Erratum: The habenula is crucial for experience-dependent modification of fear responses in zebrafish

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In the version of this article initially published online, there was an error on page 2, right column, second paragraph, 14th line. Here, 'Mtz' should read 'metronidazole.' The supplementary material file was also missing some information from the images. Both errors have been corrected for the print, PDF and HTML versions of this article.
The habenula is crucial for experience-dependent modification of fear responses in zebrafish

Masakazu Agetsuma¹, Hidenori Aizawa¹, Tazu Aoki¹, Ryoko Nakayama¹², Mikako Takahoko¹², Midori Goto¹, Takayuki Sassa¹, Ryunosuke Amo¹³, Toshiyuki Shiraki¹², Koichi Kawakami⁴, Toshihiko Hosoya¹, Shin-ichi Higashijima⁵ and Hitoshi Okamoto¹⁶

¹RIKEN Brain Science Institute, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan. ²Research Resource Center, RIKEN RIKEN Brain Science Institute, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan. ³Department of Life Science and Medical Bioscience, Graduate School of Advanced Science and Engineering, Waseda University, Shinjuku-ku, Tokyo 162-8480, Japan. ⁴Division of Molecular and Developmental Biology, National Institute of Genetics, and Department of Genetics, The Graduate University for Advanced Studies (SOKENDAI), 1111 Yata, Mishima, Shizuoka 411-8540, Japan. ⁵National Institutes of Natural Sciences, Okazaki Institute for Integrative Bioscience, National Institute for Physiological Sciences, Okazaki, Aichi 444-8787, Japan. ⁶Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Sanbancho Bldg., 5, Sanbancho, Chiyoda-ku, Tokyo 102-0075, Japan. ⁷Present address: Laboratory of Biochemistry, Faculty of Pharmaceutical Sciences, Hokkaido University, Kita 12-jo, Higashi 6-chome, Kita-ku, Sapporo 060-0812, Japan.
Supplementary Figure 1

Asymmetric pathways from dHb to IPN and parallel pathway from vHb to MR. a, Dorsal oblique view. b, Sagittal view. Red, dHbL-d/iIPN pathway; green, dHbM-v/iIPN pathway; blue, vHb-MR pathway. The larger dHbM shown on the right innervates the ventral (vIPN) and intermediate IPN (iIPN), together with axons from the smaller dHbM on the left. Conversely, the larger dHbL shown on the left is the predominant source of axons innervating the dorsal IPN (dIPN) and iIPN, together with axons from the smaller dHbL on the right. Dotted gray lines indicate the positions of the coronal and horizontal sections used in the panels of Figure 1. Cbll, cerebellum; GC, griseum centrale; Hb, habenula; IL, inferior lobe of the hypothalamus; MR, median raphe; OB, olfactory bulb; P, pineal organ; PP, parapineal organ; Tel, telencephalon; TeO, optic tectum.
Supplementary Figure 2

In situ hybridization of tph2, a marker for serotonergic neurons, in a sagittal section of adult zebrafish brain. Representative trajectory from the dIPN to the griseum centrale (GC) shown by the tracer experiments is indicated by the red arrows, suggesting the possible termination onto the serotonergic neurons in the putative dorsal raphe (DR). D, dorsal; MR, median raphe; P, posterior. Scale bar, 200 µm.
**Supplementary Figure 3**

**a, b,** The axonal terminals in the GC (a) and MR (b) were anterogradely labeled by injection of Neurobiotin into the dIPN and vIPN, respectively. Putative synaptic boutons are indicated by arrowheads. **c, d,** The cell bodies in the GC (c) and MR (d) were retrogradely labeled by the injection of Neurobiotin into the dIPN and the vIPN, respectively. Labeled neurons are indicated by arrows. Ve, rhombencephalic ventricle; MLF, medial longitudinal fascicle. Scale bar in a, 50 µm; b and d, 30 µm. c, 25 µm.
Supplementary Figure 4

The retrograde labeling from the MR suggested that the main afferent to the MR comes from the vIPN rather than from the d/iIPN. For retrograde labeling from the MR to the IPN, a small amount of DiI was injected to restrict the distribution of the dyes within the MR. A glass micropipette was used for the iontophoretic injection of a solution of 0.5% DiI dissolved in ethanol by applying 60 – 80 µA direct current for 1 – 4 min. a, Coronal section showing the site of DiI injection (red) in the MR. b, Representative coronal section of the IPN of Tg(brn3a-hsp70:GFP) (green) counterstained with SYTOX orange (red), suggesting the distribution of the cells in the IPN. c, An example of retrograde labeling of the IPN neuron from the MR (red). A projecting neuron retrogradely labeled from the MR is indicated in the panel (arrow). d, Positions of labeled cell bodies (black dots) were summarized by plotting on a diagram of a representative section (c). Scale bars in a, 200 µm; b and c, 50 µm.
Supplementary Figure 5

*narp* mRNA expression in the Tg(*brn3a-hsp70:GFP*) adult brain. The intrinsic *narp* gene showed strong expression in the dHb\_L, although the expression was observed in a broader region of the habenula including the vHb than the region in the dHb\_L alone. **a**, *narp* mRNA expression. **b**, GFP expression of the Tg(*brn3a-hsp70:GFP*) fish. **c**, merged images. Scale bar, 100 µm.
Supplementary Figure 6

TeTxLC mRNA expression in larval Tg(narp:GAL4VP16;UAS:TeTxLC) fish. Lateral view (a) and dorsal view (b, c) of the whole-mount 7-day post-fertilization (dpf) larvae. Arrowheads, dHbL; open arrowheads, expression near otic vesicles. Scale bar in a, 400 µm, in b and c, 100 µm.
Serial in situ hybridization sections for TeTxLC mRNA of the whole brain of an adult Tg(narp:GAL4VP16;UAS:TeTxLC). Most of the dark stains in peripheral regions of each slice and in blood vessels are not signals, except for the dHbt. signals indicated by arrowheads. Thickness of the slices, 100 µm. Scale bar, 400µm.
Supplementary Figure 8

Procedure for the cued fear conditioning of adult zebrafish.
Locomotor changes against the first CS application during the retrieval session. Each bin corresponds to the locomotor speed for every 0.1 sec. Time zero suggests the onset of the CS, and it was applied for 8.5 sec (from 0 to 8.5, red). a, An example of one individual control fish, the behavior of which is shown in Supplementary Movie 1. This individual showed the sudden increase in locomotion immediately after the onset of the CS, and after the CS off, started to show normal locomotor activity. b, An example of one individual dHbL-silenced fish, the behavior of which is shown in Supplementary Movie 2. This fish showed the increase in locomotion about two seconds after the CS on, and during the CS exposure, started to slow down, followed by the complete and continuous immobility. Gray bar in Each panel shows the result in the other panel.
Supplementary Figure 10

Supplementary Figure 10
Ratio of freezing individuals at each trial during the adaptation session (a) and the retrieval session (b).
Supplementary Figure 11
The locomotion trajectory of one dHbLT-silenced fish which initiated persistent rotation upon the presentation of the CS in the retrieval session, before (20 sec, red dotted lines), during (8.5 sec, red solid lines) and after (20 sec, blue lines) the CS exposure. Arrow, initial position; open arrowhead, final position. Scale bar, 20 mm.
Supplementary Figure 12

Innate flight behaviors against CS during the adaptation session were not significantly different between control and dHbL-silenced fish. Mann-Whitney U-test was performed for the comparison between control and dHbL-silenced. Middle line, median; box edges, quartiles; vertical bars, range. ns, not significant ($P > 0.05$).
Supplementary Figure 13

The projection patterns from the dHbL to the d/iIPN were not different between adult control fish and dHbL-silenced fish. Both Tg(narp:GAL4VP16;UAS:TeTxLC) (a–d) and Tg(narp:GAL4VP16) (e–h) were crossed with the Tg(UAS:DsRed2) to visualize the projection patterns. The DsRed2 signals were enhanced by the antibody in combination with the secondary antibody fused to Alexa 546. a and e, DsRed2-expressing fibers projected to the d/iIPN. b and f, transmitted light images. c and g, merged images. d and h, stacked images (50 µm section). Scale bar, 100 µm.
Supplementary Figure 14

Nitroreductase-Mtz-based inducible and specific cell ablation of the dHbL neurons. a, b Expression patterns of the 5-dpf Tg(narp:GAL4VP16;UAS:nfsB-mCherry) larvae. Dorsal views of the habenula (a) and the IPN (b). c, Expression pattern of nfsB-mCherry (red) in the 6-dpf Tg(narp:GAL4VP16;UAS:nfsB-mCherry) larvae after a 16-hr Mtz treatment. Dorsal view of the habenula. d–f, TUNEL staining of 6-dpf Tg(narp:GAL4VP16;UAS:nfsB-mCherry) larvae at 10 hr (d), 14 hr (e) and 16 hr (f) of the treatment with Mtz. Dorsal views of the habenula. g–j, Coronal sections of the Tg(narp:GAL4VP16;UAS:nfsB-mCherry) adult fish before (g, i) and one week after the 24-hr Mtz treatment (h, j). Dotted line in g, h, dHb; dotted line in i, j, IPN. Scale bars in a, and c–f, 50 µm; in b, 25 µm; in g–j, 100 µm.
Supplementary Figure 15

Specific expression of the nitroreductase of the habenula-IPN pathway in serial sections of the whole brain of an adult Tg(narp:GAL4VP16;UAS:nfsB-mCherry). (Upper panel) Expression patterns of the nfsB-mCherry. (Lower panel) Transmitted light images merged with mCherry expression. Signals in peripheral regions of each slice and in blood vessels are autofluorescence. Thickness of the slices, 100 µm. Scale bar, 500µm. Arrowheads, dHbL; arrows, d/iIPN.
Supplementary Figure 16

The Mtz-induced specific cell death in dHbl neurons of Tg(narp:GAL4VP16; UAS:nfsB-mCherry) (left panel) was not shown in DMSO-treated Tg(narp:GAL4VP16; UAS:nfsB-mCherry) (middle panel) or in Mtz-treated sibling fish that did not express nfsB-mcherry (right panel). This nfsB-mCherry negative fish treated with Mtz are same with the control fish in the dHbl- ablation experiments. Scale bar, 50 µm.
Supplementary Figure 17

Mtz-treated control sibling (n = 11) and dHbI-ablated fish (n = 13) were used for the behavioral experiments. ns, not significant (P > 0.05). a, Total average of freezing ratios of the adult fish, before (adaptation session) and after (retrieval session) the fear conditioning. Mean +/- s.e.m. is plotted. Two-way repeated-measure ANOVA, Habenula state x Conditioning, F = 2.71, P = 0.11. ***P < 0.001, Fisher's exact test to compare control and dHbI-ablated fish with using total number of freezing and non-freezing responses at each session. b, Total average of immobility times (sec). Mean +/- s.e.m. is plotted. Two-way repeated measure ANOVA, Habenula state x Conditioning, F = 3.85, P = 0.06. **P < 0.01, Mann Whitney U-test. C, Normalized value of the change in the turning frequency before and after the onset of the CS presentation at each session. The values are normalized to the averages of the adaptation sessions. Mean +/- s.e.m. is plotted. *P < 0.05, Wilcoxon signed rank test (comparison with the adaptation session). D, The US-triggered locomotor activity at each trial of the first (1–5) and second (6–10) conditioning sessions. Mean +/- s.e.m. is plotted. Two-way repeated-measure ANOVA, the main effect of Habenula state, F = 0.80, P = 0.38. e, Exploration time spent in the center of the field. Mann–Whitney U-test, P = 0.38. Middle line, median; box edges, quartiles; vertical bars, range. f, The locomotor activity of the dHbI-ablated adult fish during the last 5 min before the onset of the retrieval session. **P < 0.01, Mann-Whitney U-test. Middle line, median; box edges, quartiles; vertical bars, range.
Supplementary Figure 18

The time course analysis of the locomotor activity of the dHbL-ablated adult fish before, during, and after the CS exposure. The average locomotion of each 10 sec for each individual was calculated, and normalized to the individual average locomotion of 10 sec before the CS. Mean +/- s.e.m. of each group is plotted. Wilcoxon signed rank test was used for the comparison of the locomotion before and after fear conditioning (a and b). Mann-Whitney U-test was performed for the comparison between control fish and dHbL-ablated fish (c and d). *P < 0.05; **P < 0.01. ns, not significant (P > 0.05).
Supplementary Figure 19

Flight behaviors and freezing behaviors of control fish and dHbL-ablated adult fish during the retrieval session. Each circle corresponds to the result of each individual (control fish, n = 11; dHbL-ablated fish, n = 13).
Supplementary Figure 20

Behavioral analysis with dHbL-ablated larval fish. a, Light preference index of control (n = 8) and dHbL-ablated (n = 8) larvae. The light-preference index was plotted against each 30-min time window. Two-way repeated-measure ANOVA, Habenula state (control, dHbL-ablated) x time windows, F = 1.09, P = 0.37; Habenula state, F = 0.24, P = 0.63. b, Average locomotor activities of the dHbL-ablated (n = 41) and control larvae (n = 50) in an environment with cycling changes of 30-min dark and 30-min light. Two-way repeated-measure ANOVA was used against whole data from each dark period, and P values for the interaction (Habenula state x time windows) are shown (See also Supplementary Data 5).
Supplementary Figure 21

Experience-dependent modification of locomotion during light off-on cycles was impaired in the dHbL-ablated fish. Repetition of the dark-light cycle affected the total locomotor activity from 14 min through 24 min after the onset of the 30-min dark cycle. The total activity of the control fish significantly decreased from the second cycle, while that of dHbL-ablated larvae remained high until the sixth cycle. Repeated Measures ANOVA for control fish, \( P < 0.001 \); for dHbL-ablated fish, \( P < 0.01 \). The results of the post-hoc test (Bonferroni's Multiple Comparison Test) for the comparison between 1st cycle and the others are shown in the figure. *\( P < 0.05 \); **\( P < 0.01 \), ***\( P < 0.001 \). ns, not significant (\( P > 0.05 \)).
Supplementary Figure 22

Locomotor activity of the control and dHbl-ablated larval fish during light off-on cycles. Open circle, control fish; filled circle, dHbl-ablated fish. a, Total locomotor activity of first 10 min during the dark cycle. b, Total locomotor activity from 14 min through 24 min after the onset of the dark cycle. c, Total locomotor activity during the 30-min bright period. Differences between control and dHbl-ablated larvae were verified using the Mann-Whitney U-test. *P < 0.05; **P < 0.01. ns, not significant (P > 0.05).
Supplementary Figure 23

Exponential decay fit against 5 – 28 min data of average locomotor activities during the dark periods of the light-dark cycles. **a**, The average locomotor activity at each cycle (open circle) was fitted to the curve with the equation, “y=\text{Aexp}(–t/\tau)”, as explained in the Supplementary Methods. (left panel) Results of control fish. R square of fitting: 1st cycle, 0.98; 2nd cycle, 0.98; 3rd, 0.98; 4th, 0.96; 5th, 0.98; 6th, 0.98. (right panel) Results of dHbL-ablated fish. R square: 1st, 0.99; 2nd, 0.97; 3rd, 0.96; 4th, 0.92; 5th, 0.97; 6th, 0.94. **b**, Exponential decay time constant (τ) calculated above. Larger τ means a slower decline in locomotor activity. Bootstrap method was also performed to calculate s.e.m. of τ. Open circle, control fish; filled circle, dHbL-ablated fish.
Supplementary Figure 24

The average ratios of the successful escape behaviors against repeated tail touch were not significantly different between control and dHbL-ablated larvae, suggesting no difference in the endurance capacity of swimming. Two-way repeated-measure ANOVA, the main effect of Habenula state, $F = 0.38, P = 0.54$; Habenula state x time windows, $F = 0.68, P = 0.69$. 
**Supplementary Table 1**
The raw data of immobility times for each individual at each trial during the retrieval session. RT1 – 5 means trial 1 – 5 during the retrieval session. Orange cell, 15 or more than 15 sec immobility; yellow, 1 – 14 sec immobility; blue, zero sec immobility.
Supplementary Results

**Nitroreductase-Mtz-based inducible and specific cell ablation of the dHbl neurons**

Nitroreductase induces cell-autonomous apoptosis following treatment with the substrate, metronidazole (Mtz)\(^1\)\(^,\)\(^2\). The Tg(narp:GAL4VP16;UAS:nfsB-mCherry) fish expressed nitroreductase/mCherry fusion protein specifically in the dHbl neurons at both the larval and adult stages (Supplementary Fig. 14a, b, g, i and 15). As in the adult Tg(narp:GAL4VP16;UAS:TeTxLC) fish (Supplementary Fig. 7), no fusion protein was detected outside the habenula-IPN pathway at the adult stage (Supplementary Fig. 15). These fusion protein-positive neurons were mostly eliminated in the Tg(narp:GAL4VP16;UAS:nfsB-mCherry) larvae after a 16-hour (hr) incubation in 10 mM Mtz (Supplementary Fig. 14c), and the induced apoptosis was detected specifically in the dHbl with a peak around 14 hr of incubation (Supplementary Fig. 14d–f and 16). In the adult Tg(narp:GAL4VP16;UAS:nfsB-mCherry) fish, the dHbl neurons and axons expressing the nitroreductase-mCherry fusion protein were also effectively eliminated in the dHbl (Supplementary Fig. 14g–j). This approach enabled the specific elimination of the dHbl neurons in the adult brain by treatment with Mtz.

**Cued fear conditioning task with Nitroreductase-Mtz-based specific and acute ablation of the dHbl neurons**

In the cued fear conditioning of the ablated fish (n = 13) and the Mtz-treated control fish (n = 11), the dHbl-ablated fish alone showed enhanced immobility (Supplementary Fig. 17a and b), and did not show significant increase in flight behaviors (Supplementary Fig. 17c) (See also Supplementary Data 3 and 4 for further statistical analysis of behaviors). The dHbl-ablated fish exhibited no significant abnormalities in electrical shock sensitivity (Supplementary Fig. 17d) or exploratory behavior (Supplementary Fig. 17e). Although they showed lower locomotor activity than control fish just before the first CS presentation in the retrieval session (Supplementary Fig. 17f), their locomotor activity normalized to the level before the CS presentation was also significantly reduced after the CS presentation in the retrieval sessions (Supplementary Fig. 18b; see also Supplementary Fig. 18a, c and d for the comparison
with the control fish). These analyses revealed that the specific and acute ablation of dHbL also impaired the experience-dependent behavioral modification. Unlike dHbL-chronically-silenced fish, the group of the dHbL-ablated fish showed slight but statistically insignificant increase in flight behaviors on the average during the retrieval session (Supplementary Fig. 17c). However, as far as those dHbL-ablated fish which showed marked increase in freezing are concerned, no increase in the flight behaviors was observed, suggesting that fish tend to make alternative and mutually exclusive choice of taking freezing or flight responses (Supplementary Fig. 19).

**Behavioral analyses of the dHbL-ablated larval fish**

To see whether the dHbL-d/iIPN-GC pathway is involved in responses to stimuli of other sensory modalities than electrical shock, we examined the effect of dHbL ablation in the larval fish on adaptation to sudden environmental shifts to darkness. Both the dHbL-ablated larvae and the control larvae treated with Mtz alone showed a similar degree of light preference (Supplementary Fig. 20a), suggesting that the dark environment is potentially aversive for both fish similarly.

Then we placed them in an environment with cycling changes of 30-min dark and 30-min light. Following the first sudden shift to darkness, both dHbL-ablated and control larvae showed a similar time course of abrupt activation and then gradual decrease in locomotion (Supplementary Fig. 20b). From the second light-to-dark shift onwards, the control larvae showed a more rapid recovery from the state of increased activity, compared with the responses observed in the first shift to darkness (Supplementary Fig. 20b and 21). In contrast, the dHbL-ablated larvae did not show a similar rapid recovery until the sixth shift to darkness (Supplementary Fig. 20b and 21). Comparison of the 14–24 min locomotor activities between cycles revealed that the experience-dependent behavioral modulation was seen in the control fish, which was affected in the dHbL-ablated larvae (Supplementary Fig. 21). For the abrupt activation periods of the first 10 min, there were no significant differences between the ablated and control larvae at each cycle (Supplementary Fig. 22a), while, for the gradual declining periods (14–24 min locomotion), there were significant differences from the second cycle (Supplementary Fig. 22b). Exponential decay fitting to the locomotor activities suggested that the decline in the locomotor activity from the second cycle was more robust in the control fish (Supplementary Fig. 23). This difference was seemingly not
derived from the abnormally increased endurance capacity of swimming, because they both showed a similar time course of fatigue in the touch response (Supplementary Fig. 24). Locomotor activities to the bright phase were almost similar each other (Supplementary Fig. 20b and 22c). These results support the hypothesis that the experience-dependent modification of the innately encoded initial response strategy is affected in the dHbt-silenced fish.
Supplementary Data

Supplementary Data 1
We verified the difference in the CS-evoked immobility measured before and after the conditioning, by using the Fisher’s exact test for the freezing ratios, or by using the Two-way ANOVA of the data for the immobility times. The control fish showed a significant decrease following conditioning ($P = 0.21$, freezing ratios; $F = 4.43$ and $P = 0.04$, the main effect of conditioning for immobility times). In contrast, for the dHbt-silenced fish, there were substantially significant increases ($P < 0.01$, freezing ratios; $F = 16.77$ and $P < 0.0001$, the main effect of conditioning for immobility times).

Supplementary Data 2
During the adaptation session, in which only the CS was exposed to fish, some of both control and dHbt-silenced fish innately showed flight behaviors against the CS. Wilcoxon signed rank tests with a hypothetical value suggested that the degrees of the innate flight behaviors during the adaptation sessions were significantly different from zero in the control fish ($P = 0.03$; mean $\pm$ s.e.m., $0.88 \pm 0.43$) and the dHbt-silenced fish ($P < 0.01$; $1.87 \pm 0.57$). We also compared the innate flight behaviors between them, and there were no significant differences (Supplementary Fig. 5).

Supplementary Data 3
For the data from the experiments of the nitroreductase-based dHbt ablation, the differences in the immobile states before and after conditioning were verified by the Fisher’s exact test for the freezing rations, or by the Two-way ANOVA for the immobility times. For the control fish, there were no significant differences ($P = 1.00$, freezing ratios; $F = 1.38$ and $P = 0.24$, the main effect of conditioning for immobility times), while for the dHbt-ablated fish, there were significant increases ($P = 0.03$, freezing ratios; $F = 3.94$ and $P = 0.049$, the main effect of conditioning for immobility times).

Supplementary Data 4
In the experiment of the nitroreductase-Mtz based ablation, the averages of the flight behaviors during the adaptation session were almost zero in both control and dHbl-ablated fish (mean +/- s.e.m. of control fish, 0.0909 +/- 0.2402; Wilcoxon Signed Rank Test of control with 0.0 as a theoretical median, \( P = 0.70 \)) (mean +/- s.e.m. of dHbl-ablated fish, 0.1846 +/- 0.4191; Wilcoxon Signed Rank Test, \( P = 0.41 \)). This difference from the TeTxLC-based experiments might be derived from the additional handling and/or stress during the Mtz treatment.

**Supplementary Data 5**

We compared locomotor activities of the dHbl-ablated (n = 41) and control larvae (n = 50) during light off-on cycles. Two-way repeated-measure ANOVA was used against whole data from each dark period: first cycle, Habenula state x time windows, \( F = 0.51, P = 0.99 \); Habenula state, \( F = 0.93, P = 0.34 \); second, Habenula state x time windows, \( F = 1.62, P = 0.02 \); Habenula state, \( F = 4.56, P = 0.04 \); third, Habenula state x time windows, \( F = 1.01, P = 0.45 \); Habenula state, \( F = 1.72, P = 0.19 \); fourth, Habenula state x time windows, \( F = 1.70, P = 0.01 \); Habenula state, \( F = 3.18, P = 0.08 \); fifth, Habenula state x time windows, \( F = 1.95, P < 0.01 \); Habenula state, \( F = 3.18, P = 0.08 \); sixth, Habenula state x time windows, \( F = 0.43, P = 1.00 \); Habenula state, \( F = 3.47, P = 0.07 \).
Supplementary Methods

Animals

Zebrafish (Danio rerio) were bred and raised under standard conditions. Embryos for behavioral studies were raised at 28.5°C under standard 14-hr light/10-hr dark cycling (lights on at 9:00 and off at 23:00). Juvenile and adult zebrafish were maintained in 1- and 7-l tanks, respectively, with continuous water exchange under the same light/dark cycles and temperature. Occasionally, we added 0.002% of phenylthiourea to the fish water from 12 hours post-fertilization to prevent larval pigment formation and to observe the fluorescent protein expressed in live larvae. All protocols were reviewed and approved by the Animal Care and Use Committees of the RIKEN Brain Science Institute.

Tract tracing in fixed fish with carbocyanines

Tg(brn3a-hsp70:GFP)TW0110b fish express GFP specifically in the dHbM, which sends axons to the ventral and intermediate parts of the IPN in the ventral midbrain. Transgenic fish were anesthetized by immersion in water containing 150 mg/l 3-aminobenzoic acid ethylester and then decapitated. The brain was removed and immersed in 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS; pH 7.4) at 4°C for 16 hr. For the anterograde labeling from the IPN, we inserted a crystal of 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI; Invitrogen, Carlsbad, CA) with a fine glass needle into either the dIPN under guidance of the GFP signal. For injections from the rostral aspect of dIPN, we embedded brains in 2% agarose and cut coronally using a vibrating microtome (Dosaka EM, Kyoto, Japan) from the rostral side until the GFP signal in the vIPN was apparent. We incubated injected brains at 37°C and then cut them into 100-µm sections on the same vibrating microtome. For the retrograde labeling from the MR, we injected iontophoretically 0.5% DiI dissolved in ethanol with using a glass micropipette while applying 60–80 μA of direct current for 1–4 min. We counterstained some sections with Neurotrace Fluorescent Nissl stain (Invitrogen). We observed fluorescence with a confocal laser scanning microscope (LSM510META; Zeiss, Jena, Germany).
Tract tracing in live fish

Fish were anaesthetized by immersion in water containing 150 mg/l 3-aminobenzoic acid ethylester and then positioned in a physical restraint device. During surgery, the gills were perfused with anesthetic in fish water to keep the fish immobile and to enable artificial respiration. We removed manually a dorsal portion of the cranium, and injected 2% Neurobiotin (Vector Laboratories, Burlingame, CA) iontophoretically using a glass micropipette filled with the dye dissolved in 0.5 M NaCl. We positioned the electrode tip 1.2 mm posterior to the pineal gland, 0 mm from the midline, and 0.7 mm from the brain surface using a stereotaxic apparatus (Narishige, Tokyo, Japan), and passed direct current (5 μA) through the electrode for 5 min (4 sec on/4 sec off). After removal of the electrode, the fish were perfused with fish water without anesthetic for recovery, and then maintained postoperatively in a tank with fish Ringer’s solution for 8–12 hr, before being deeply anaesthetized and then decapitated. The brains were immersed in 4% PFA in PBS for 16 hr and 60-μm sections were cut with a vibrating microtome for staining by the ABC technique (Vector Laboratories) and neutral red counterstaining. Images were taken on a Sony DKC-5000 camera (Sony, Tokyo, Japan) attached to a Zeiss Axioplan2 compound microscope (Zeiss).

Transgenesis

The narp-containing BAC clone zc284F1 was identified by blasting the narp (or neuronal pentraxin II, Genbank accession number NM_001109768) cDNA sequence against zebrafish genomic sequences of Ensemble (v38, http://apr2006.archive.ensembl.org/index.html), which provides the BAC end information. The GAL4VP16/poly(A) sequence and the kanamycin-resistance gene were inserted into the multiple cloning sites of the pBluescript (stratagene) in tandem, to generate pGAL4VP16-Km. Using pGAL4VP16-Km, a BAC plasmid containing the GAL4VP16 sequence (pNarpB:GAL) at the ATG site of the narp gene was generated by homologous recombination of zc284F1, as described previously. We injected purified pNarpB::GAL into zebrafish embryos as described previously, and obtained three transgenic lines. One line showed very specific expression pattern in the dHbt, and thus was used for the present study, while other two lines showed similar expression patterns.
with more nonspecific expression.

To visualize GAL4VP16 expression, we generated a transgenic line encoding the DsRed2 gene under the UAS promoter using the pDsRed2-1 plasmid (Clontech). This transgenic line Tg(UAS:DsRed2)$^{rw0135}$ was crossed with Tg(narp:GAL4VP16)$^{rw0143a}$ and Tg(brn3a-hsp70:GFP)$^{rw0110b}$ 4. Tg(UAS:nfsB-mCherry)$^{rw0144}$ was generated using a plasmid containing the nfsB-mCherry sequence under the UAS promoter sequence7, kindly provided by Dr. Micheal Parsons. All transgenic lines were generated with the Riken-Wako strain, and Tg(UAS:DsRed2)$^{rw0135}$ and Tg(UAS:nfsB-mCherry)$^{rw0144}$ were generated using the Tol2 transposon-mediated system9 for higher efficiency of transgenesis. Tg(brn3a-hsp70:GFP)$^{rw0110b}$ 4 and Tg(UAS:TeTxLC)$^{10,11}$ were generated previously.

**Cued fear conditioning task**

We used adult female zebrafish, 10–12 months old, for the behavioral experiments. We designed the experiments involving fear conditioning tasks based on similar studies in medaka (Oryzias latipes)$^{12}$, and performed them in a soundproof chamber (Muromachi Kikai Co., Ltd., Tokyo, Japan) in a soundproof room. We used a white opaque acrylic box (dimensions: 100 mm W x 200 mm D x 100 mm H, water level: 37 mm) divided into two compartments of equal size to monitor the behaviors of two individual fish simultaneously. The bottom of the box was constantly illuminated with the white LED lamps (550 lux). The US (a 1.0 sec, 5-V AC shock) was delivered through stainless-steel mesh electrodes positioned on the longer sides of the box. The CS lights were delivered by 6-V, 19-mA red LED lamps through transparent small windows positioned on the shorter sides. When the CS lights were turned off, 6-V, 24-mA white LED lamps were exposed through the opaque wall on the same side as the CS lamps, to diminish the difference in brightness during the behavioral tasks. The onsets and durations of the CS and the US were controlled using a system purchased from Neuroscience (Tokyo, Japan). After 10 min in the compartment, fish were subjected to an adaptation session towards the CS, wherein the CS was delivered for 8.5 sec, and fish received five CS-only exposures in total. After a 10-min intersession interval, fish were subjected to the conditioning sessions. In each trial, the CS was delivered for 8.5 sec, with the last 1.0 sec overlapping with the US. The intertrial
interval within a session of 5 trials was 180 sec. After two such sessions, which were
separated by a 30-min intersession interval, fish were subjected to the 30-min
intersession interval, followed by the retrieval session. In the retrieval session, fish were
exposed to the CS for 8.5 sec five times, with a 51.5-sec intertrial interval. Fish
behaviors were monitored from above, and the movies were analyzed offline with
Move-tr/2D (Library Ltd, Tokyo) and Excel (Microsoft). The centre of gravity of the
fish at each time point was defined as the fish position for the measurement of fish
locomotion. Fish angle was also measured from the movie with Move-tr/2D.

The exploratory behavior (preference for the centre of the tank)\textsuperscript{13} was defined as
time spent in the central 48 mm x 48 mm field in each compartment.

The US-triggered locomotion was calculated from the locomotion distance during
the 1.0-sec US exposure, to determine the US sensitivity.

To analyze the freezing response, locomotion data were divided into 1.0-sec bins,
and a bin with less than 2.5 mm locomotion was defined as a “no movement” bin. The
animals were considered to be freezing if no movement was detected in 15 or more bins
for the 50 sec following the US exposure (from 10 to 60 sec after the US). Immobility
time was also calculated by the summation of the number of the no movement bins at
each trial.

Turning reactions during the CS presentation were determined by measuring the
difference of the fish angle between one frame and another frame taken 0.2 sec later. A
difference of more than 90° was counted as a turn. The turns for 7 sec before and after
each CS onset were counted, and the difference (“After” minus “Before”) was defined
as CS-evoked flight behavior (change in turning frequency) for each trial.

\textit{Nitroreductase-metronidazole mediated cell ablation in larvae}

Nitroreductase-metronidazole-mediated cell ablation is known to effectively induce
cell death in nitroreductase-expressing cells in zebrafish\textsuperscript{1, 2, 14, 15}. Metronidazole (MtZ,
M3761, Sigma) was administered to the 5-day post-fertilization (dpf) larval fish at 10
mM in the fish water for 24 hr in the dark, followed by several washes in the fish water.
Two hours after completion of the washes, the fish were used for the periodical
dark-light change experiments. For the light preference test, fish were used one day
after the washes.
Nitroreductase-metronidazole mediated cell ablation in adult fish

Fresh 10 mM Mtz was dissolved in fish water with 0.2% DMSO, and fish were treated with the solution for 24 hr in the dark without feeding. Three fish were treated in one tank (500 ml solution). After the treatment, 500 ml fish water was added to the tank, and then the solution in the tank was replaced with fresh fish water. One hour after the replacement, fish were washed again, in total three times. Then they started to be fed, and were placed in fresh fish water overnight without water circulation. After a further wash, the fish were placed under water circulation. The time for recovery before fear conditioning tasks was seven days in total after the Mtz treatment. During the recovery, the groups of three fish were kept in the same tank, and then were used for the behavioral tests. Control fish (nitroreductase-mCherry signal negative sibling fish) were simultaneously treated with Mtz, and tested in a same time course.

TUNEL assay

Mtz-treated larvae were fixed with 4% PFA in PBSTw, and the brains were isolated for TUNEL assays. Following a methanol permeabilization, samples were treated with 3% H$_2$O$_2$ in methanol for 15 min, and then rehydrated. Samples were then incubated in PBSTw containing 10 mg/ml Proteinase K for 10 min, treated with 2 mg/ml glycine in PBSTw for 2 x 5 min, incubated in 4% PFA, and finally washed several times in PBSTw. Samples were then incubated in TUNEL reaction mixture (In Situ Cell Death Detection Kit – POD, Roche Diagnostics) for 60 min at 37°C in the dark. After several washes in PBSTw and incubation in PBSTw/1% Blocking Reagent (Roche Diagnostics), samples were treated with anti-fluorescein-POD (1:1000, Roche Diagnostics) overnight. Samples were washed several times in PBSTw, and stained in 0.5 mg/ml 3,3'-diaminobenzidine (DAB) with 0.0003% H$_2$O$_2$ in PBSTw. Finally, pigments on the sample surfaces were bleached by overnight incubation in 1% H$_2$O$_2$ and 0.25% formamide in 0.0128 x SSC solution.

Behavioral analysis of larval fish

We performed behavioral analysis of the larval fish as described previously. A single larva was placed in each well of a 96-well plate and locomotor activity was
monitored by an automated video-tracking system (Videotrack; ViewPoint Life Sciences, Quebec, Canada)\textsuperscript{16}, with the movement of each larva recorded in the Videotrack quantitation mode. The temperature of the experimental room was maintained at 26–28°C. The Videotrack quantitation parameters were set as follows: detection threshold, 140–190; bin size, 60 sec. Fish locomotion was detected with the threshold for the fish speed (0.5 for inactive/small). To analyze the adaptation of larval fish to darkness, 6-dpf fish placed in 96-well plates were observed in the light starting at 14:00 and exposed to six consecutive 30-min light-off and -on cycles from 16:00, using an automated timer to regulate each transition. The data were further analyzed using Excel (Microsoft, Seattle, WA), and the statistical analyses were performed using Prism 4 (GraphPad Software) or Matlab (MathWorks). The average of each trial’s declining locomotor activity during a 5–28 min period in the dark phase was fitted by a single exponential function as described below, with a decay time constant $\tau$.

$$y = A \cdot e^{-t/\tau}$$

$y$, locomotor activity  
$A$, initial amplitude  
$t$, time after the onset of the dark phase  
$\tau$, decay time constant

Standard error of $\tau$ was estimated by bootstrapping (1000 samples).

To analyze the light-dark preference, we used 7-dpf naive fish and performed the experiments during the daytime (from 9:00 am to 20:00 pm). Fish were placed in a round field (internal diameter: 21 mm, water level: 8 mm), which was divided at the middle into two compartments, bright and dark. The bottom of the bright compartment was transparent, while the dark compartment was covered with infrared-transparent black acrylic board, which allowed infrared radiation to pass through the bottom and thus enabled the detection of fish locomotion. The behaviors of 8 individuals were detected simultaneously as described above. The light preference index (LPI) was
calculated by the following equation:

\[ LPI = \frac{L - D}{L + D} \]

L, time spent in the light compartment
D, time spent in the dark compartment

To test endurance capacity for swimming, we performed consecutive touch experiments. The 6-dpf fish used for the light-dark cycle experiments were used after 14 hr of continuous incubation in the dark and consecutive light period for more than 7 hr. Fish were placed in the center of the round field (internal diameter: 39 mm, water level: 2 mm), under the video camera for recording. We then touched the tail of the fish to force swimming, about every 1.5 sec, and 80 times in total. The average ratios of the successful escape behaviors against every ten repeated tail touch were scored.

Brightness during each larval experiment was measured by the luminometer (ASONE, Osaka, Japan). In the light-dark cycle, illumination during the light period was 50.0 lux at the center of the recording field, while it was 0.0 lux during the dark period. During the experiments to assess the endurance capacity for swimming, the field was illuminated by about 550.0 lux white LED light. In testing the light-dark preference, illumination toward the bright compartment was 50.0 lux, while the black acrylic board used for the dark compartment completely blocked the illumination (0.0 lux), although fish might have detected some light from the bright compartment.

**In situ hybridization**

*In situ* hybridization for embryos and larvae, and vibratome sectioning of the adult brain, were performed as described previously\(^3,17\), with samples prepared and observed as described previously\(^18\). *narp* (NP_001103238) and *tetanus toxin light chain*\(^19\) were used as probes.

**Statistical analysis**

All statistical analyses were two-sided, and conducted as indicated in the text, figure legends, and supplementary materials. Statistical analyses were mainly performed with
Prism 4 (GraphPad Software). For the curve fitting and the bootstrap method, Matlab (MathWorks) was used.
Supplementary References

12. Eisenberg, M., Kobilo, T., Berman, D. & Dudai, Y. Stability of retrieved


Supplementary Movie Legends

**Supplementary Movie. 1**
Response of the control fish at the first trial of the retrieval session.

**Supplementary Movie. 2**
Response of the dHbL-silenced fish at the first trial of the retrieval session.

**Supplementary Movie. 3**
Persistent rotation of the dHbL-silenced fish at the first trial of the retrieval session.