Supporting Information

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SI Materials and Methods

***Drosophila* Culture and Transformation.** Fly stocks and crosses were raised at 25 °C on standard corn meal-yeast-agar medium supplemented with methyl-4-hydroxy-benzoate as a mold protector under a 12-h/12-h light/dark cycle and at around 50% humidity. *Drosophila* transformants were obtained by standard procedure: w^{1118} embryos were microinjected in their posterior pole with 375 μg/mL transformation vector and 80 μg/mL helper plasmid pπ25.7wc (1) as a source of transposase.

***Drosophila* Strains.** The following fly strains were used: Canton S for behavior tests and w^{1118} for HPLC assays as WT flies, *TH-GAL4* (2), *Ddc-GAL4* (3), *UAS-DTHg* (4), *UAS-DTHg*$^{FS+}$ and *UAS-DTHg*$^{FS\pm}$ (see below). The *ple*^{2cl} chromosome (2), which contains the null *DTH* mutant allele *ple*^{2} (5) cleared from other chromosomal mutations, was kept balanced by the TM6B (*Tb*) chromosome. *DTHg*- and *DTHg*$^{FS\pm}$-rescued homozygous *ple Drosophila* were generated by crossing a line containing the combined *TH-GAL4* and *Ddc-GAL4* drivers with the respective *UAS* transgene, each in a heterozygous *ple* mutant background. Because both male and female flies are fertile, *DTHg*; *ple* and *DTHg*$^{FS\pm}$; *ple* can be maintained as stable lines. To reduce the risk of genetic compensation over generations, however, we preferred to generate them by crossing heterozygous *ple* progenitors before each experiment. The *DTHg*$^{FS\pm}$; *ple*/+ flies generated in these crosses were used as heterozygous *ple* controls in Fig. 1*B* and Fig. S6. If not stated otherwise, all experiments were performed with 3- to 5-d-old adult female flies.

DNA Constructs. Frameshift mutations were introduced in vitro in the *DTH* gene by a modification of the three-primer PCR mutagenesis procedure (6) as described previously (7). Mutagenizing antisense *DTH* primers OTH^{+} (5′-GCTGTTGTGTTGCCTTCTGG) and OTH^{-} (5′-CCAACAAAA_CTCGTCCTCGG) were used, respectively, to introduce a base near the end of exon d or to remove a base at the beginning of exon e (Fig. S2*B*). Mutated *DTHg*$^{FS+}$ or double-mutated *DTHg*$^{FS\pm}$ segments were then inserted in place of the corresponding WT sequence in the *pUAS-DTHg* vector (4), generating *pUAS-DTHg*$^{FS+}$ and *pUAS-DTHg*$^{FS\pm}$. These mutations were checked by PCR and confirmed by double-stranded DNA sequencing before *Drosophila* transformation. *DTHg*$^{FS+}$ produces a mutated hypodermal enzyme isoform (DTH2^{FS+}) that is truncated before the catalytic domain and terminated by a nonsense mutation (Fig. S1 *C* and *D*) and a normal neural DTH1 isoform. *DTHg*$^{FS\pm}$ produces a mutated hypodermal enzyme isoform (DTH2$^{FS\pm}$) that contains 15 missense amino acids in a noncritical region between the regulatory and catalytic domains (Fig. S1 *C* and *D*) and a truncated inactive CNS splice isoform.

***ple* Rescue.** To compare the *ple* rescue capability of different *GAL4* driver/*UAS-DTH* combinations, the following procedure was used as described previously (4). First and second chromosome insertions of the *X-GAL4* (i.e., *Ddc-GAL4*, *TH-GAL4*, or both) and *UAS-DTH* transgenes were homozygosed in *ple*^{2cl}/TM6B (Tb, Hu, e) background, and third chromosome insertions were recombined to the *ple*^{2cl} chromosome and balanced by TM6B. *X-GAL4*, *ple*/TM6B virgin female flies were mated to *UAS-DTH*, *ple*/TM6B male flies, and reciprocal crosses were set up (i.e., *UAS-DTH*, *ple*/TM6B virgin female flies mated to *X-GAL4*, *ple*/TM6B male flies). Rescue from *ple* embryonic lethality was determined by scoring the relative number of homozygous *ple* (i.e., *Tb*$^{+}$, *Hu*$^{+}$) pupae and adults recovered in the progeny of these crosses.

RT-PCR. RNA was extracted from 15 adult heads or 25 dissected adult brains by the TRIzol (Invitrogen) procedure (8). The final RNA pellets were washed in 75% (vol/vol) ethanol before resuspension in diethylpyrocarbonate-treated water. After annealing with a *DTH* antisense primer (OTH5, exon e, 5′-ATTGCGATCTCGGCAATTTCCTTGCGACGCTGGC), RT was carried out in the presence of SuperScript II RNase H-Reverse Transcriptase (Invitrogen). PCR primers (OTH2, *DTH* antisense exon e, and OTH3, *DTH* sense exon a) and reaction conditions were as previously described (9), leading to the amplification of a 195-kb fragment and a 408-kb fragment from the DTH1 and DTH2 cDNAs, respectively.

Immunohistochemistry. According to the experiments, the secondary antibodies were goat anti-mouse, anti-rabbit, or anti-rat IgG conjugated to Alexa Fluor 488 or 555 (1:500; Invitrogen Molecular Probes). For TH, Ddc, serotonin, and GFP immunostaining, brains were dissected in *Drosophila* Ringer's solution [130 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 0.3 mM Na_2HPO_4, 0.35 mM KH_2PO_4 (pH 7.5)] and fixed for 2 h on ice in 4% (wt/vol) paraformaldehyde in PBS (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM KH_2PO_4), followed by three 20-min washes in PBS. After 2 h of preincubation in PBS containing 0.5% Triton X-100 (PBT) plus 2% (wt/vol) BSA, brains were incubated overnight at 4 °C with the primary antibodies diluted in the same solution. This was followed by three 20-min washes in PBT, a 2-h incubation with the secondary antibodies at room temperature, three 20-min washes in PBT again, and one final wash in PBS. For DA immunostaining, brains were dissected in Ca^{2+}-free *Drosophila* Ringer's solution supplemented with 1.25% (wt/vol) glutaraldehyde plus 1% sodium metabisulfite ($Na_2S_2O_5$) (Sigma) to protect DA from oxidation and then were fixed in the same solution for 3 h on ice. After three 20-min washes in PBS, brains were incubated for 20 min in PBS containing 0.5% sodium borohydride ($NaBH_4$) to saturate double bonds, followed by 20 min in PBS plus 1% (wt/vol) $Na_2S_2O_5$. After overnight preincubation in blocking solution (PBS + 2% (wt/vol) BSA), brains were incubated for 2 d at 4 °C with the primary antibody diluted in the same solution and then washed three times in PBS at room temperature before being incubated for 2 d with the secondary antibody in PBS. Brains were finally washed in PBS for 2 d at 4 °C to reduce background. Tissues were mounted in Vectashield (Vector Laboratories). Confocal microscopy was performed with a Zeiss LSM 510 microscope.

Lifespan Analysis. Survival in standard conditions was determined at 25 °C on a population of 100–125 female flies of each genotype divided in five batches. Each batch was treated independently. Newly eclosed flies were placed in vials and transferred every 2–3 d into fresh vials to minimize death caused by bacterial infection or moist medium. The number of surviving flies was recorded every 2 d. Survival curves represent the percentage of surviving flies as a function of time.

Locomotor Behavior. Two methods were used to test for spontaneous locomotor behavior of 2- to 5-d-old adult *Drosophila*. First, walking speed and covered distance were computed from video-based recordings of individual flight-disabled flies (wings cut to one-third of length 1 d before the experiment under cold anesthesia) walking freely for 15 min in an open arena (10). Path lengths were calculated for five successive 3-min intervals of undisturbed walking and averaged over individuals. Walking speeds

were calculated for every transition between the landmarks. Because some data were not normally distributed (Shapiro–Wilks W test), we used the Mann–Whitney U test for pairwise comparison ($n = 13$ flies). Alternatively, activity was monitored by recording infrared beam crossings in glass tubes (6.5-cm length, 3-mm inside diameter). In this case, events were scored at 1-min intervals for 3 d (with light/dark or dark/dark circadian cycles) using a *Drosophila* activity monitoring system (DAM; TriKinetics) (11). To analyze the effect of drugs on locomotor activity, 2- to 3-d-old male flies were placed in monitor tubes in which regular food was replaced by either sucrose alone (control) or sucrose supplemented with 0.5 mg/mL caffeine or 3 mM 3IY, a TH inhibitor.

Negative Geotaxis. Startle-induced negative geotaxis was assayed as previously described (2). Groups of 10–20 flies were placed in a vertical column (25 cm long, 1.5 cm in diameter) with a conical bottom end. They were suddenly startled by gently tapping them down, to which *Drosophila* respond by climbing up. After 1 min, flies having reached the top of the column (above 20.5 cm) and flies remaining at the bottom end (below 6.5 cm) were separately counted. Three trials were performed at 1-min intervals. The scores are the mean of the numbers of flies at the top (n_{top}) and bottom (n_{bot}), expressed as percentages of the total number of flies (n_{tot}). Results are the mean ± SEM of the scores obtained in three independent experiments. The PI is defined as $1/2((n_{tot} + n_{top} - n_{bot})/n_{tot})$. To test for the effect of aging on negative geotaxis, 30 flies of each condition were evaluated each week throughout a period of 8 wk. Dead flies were replaced by substitutes of the same age. Statistical analysis was performed on the PIs with the Student's t test.

Sleep and Arousal. Circadian activity was followed with a DAM system for 72 h at 18 °C in a 12-h/12-h light/dark cycle. Individual flies were placed in small glass tubes (5-cm length, 3-mm inside diameter) containing food medium with an infrared laser beam passing through the midline of the tubes. Apparent sleep was defined as periods of 5 min or longer without laser beam breaks. To monitor sleep by direct observations, individual flies placed in similar tubes were visually observed for 30-min periods during day [Zeitgeber time (ZT) 8–10] or night (with a red light, ZT 14–15) to determine the duration of quiescent intervals. Sleep was defined as periods of 5 min or longer without any movement. Similar results were obtained with three independent batches of flies. Arousal state was evaluated by applying mechanical stimulations during sleep as previously described (11). Mild and moderate stimuli were delivered by quickly removing 7- or 16-mm thick plastic cushions, respectively, from below the monitors. Flies maintained in 12-h/12-h light/dark cycles were stimulated every 2 h with mild stimuli on days 1 and 3 and with moderate stimuli on days 2 and 4. Results were classified in three categories: "awake" (flies that moved at least once during the 10 min that preceded stimulation), "responding" (flies that started moving within 1 min after stimulation and crossed the laser beam at least twice), and "not-responding" (flies that did not move or moved only once after stimulation).

Olfactory Conditioning. To test for aversive olfactory learning, groups of about 35 flies were conditioned in a barrel-type machine as described (12) by sequential exposure to two odors, octanol (OCT) and methylcyclohexanol (MCH), for 60 s with 45-s rest intervals between odor presentation. Exposure to the first odor was paired with electrical shocks (twelve 1.5-s pulses of 60 V dc with 5-s intervals between pulses). Flies were tested for odor avoidance by simultaneous exposure to both the shocked and control odors in a T-maze (13), either immediately ($t = 0$) or 2 h after conditioning. After 1 min, the flies trapped in either T-maze arm were scored. In each experiment, a reciprocal conditioning was run with different flies of the same genotype, by applying electrical shocks during exposure to the second odor, to rule out bias from odor preference. The results are the mean of 6 to 11 independent experiments. The PI corresponds to an averaged and normalized probability of the correct answer, such that a 50:50 distribution (no memory) would yield PI = 0 and total avoidance of the shock-paired odor would yield PI = 1 (13).

Odor and Electrical Shock Avoidance. Both OCT or MCH are naturally slightly aversive to unconditioned flies. To test for olfactory acuity, the odor avoidance responses were quantified by giving naive flies a choice between odor and air in a T-maze. After 1 min, the flies were trapped in their respective tubes and counted. The PI was calculated as the normalized percentage of flies avoiding the odor. Two groups of the same stock were run successively, and the side of the test tube with odor was alternated. To remove odor traces from the previous run before each experiment, odor and fresh air, respectively, were aspirated through the relevant test tubes for 1 min in the absence of flies. Electrical shock avoidance of untrained flies was quantified by giving them a choice between "shock" and "no shock" for 1 min in a T-maze. Two barrels, identical to those normally used for conditioning, were connected to each other by a Plexiglas dish. Flies were trapped in the center of the compartment and allowed to choose for 1 min between the two barrels, one of which was electrified with pulses of 60 V DC identical to those normally used for conditioning. The PI was determined as for olfactory avoidance.

L-DOPA Treatment. Flies were incubated for 72 h at 25 °C on a piece of Whatman 3MM paper soaked with a solution of 5% (wt/vol) sucrose, 2% (wt/vol) yeast, and 10 mM sulforhodamine B (Sigma) containing or not containing (control) 1 mg/mL L-DOPA (Sigma). Before the behavior tests, flies were visually selected under CO_2 for sulforhodamine B staining in the abdomen to check for feeding and left to recover from anesthesia for at least 4 h in vials containing Whatman 3MM paper soaked with the same sucrose solution with or without L-DOPA.

Sugar Preference. Flies were starved for 21 h at 25 °C and 80% humidity. Their responses to sugar were quantified by giving them a choice between sucrose and an empty tube in a T-maze as described previously (14). After 1 min, the flies were trapped in their respective tubes and counted.

Phototaxis. Phototactic behavior was tested by mass and single-fly assays. For both assays, flies were raised in 12-h/12-h dim light/darkness conditions. Before the tests, 3- to 5-d-old adult female flies were selected under CO_2 and allowed to recover in fresh food vials for at least 3 h in darkness. The mass assay was carried out in a dark room with the countercurrent procedure described by Benzer (15). The apparatus consists of two rows of six relocatable horizontal tubes, one row proximal (labeled A1–6) and one distal (labeled B1–6) to the light source (a 4-V blue light diode was placed at a distance of 15 cm from the apparatus). Any tubes of each row can be connected to form double tubes. To start the experiment, 10 female flies were placed inside the A6–B1 double tube and brought to the distal end of tube B1 by gentle tapping. They were allowed to distribute freely for 1 min. Thereafter, swift sliding of the rows let tube A6 join tube B2 and tube A5 connect to tube B1. Again, the flies were gently tapped to the distal end of the two connected double tubes and then were allowed to distribute freely for 1 min. After six cycles, all the tubes were connected and the number of flies present in each double tube (e.g., A1–B1, A2–B2) was scored. In each experiment, the results were the mean of the scores from five trials. Data were statistically analyzed with a one-way ANOVA (Bonferroni-corrected). The single-fly phototaxis assay was performed using a T-maze system in which flies choose freely between an illuminated vial (12-V blue light) and a dark vial, as described by

Le Bourg and Buecher (16). Individual flies were transferred without anesthesia into a 1-mL syringe connected to the apparatus and gently pushed to the starting point of the maze. A trial ended when the fly reached one of the vials, and it was then recovered. The test was repeated five times with each fly (n = 16), and the percentage of photopositive choices was scored. Data were analyzed with the Wilcoxon signed-rank test, mean-tested against the reference constant (50% for equal distribution).

Electroretinograms. Electroretinograms (electrophysiological recordings from the surface of the eye) were performed on 3-d-old flies, attached to a holder by dental cement (Protemp). Borosilicate glass microelectrodes (Clark) filled with physiological saline solution (200 mM NaCl, 2 mM $CaCl_2$, 5 mM KCl) were placed in the thorax (reference electrode) or just beneath the cornea in the center of the eye (recording electrode, tip resistance ~10–15 MΩ). Recordings were performed at 19 °C after at least 20 min of dark adaptation under dim red light. A lamp equipped with a mechanical shutter was used for stimulation.

Optomotor Assay. Visual movement responses of walking flies were measured as described (17). In this paradigm, tethered flies walked on an 8-mm Styrofoam sphere supported by a gentle stream of humidified air in the center of a rotating striped drum (20 black stripes, spatial wavelength = 18°). The rotations of the Styrofoam ball were recorded optoelectronically, providing a quantitative measure of the turning responses. Performance depends on the contrast in the environment and increases with increasing light intensity.

Visual Fixation and Orientation. Orientation toward a landmark was analyzed in the Buridan's paradigm (10, 18) on freely walking 5-d-old *Drosophila* with shortened wings. Single flies were confined to an elevated circular arena (8.5-cm diameter) surrounded by a water-filled moat and, beyond, a white cylindrical screen containing two opposing vertical black stripes. In this situation, WT flies spontaneously walk from one of these inaccessible landmarks to the other. Trajectories were recorded from above at 200 frames per second with a video-scanning device. The angular deviation between fly trajectory and the approached target was measured every 0.2 s for 15 min. The curve for random orientation was calculated as described by Strauss and Pichler (10).

Detour Paradigm. Spatial orientation memory was tested in the detour paradigm as recently described (19). The device was comparable to that of the Buridan's test with a larger arena (16-cm diameter). As soon as the fly had traveled once between the two inaccessible vertical stripes and crossed the midline again, the landmarks disappeared and, simultaneously, a vertical distracter stripe appeared laterally at a 90° angle to the fly. After the fly approached for 1 s toward this landmark, the distracter stripe also disappeared and no stripes were visible thereafter. In this situation, a WT fly will turn back to the previous, still invisible, landmark, demonstrating memory of its anterior spatial orientation. Such a behavior was quantified by measuring the angle deviation between the actual trajectory of the fly and the direction of its initial target [further details are provided by Neuser et al. (19)]. Ten consecutive trials of at least 10 flies per genotype were recorded. Because some data were not normally distributed (Shapiro–Wilks W test), we used the Mann–Whitney U test for statistical analysis.

Food Intake Assay. For each determination, ten 3- to 5-d-old flies were starved for 1 h at 25 °C before being incubated for 20 min on a piece of Whatman 3MM paper soaked with a solution of 5% (wt/vol) sucrose, 2% (wt/vol) yeast, and 10 mM sulforhodamine B supplemented or not with 1 mg/mL L-DOPA. They were frozen at −20 °C for 2 h; then the heads were removed to prevent contamination by eye pigments, and the bodies were homogenized in 0.5 mL of PBS. After centrifugation at 17,000 × g in an Eppendorf 5415 R microcentrifuge for 7 min at 4 °C, the supernatant was collected, mixed with 0.2 mL of chloroform, and microcentrifuged again for 6 min. Optical density of the supernatant was determined with a NanoDrop (ThermoScientific) at 570 nm. Results are the mean of three independent determinations of each genotype.

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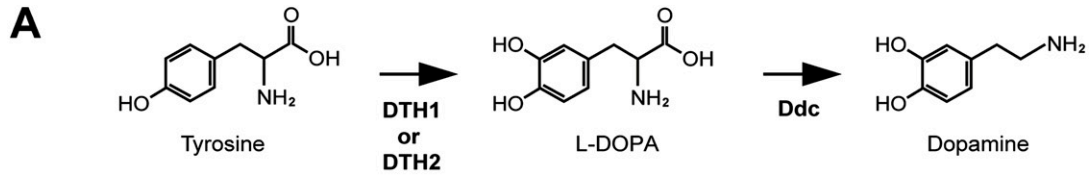

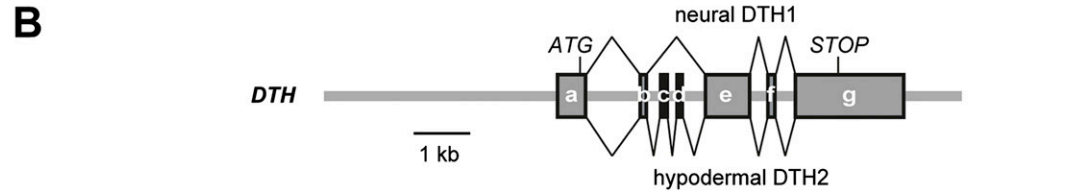

C

		Active TH expression	
		CNS	HYP
DTHg	UAS	yes	yes
DTHg^{FS+}	UAS +	yes	no
DTHg$^{FS\pm}$	UAS + -	no	yes

D

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DTH2..........MMAVAAAQKNREMFAIKKSYSIENGYPSRRRSLVDDARFETLVVKQTKQTVLEEARSKANDDSLEDCIVQAQEHIPSEQDVELQDEHANLENLPLEEYVP
DTH2FS±.......MMAVAAAQKNREMFAIKKSYSIENGYPSRRRSLVDDARFETLVVKQTKQTVLEEARSKANDDSLEDCIVQAQEHIPSEQDVELQDEHANLENLPLEEYVP
DTH2FS+.......MMAVAAAQKNREMFAIKKSYSIENGYPSRRRSLVDDARFETLVVKQTKQTVLEEARSKANDDSLEDCIVQAQEHIPSEQDVELQDEHANLENLPLEEYVP

DTH2 .........VEEDVEFESVEQEQSESQSQEPEGNQQPTKNDYGLTEDEILLANAASESSEAEAAMQSAALVVRLKEGISSLGRILKAIETFHGTVQHVESRQSRVEGVD
DTH2FS±.......VEEDVEFESVEQEQSESQSQEPEGNTTADQERLRSHRGRVLLANAASESSEAEAAMQSAALVVRLKEGISSLGRILKAIETFHGTVQHVESRQSRVEGVD
DTH2FS+.......VEEDVEFESVEQEQSESQSQEPEGNTTADQERLRSHRGRDFVGQCRLRILGSRGCHAECRFGGPPQGGHLLLGSHPQGHRNLPRHRPACGVPSVARGGRG

DTH2 .........HDVLIKLDMTRGNLLQLIRSLRQSGSFSSMNLMADNNLNVKAPWFPKHASELDNCNHLMTKYEPDLDMNHPGFADKVYRQRRKEIAEIAFAYKYGDPIPF
DTH2FS±.......HDVLIKLDMTRGNLLQLIRSLRQSGSFSSMNLMADNNLNVKAPWFPKHASELDNCNHLMTKYEPDLDMNHPGFADKVYRQRRKEIAEIAFAYKYGDPIPF
DTH2FS+.......PRCPHQVGHDPWQSAAADPLPQAVGLLQQHESDGRQ

DTH2 .........IDYSDVEVKTWRSVFKTVQDLAPKHACAEYRAAFQKLQDEQIFVETRLPQLQEMSDFLRKNTGFSLRPAAGLLTARDFLASLAFRIFQSTQYVRHVNSPY
DTH2FS±.......IDYSDVEVKTWRSVFKTVQDLAPKHACAEYRAAFQKLQDEQIFVETRLPQLQEMSDFLRKNTGFSLRPAAGLLTARDFLASLAFRIFQSTQYVRHVNSPY

DTH2 .........HTPEPDSIHELLGHMPLLADPSFAQFSQEIGLASLGASDEEIEKLSTVYWFTVEFGLCKEHGQIKAYGAGLLSSYGELLHAISDKCEHRAFEPASTAVQP
DTH2FS±.......HTPEPDSIHELLGHMPLLADPSFAQFSQEIGLASLGASDEEIEKLSTVYWFTVEFGLCKEHGQIKAYGAGLLSSYGELLHAISDKCEHRAFEPASTAVQP

DTH2 .........YQDQEYQPIYYVAESFEDAKDKFRRWVSTMSRPFEVRFNPHTERVEVLDSVDKLETLVHQMNTEILHLTNAISKLRRPF
DTH2FS±.......YQDQEYQPIYYVAESFEDAKDKFRRWVSTMSRPFEVRFNPHTERVEVLDSVDKLETLVHQMNTEILHLTNAISKLRRPF
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Fig. S1. Structures of the DTH gene, derived transgenes, and mutant isoforms. (*A*) DA biosynthesis pathway in *Drosophila*. Two tissue-specific isoforms of TH (neural DTH1 and nonneural DTH2) hydroxylate L-tyrosine to produce L-DOPA, which is decarboxylated in a second step to DA by Ddc. (*B*) Exon-intron structure and alternative splicing of the *DTH* gene (*DTH*). (a–g) Exons are represented as wide boxes separated by introns. Gray-filled boxes represent constitutive exons (a, b, e, f, and g), and black-filled boxes represent the two cassette exons (c and d). Exons c and d are skipped in the CNS but are normally spliced in nonnervous tissues, leading to expression of the neural DTH1 and hypodermal DTH2 isoforms, respectively. ATG and STOP represent the initiation and arrest of translation. (Scale bar: 1 kb.) (*C*) *UAS-DTH* transgenes. The 6.7-kb coding part of *DTH* with introns was fused downstream of GAL4-binding UASs. *DTHg*, WT *DTH*; *DTHg*$^{FS+}$, frameshift mutant with one supplementary base inserted in exon d; *DTHg*$^{FS\pm}$, frameshift mutant with one supplementary base in exon d and one compensatory deleted base in exon e. On the right is indicated in which tissue, either nervous (CNS) or hypodermal (HYP), these transgenes are expected to give an active or inactive form of TH after alternative splicing. (*D*) Amino acid sequence alignments of native DTH2 and mutant DTH2^{FS+} and DTH2$^{FS\pm}$ isoforms. Amino acids of the acidic segment encoded by exons c and d are shown in green. The sequences generated by the mutagenesis and unrelated to *DTH* are in shown

Legend continued on following page

red. Stars indicate identical amino acids. The *DTH2*$^{FS+}$ cDNA contains an additional base at the end of exon d, which generates a frameshift mutation leading to translation of a truncated and inactive enzyme. The *DTH2*$^{FS\pm}$ cDNA contains both the additional base in exon d and a nearby compensatory base deletion in exon e, leading to translation of an active enzyme. Indeed, DTH2$^{FS\pm}$ only differs from the native DTH2 by a short 15-aa segment at the boundary of exons d and e. This segment is not conserved in vertebrate TH and is not essential for enzyme activity.

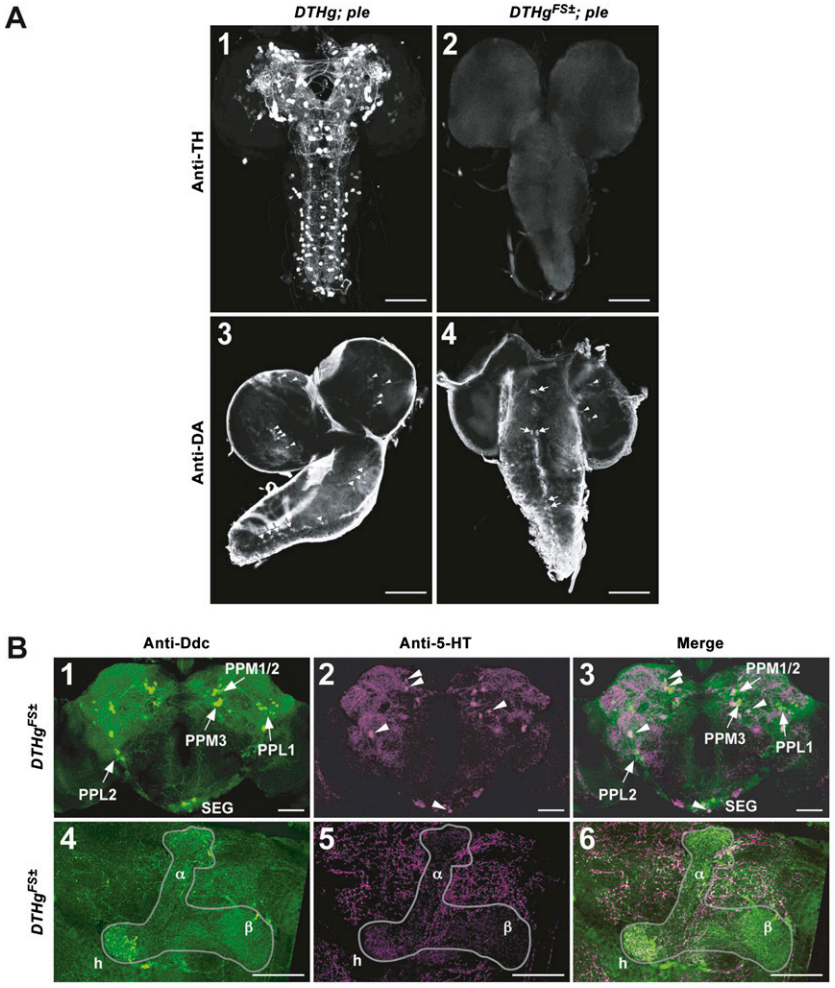

Fig. S2. Lack of TH does not hamper development of the brain dopaminergic system. (*A*) Anti-TH and anti-DA immunostaining on whole-mount third instar larval CNS of *DTHg*- or *DTHg*$^{FS\pm}$-rescued *ple* mutants. TH immunoreactivity was detected in the ventral nerve cord and larval brain of *DTHg*; *ple* (1) but not of *DTHg*$^{FS\pm}$; *ple* (2). In contrast, DA-immunopositive neurons could be observed in the larval nerve cord (arrows) and brain (arrowheads) of both *DTHg*; *ple* (3) and *DTHg*$^{FS\pm}$; *ple* (4). The number of DA-positive cells appeared reduced in the brain hemispheres of the *DTH*-deficient mutants compared with the *DTHg*-rescued larvae. (*B*) Double immunostaining with anti-Ddc (green) and antiserotonin (magenta) in whole-mount adult brains of *DTHg*$^{FS\pm}$-rescued *ple* mutants. (*Right*) Colocalizations appear in white in the merged pictures. (1–3) Regular pattern of dopaminergic neurons, which express *Ddc* but not *DTH*, can be recognized in the adult brain of *DTHg*$^{FS\pm}$; *ple* flies. Specific dopaminergic clusters are indicated (1 and 3, arrows) that are distinct from *Ddc*-expressing serotoninergic cells (2 and 3, arrowheads). (4–6) Magnification of the mushroom body lobe region reveals *Ddc*-positive and serotonin-negative projections on the vertical lobe (α), the tip of the horizontal lobes (β), and the heel (h). This indicates preservation of the characteristic innervation pattern of the adult mushroom body by dopaminergic neurons in the absence of *DTH* and DA. PPL, lateral posterior protocerebral neurons; PPM, medial posterior protocerebral neurons; SEG, subesophageal ganglion neurons. (Scale bars: 50 μm).

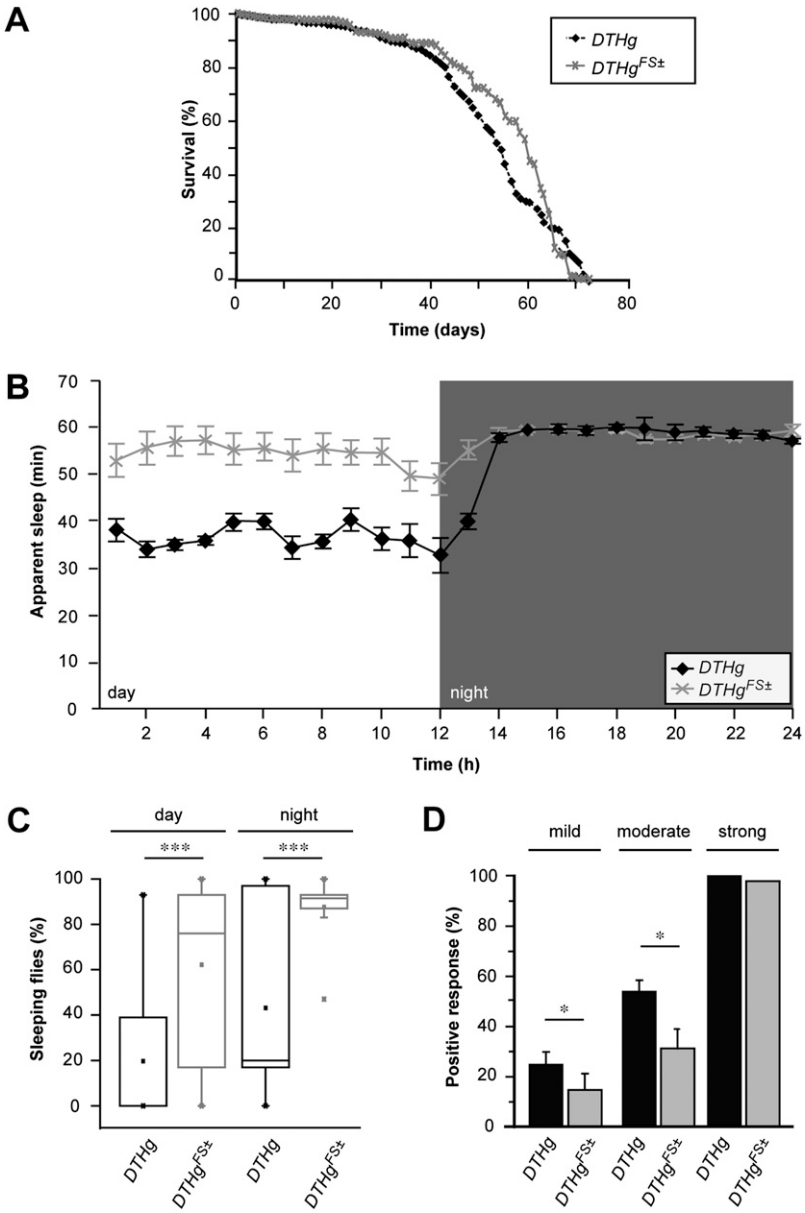

Fig. S3. Lifespan, sleep, and arousal in the absence of neural DA. (*A*) Survival curves of adult *DTHg*- and *DTHg*$^{FS\pm}$-rescued *Drosophila* maintained at 25 °C in standard conditions. The maximal lifespan of the rescued flies was ~70 d, which was indistinguishable from that of WT animals. (*B–D*) Neural DA-deficient flies show increased sleep and arousal thresholds. (*B*) Activity of 5-d-old female adult flies monitored in a DAM system for 24 h. Apparent sleep was defined as periods of quiescence without laser beam breaks lasting for 5 min or longer. The DA-deficient *DTHg*$^{FS\pm}$; *ple* flies appear less active than the neural DA-expressing *DTHg*; *ple* flies during the day. Direct observation indicated that inactive flies of both strains were frequently not actually asleep but just did not move enough to break the laser beam. (*C*) Box-and-whisker plots showing results of direct visual observation of individual flies for 30-min periods during day (ZT 8–10, $n = 21$) or night (ZT 14–15, $n = 9$). Flies not moving at all for at least 5 min were considered to be sleeping. The *DTHg*$^{FS\pm}$; *ple* flies do spend more time asleep during both day and night compared with the *DTHg*; *ple* flies (day, $^{***}P < 0.001$; night, $^{***}P < 0.005$.) Plots represent the median (horizontal line), mean (square), 25% and 75% quartiles (box), 10% and 90% quantiles (whiskers), and extreme values (crosses) (Student's *t* test). (*D*) Response of sleeping flies to mechanical stimulations. Mild, moderate, and strong stimuli were applied as described in *Materials and Methods*. Flies awake before the stimulation were excluded. The percentage of sleeping *DTHg*$^{FS\pm}$ flies responding to mild and moderate stimuli was significantly reduced compared with the *DTHg* flies, suggesting a higher arousal threshold ($^{*}P < 0.05$, χ^2 test).

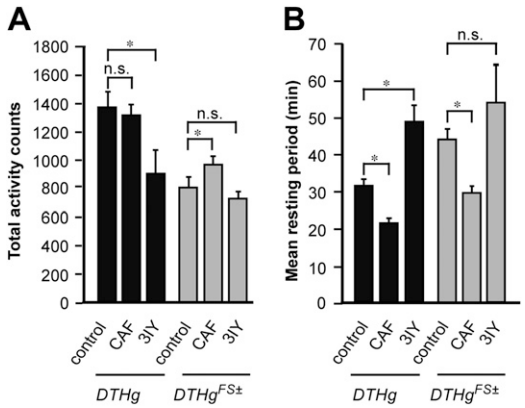

Fig. S4. Effect of TH inhibitors and caffeine on the activity of rescued *ple* flies. Total locomotor activity [i.e., infrared beam crossings (*A*), mean resting periods (*B*)] of adult *DTHg*; *ple* and $DTHg^{FS\pm}$; *ple*. Spontaneous locomotion was recorded over a 3-d period in activity monitor tubes containing either sucrose alone (control) or sucrose supplemented with 0.5 mg/mL caffeine (CAF) or 3 mM 3IY, a TH inhibitor. After CAF ingestion, $DTHg^{FS\pm}$; *ple* show increased locomotor activity and both mutants show a decrease in mean resting period. 3IY decreases total activity and increases the mean resting period of *DTHg*; *ple*, making activity of these flies indistinguishable from that of $DTHg^{FS\pm}$; *ple*. In contrast, 3IY has no effect on the activity or resting periods of neural TH-deficient $DTHg^{FS\pm}$; *ple* (*$P < 0.05$, Student's *t* test). n.s., not significant.

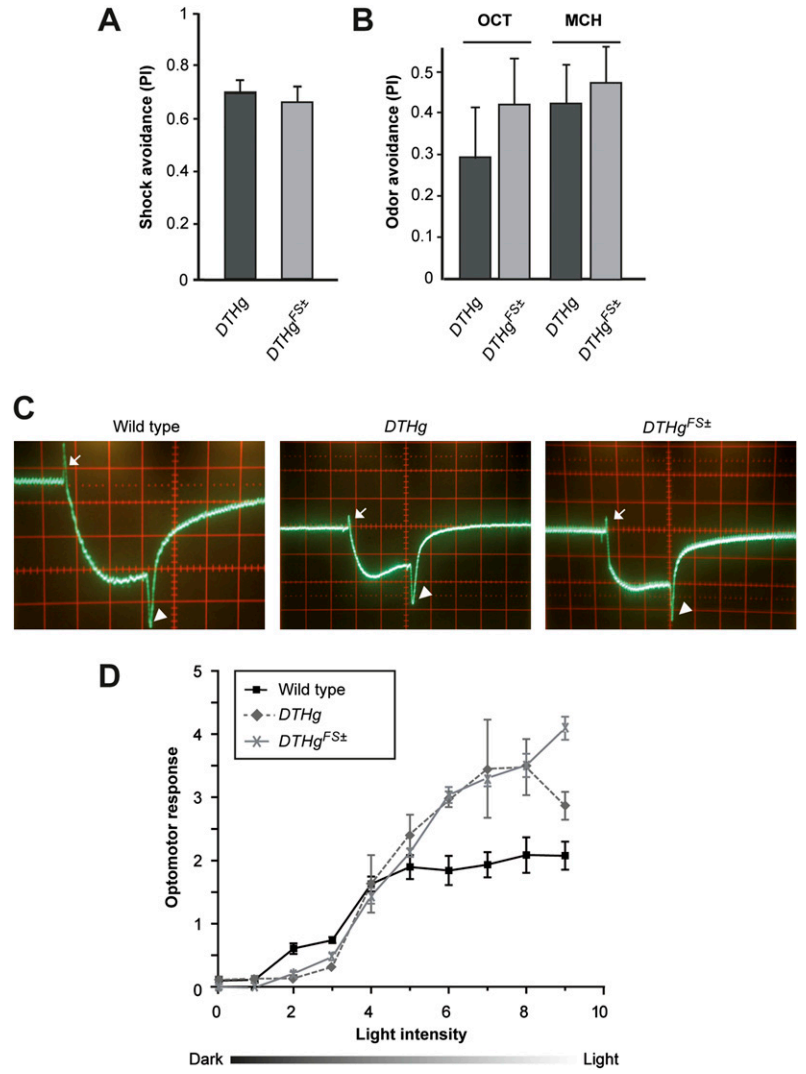

Fig. S5. Neural DA deficiency does not alter sensory perceptions and optomotor response. Electrical shock (*A*) and odor (*B*) avoidance behaviors of untrained flies appear normal both for *DTHg*; *ple* and $DTHg^{FS\pm}$; *ple*. (*C*) Representative electroretinogram (ERG) recordings. ERGs of $DTHg^{FS\pm}$; *ple* flies were similar to those of WT and *DTHg*; *ple* flies. The sustained negative potential represents light-evoked currents of photoreceptors. The positive on (arrows) and negative off (arrowheads) transients indicate normal activation of the postsynaptic lamina neurons in response to input from the photoreceptor neurons. (*D*) Optomotor response. The flies are fixed at the back on a Styrofoam ball centered in a rotating arena of black and white stripes. They try to compensate for the rotation of the environment by their own movements. Performance depends (arbitrary units) on the contrast in the environment and increases with increasing light intensity (arbitrary units). Either WT, *DTHg*; *ple*, or $DTHg^{FS\pm}$; *ple* show an intact capability to compensate for their environment. Plots represent 10 recordings of one representative fly per genotype.

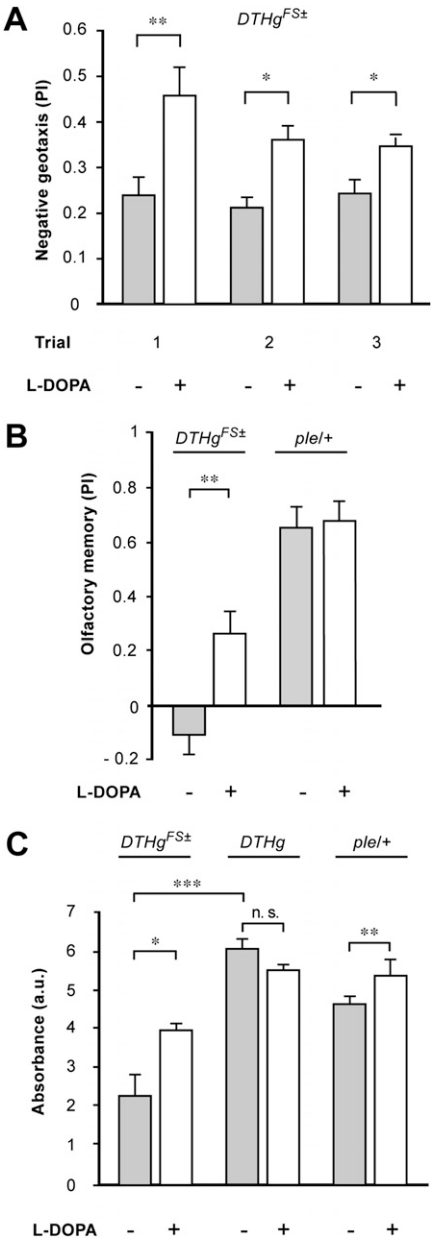

Fig. S6. L-DOPA rescue of disrupted behaviors in neural DA-deficient *Drosophila*. (*A*) Climbing abilities of 1-wk-old $DTHg^{FS\pm}$; *ple* flies fed for 3 d on sucrose alone (gray bars) or on sucrose plus 1 mg/mL L-DOPA (white bars), monitored by startle-induced negative geotaxis. Tests were repeated three times for each group (trials 1–3). The defect in negative geotactic behavior of $DTHg^{FS\pm}$; *ple* flies was significantly rescued by L-DOPA feeding at each trial (**$P < 0.02$ and *$P < 0.05$, Student's *t* test; $n = 13$). (*B*) Aversive olfactory learning. PI of flies was tested immediately ($t = 0$) after electrical shock training. (*Left*, bars) Feeding $DTHg^{FS\pm}$; *ple* flies for 3 d on sucrose plus 1 mg/mL L-DOPA significantly increased their conditioned avoidance of the aversive odor, compared with the same flies fed on sucrose alone (**$P = 0.015$, Student's *t* test; $n = 6$). (*Right*, bars) L-DOPA did not alter the learning performance of control $DTHg^{FS\pm}$; *ple*/+ flies. (*C*) Food intake measured as light absorbance of ingested sulforhodamine B. a.u., arbitrary units. Starved flies were exposed for 20 min to a sucrose solution containing the colorimetric dye and then chloroform-extracted. DA deficient $DTHg^{FS\pm}$; *ple* eat one-third as much as *DTHg*; *ple*. This aphagic phenotype is rescued by L-DOPA feeding. Food intake by control $DTHg^{FS\pm}$; *ple*/+ flies is also significantly improved by L-DOPA (*Left*), whereas *DTHg*; *ple* show no significant difference in the absence or presence of L-DOPA (*Center*). Results from three independent determinations. (*$P < 0.05$, **$P < 0.02$, and ***$P < 0.005$, Student's *t* test.)

Table S1. Rescue of *ple* mutants to adult stage with a combination of dopaminergic drivers

	Pupae*		Adult female flies†		Adult male flies†	
	Tb	*Tb*+‡	*Tb*	*Tb*+‡	*Tb*	*Tb*+‡
Ddc-GAL4§	153	0	73	0	87	0
TH-GAL4§	374	123 *(24.7)*	254	0	287	0
Male *TH/Ddc-GAL4*	324	142 *(30.5)*	219	121 *(35.6)*	278	0¶
Female *TH/Ddc-GAL4*	94	55 *(36.9)*	76	56 *(42.4)*	45	32 *(41.5)*

Male or female *ple*/TM6B (*Tb*) flies with homozygous insertion of the indicated GAL4 drivers were mated to *ple*/TM6B (*Tb*) flies with homozygous insertion of the *UAS-DTHg* transgene. Rescue from *ple* late embryonic lethality was determined by scoring the relative number of homozygous *ple* (i.e., Tb^+) pupae and adult flies in the progeny. Values indicate the number of progeny obtained in two to six independent crosses. Only the combination of one copy each of *TH-GAL4* and *Ddc-GAL4* (*TH/Ddc-GAL4* strain) led to rescue of *ple* to adult stage.
*Number of pupae scored 10 d after parent cross.
†Number of adult progeny scored from 12 to 22 d after parent cross.
‡Percentages of Tb^+ progeny are in italics.
§For *Ddc-GAL4* and *TH-GAL4* alone, either male or female flies were used with similar results.
¶No Tb^+ adult male flies were recovered with male GAL4 parents because *Ddc-GAL4* is inserted on the X chromosome.

Table S2. Rescue of *ple* by *DTH* gene frameshift mutants

	Adult male flies		Adult female flies	
	Tb	*Tb*+*	*Tb*	*Tb*+*
UAS-DTHg	49	36 *(42.3)*	50	46 *(47.9)*
UAS- DTHg^{FS+}	80	0	70	0
UAS- DTHg$^{FS\pm}$	52	37 *(41.6)*	53	31 *(36.9)*

Female *ple*/TM6B (*Tb*) flies with homozygous insertion of the *Ddc-GAL4*: *TH-GAL4* double driver were mated to male *ple*/TM6B (*Tb*) flies with homozygous insertion of the indicated *UAS* transgene. Rescue from *ple* late embryonic lethality was determined by scoring the relative number of homozygous *ple* (i.e., Tb^+) adult flies in the progeny. Values indicate the number of progeny obtained in two to three independent crosses.
*Percentages of Tb^+ progeny are in italics.

Table S3. Determination of DA levels in brain extracts by HPLC: Data from HPLC at +500-mV oxidation potential (*n* = 3)

Strain	"DA" (pg/brain)	SEM
w^{1118}	52.0	5.5
DTHg; *ple*	49.0	3.1
DTHg$^{FS\pm}$; *ple*	7.5	1.0

A standard HPLC analysis for DA performed with the fully oxidizing +500-mV electrochemical potential indicates that DA levels in the *DTHg*$^{FS\pm}$; *ple* strain are reduced to ~15% of WT levels (but see Table S4) and that the *DTHg*; *ple* line shows fully restored levels of DA.

Table S4. Determination of DA levels in brain extracts by HPLC: Relative "DA" peak area at +420- vs. +500 mV-detector potential (*n* = 3)

Strain	Relative peak area	SEM
w^{1118}	0.43	0.07
DTHg; *ple*	0.46	0.01
DTHg$^{FS\pm}$; *ple*	1.05	0.18

To determine whether the reduced peak in *DTHg*$^{FS\pm}$; *ple* was authentic DA, the HPLC was repeated using a reduced detector potential of +420 mV, near the half-maximal detector sensitivity for DA. This analysis showed that the residual peak in the *DTHg*$^{FS\pm}$; *ple* strain is unaffected by the reduced detector potential (relative peak area = 1.05), in contrast to w^{1118} and *DTHg; ple* flies. Thus, the residual peak in the neural TH-deficient strain is largely, if not totally, a non-DA contaminant. This shows that the *DTHg*$^{FS\pm}$; *ple* brain extract lacks detectable DA.

Movie S1. Proboscis extension reflex is normal in DA-deficient *Drosophila*. Representative movie successively showing two presentations of water (with a soaked standard cotton swab) followed by two presentations of 5% (wt/vol) sucrose to the foreleg tarsi of a female $DTHg^{FS\pm}$; *ple* fly. Only the sucrose solution triggers proboscis extension as in WT flies.

Movie S1