* reveals neural activity in basal and induced states. (*A*) Nominal basal *c-fos* expression in the brain of adult zebrafish (the hypothalamic region is shown, as boxed in *C*). (*B*) Strong and widespread *c-fos* expression in the brain of zebrafish subjected to intense handling stress (the hypothalamic region is shown, as boxed in *C*). (*C*) Section through the diencephalon/midbrain region highlighting the region shown in *A* and *B* [corresponding to section 173 in the zebrafish brain atlas (1)]. CIL, central nucleus of the inferior lobe; CM, corpus mamillare; Ctec, commissure tecti; Ctub, commissure of the posterior tuberculum; DIL, diffuse nucleus of the inferior lobe; DTN, dorsal tegmental nucleus; EW, Edinger–Westphal nucleus; FR, fasciculus retroflexus; Hc, caudal zone of periventricular hypothalamus; Hd, dorsal zone of periventricular hypothalamus; LLF, lateral longitudinal fascicle; LR, lateral recess of diencephalic ventricle; MLF, medial longitudinal fascicle; NMLF, nucleus of MLF; PGc, caudal preglomerular nucleus; PGZ, periventricular gray zone of the TeO; Pit, pituitary; PR, posterior recess of diencephalic ventricle; TeO, tectum opticum; TeV, tectal ventricle; TL, torus longitudinalis; TLa, torus lateralis; TPM, tractus pretectomamillaris; TSc, central nucleus of torus semicircularis; TSvl, ventrolateral nucleus of torus semicircularis; TTB, tractus tectobulbaris; Val, lateral division of valvula cerebelli; Vas, vascular lacuna of area postrema.

1. Wullimann MF, Rupp B, Reichert H (1996) *Neuroanatomy of the Zebrafish Brain* (Birkhauser, Basel).

A

	Visual Acuity		
Average LD choice index	**# of encounters**	**# of responses**	**% responses**
0.69 ± 0.04	49.33 ± 1.41	46.50 ± 1.82	94.19 ± 1.85
-0.07 ± 0.08	48.00 ± 1.52	47.00 ± 1.34	97.97 ± 0.92

P value for LD Choice index = 0.0000067
P value for % response = 0.12

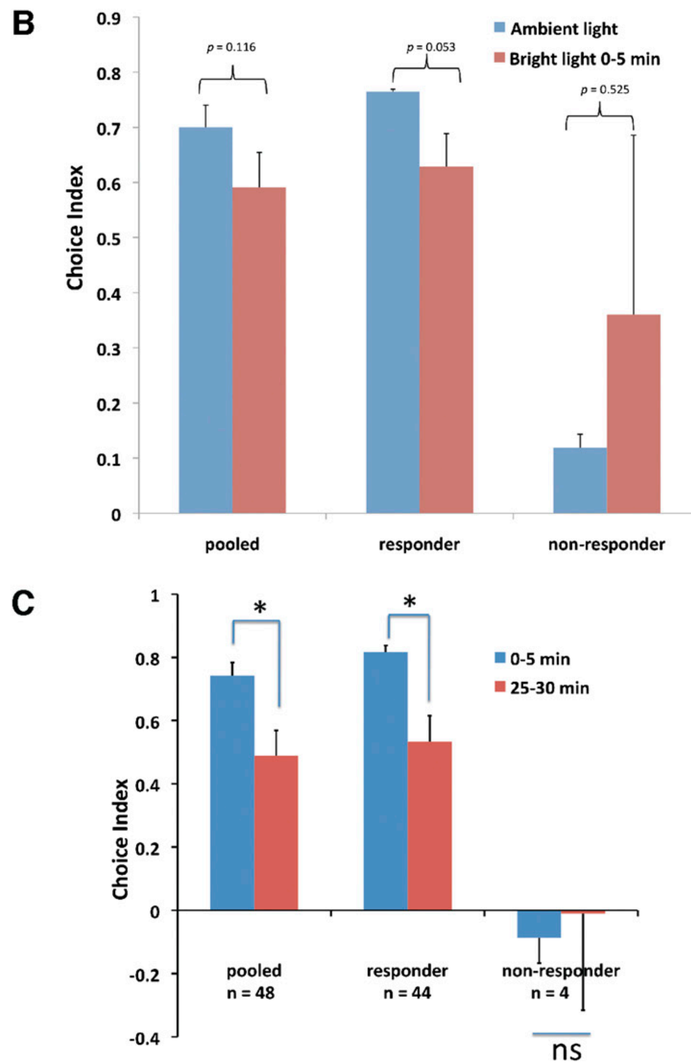


Fig. S4. Visual acuity of zebrafish that display high vs. low avoidance and the effect of increased lighting and prolonged time on light avoidance. (*A*) High light-avoidant fish (average L/D choice index: 0.69 ± 0.04, n = 7) and low light-avoidant fish (average L/D choice index: −0.07 ± 0.08, n = 6) show comparable visual capability as assessed by the % response in the visual acuity assay. (*B*) Individual zebrafish were tested for their L/D preference; afterward, the light intensity was increased to 1,000 Lux and the animal's L/D preference was tracked for an additional 5 min. There is no significant effect of bright light, either on pooled data (n = 40), the high light-avoidant fish (n = 36), or the low light-avoidant fish (n = 4). (*C*) Individual zebrafish were computed for their L/D preference for 0–5 min and 25–30 min while remaining in the L/D choice chamber. The pooled (n = 48) and the high light-avoidant (n = 44) fish showed a significant attenuation of light avoidance (*$P < 0.05$), but the low light-avoidant fish did not show an alteration.

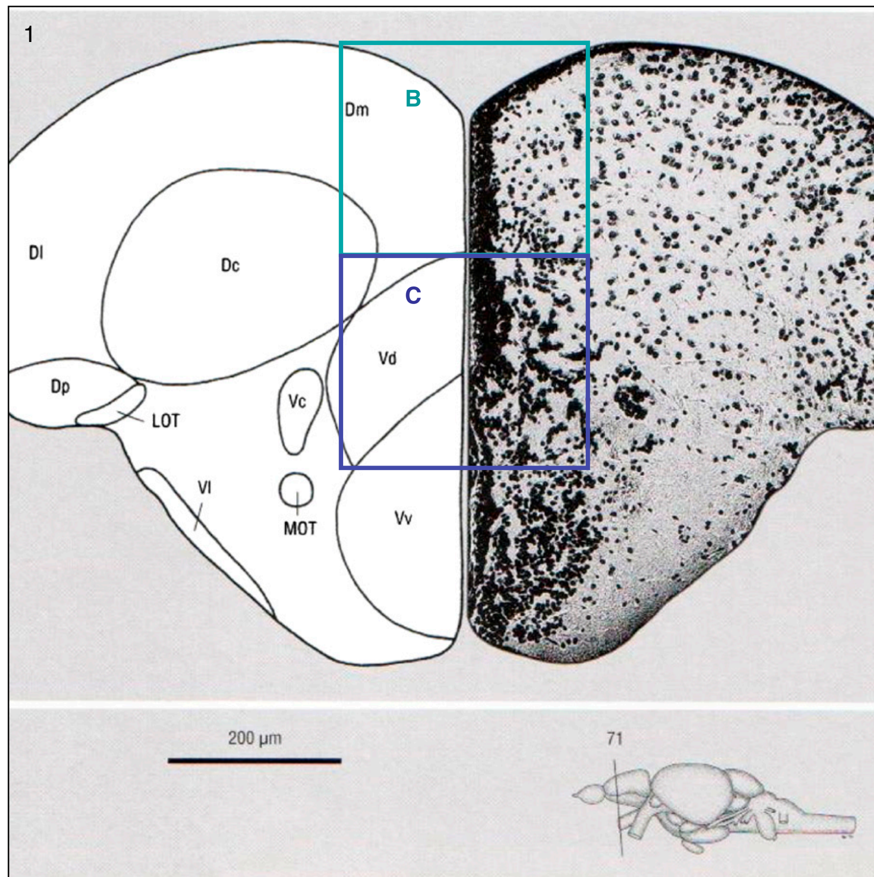


Fig. S5. (Continued)

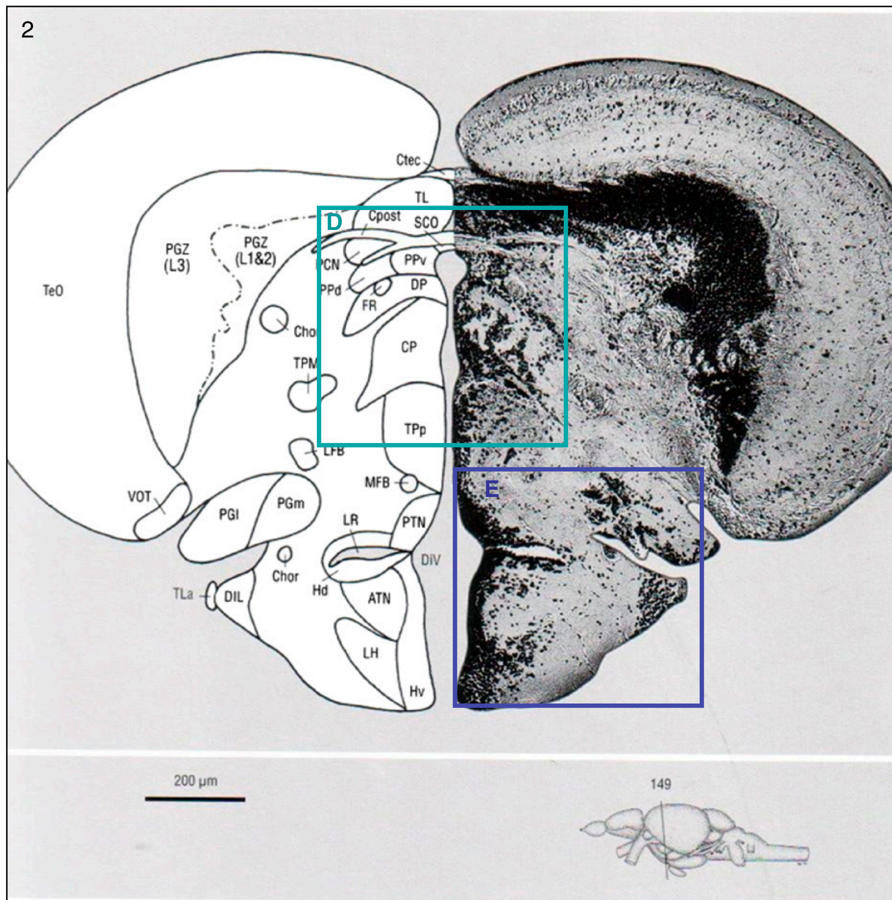


Fig. S5. (Continued)

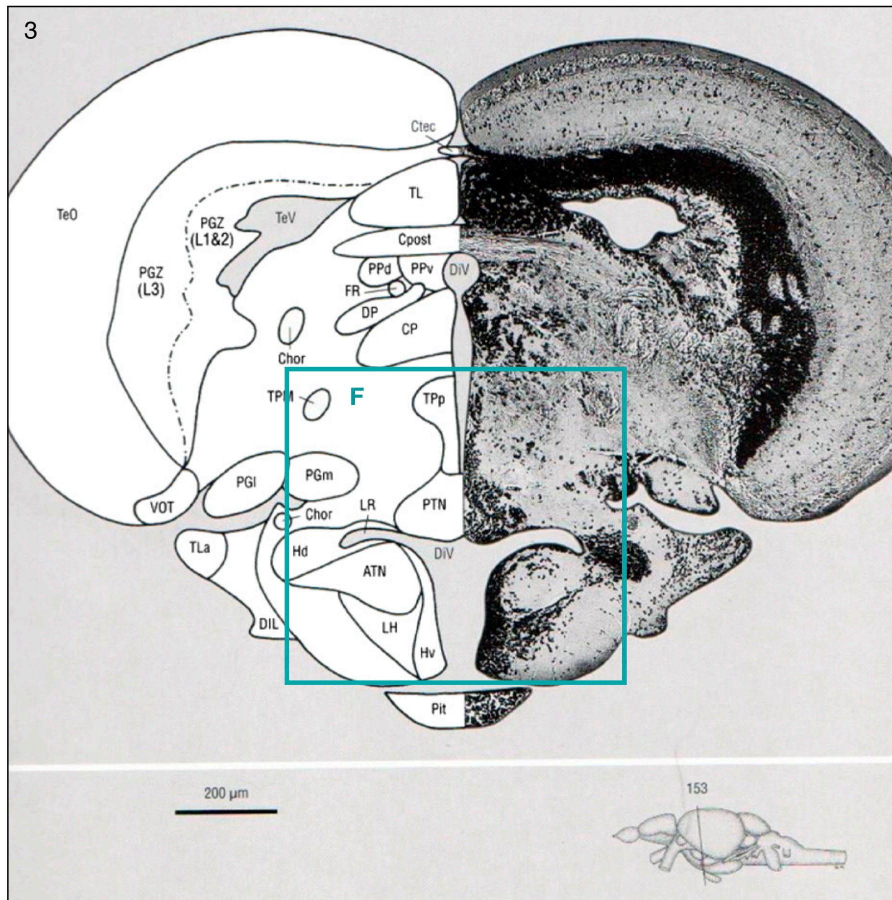


Fig. S5. Sections from the adult zebrafish brain atlas (1) highlighting the regions shown in Fig. 4 *B–F* (boxed areas). (1) Section through the telencephalic region [corresponding to section 71 in the zebrafish brain atlas (1)]. D, dorsal telencephalic area; Dc, central zone of D; Dl, lateral zone of D; Dm, medial zone of D; Dp, posterior zone of D; LOT, lateral olfactory tract; MOT, medial olfactory tract; V, ventral telencephalic area; Vc, central nucleus of V; Vd, dorsal nucleus of V; Vl, lateral nucleus of V; Vv, ventral nucleus of V. (2–3) Sections through the diencephalon/midbrain region [corresponding to sections 149 and 153 in the zebrafish brain atlas (1), respectively]. ATN, anterior tuberal nucleus; Chor, commissura horizontalis; CP, central posterior thalamic nucleus; Cpost, commissura posterior; Ctec, commissura tecti; DIL, diffuse nucleus of the inferior lobe; DiV, diencephalic ventricle; DP, dorsal posterior thalamic nucleus; FR, fasciculus retroflexus; Hd, dorsal zone of the periventricular hypothalamus; Hv, ventral zone of periventricular hypothalamus; LFB, lateral forebrain bundle; LH, lateral hypothalamic nucleus; LR, lateral recess of diencephalic ventricle; MFB, medial forebrain bundle; PCN, paracommissural nucleus; PGl, lateral preglomerular nucleus; PGm, medial preglomerular nucleus; PGZ, periventricular gray zone of the TeO; PPd, periventricular pretectal nucleus dorsal part; PPv, periventricular pretectal nucleus ventral part; PTN, posterior tuberal nucleus; Sco, subcommissural organ; TeO, tectum opticum; TL, torus longitudinalis; TLa, torus lateralis; TPM, tractus pretectomammillaris; TPp, periventricular nucleus of posterior tuberculum; VOT, ventrolateral optic tract.

1. Wullimann MF, Rupp B, Reichert H (1996) *Neuroanatomy of the Zebrafish Brain* (Birkhauser, Basel).

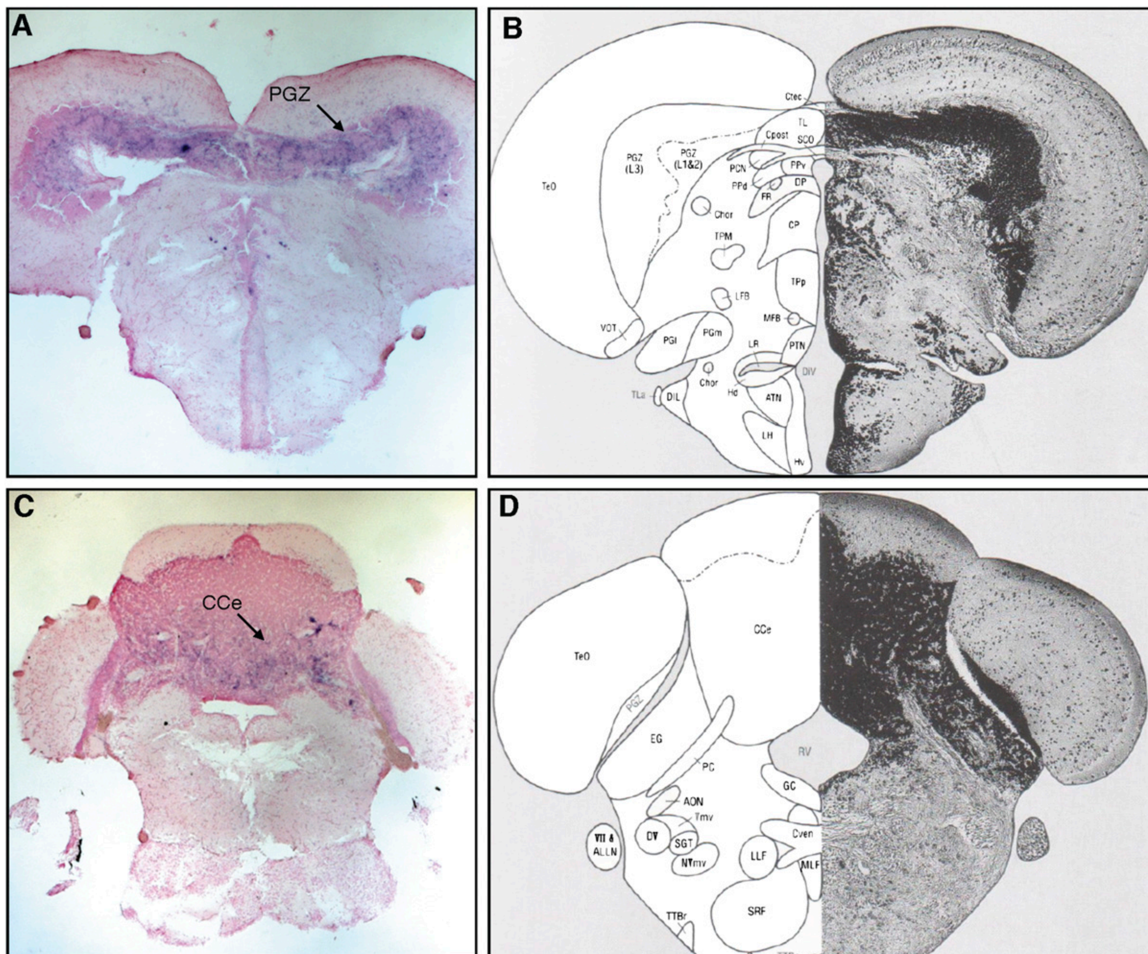
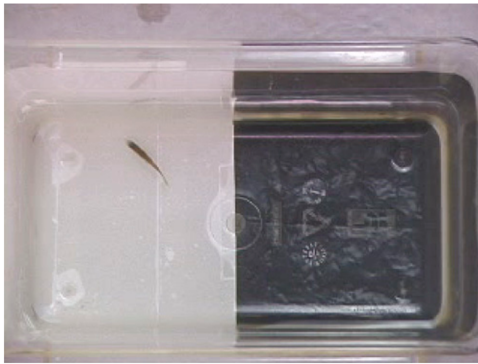


Fig. S6. Activity of *c-fos* in the mid- and hind-brain regions. (*A*) Section through the midbrain region showing the *c-fos*–positive cells in the TeO periventricular zone (PGZ). (*B*) Corresponding brain section from the adult zebrafish brain atlas (1). (*C*) Section through the hind-brain region showing the *c-fos*–positive cells in the cerebellar region, corpus cerebelli (CCe). (*D*) Corresponding brain section from the the adult zebrafish brain atlas (1).

1. Wullimann MF, Rupp B, Reichert H (1996) *Neuroanatomy of the Zebrafish Brain* (Birkhauser, Basel).

Fig. S7. Activity of *c-fos* in brain regions from animals subjected to both L/D and L/D + H. Sections through the telencephalic region (*A*) and diencephalic/midbrain regions (*C* and *E*) showing the *c-fos*–positive cells in regions, including the Dc (central zone of dorsal Tel), Dl (lateral zone of dorsal Tel), Dm (medial zone of dorsal Tel), Dp (posterior zone of dorsal Tel), Vd (dorsal nucleus of ventral Tel), Vv (ventral zone of ventral Tel), Had (dorsal habenula nucleus), VL (ventrolateral thalamic nucleus), and TPp (periventricular nucleus of posterior tuberculum). (*B*, *D*, and *F*) Corresponding brain section from the adult zebrafish brain atlas (1).

1. Wullimann MF, Rupp B, Reichert H (1996) *Neuroanatomy of the Zebrafish Brain* (Birkhauser, Basel).



Movie S1. After being netted into the light side, adult zebrafish explore the L/D choice chamber. It is noted that frequent turns are made at the border to turn away and avoid the light side.

Movie S1

Movie S2. Adult zebrafish were habituated overnight in the chambers. After computer projection of a bright light visual stimulus from underneath, the animal on the left showed a light avoidance behavior, whereas the animal on the right did not.

Movie S2