# Behavioral consequences of dopamine deficiency in the *Drosophila* central nervous system

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The neuromodulatory function of dopamine (DA) is an inherent feature of nervous systems of all animals. To learn more about the function of neural DA in *Drosophila*, we generated mutant flies that lack tyrosine hydroxylase, and thus DA biosynthesis, selectively in the nervous system. We found that DA is absent or below detection limits in the adult brain of these flies. Despite this, they have a life-span similar to WT flies. These mutants show reduced activity, extended sleep time, locomotor deficits that increase with age, and they are hypophagic. Whereas odor and electrical shock avoidance are not affected, aversive olfactory learning is abolished. Instead, DA-deficient flies have an apparently "masochistic" tendency to prefer the shock-associated odor 2 h after conditioning. Similarly, sugar preference is absent, whereas sugar stimulation of foreleg taste neurons induces normal proboscis extension. Feeding the DA precursor L-DOPA to adults substantially rescues the learning deficit as well as other impaired behaviors that were tested. DA-deficient flies are also defective in positive phototaxis, without alteration in visual perception and optomotor response. Surprisingly, visual tracking is largely maintained, and these mutants still possess an efficient spatial orientation memory. Our findings show that flies can perform complex brain functions in the absence of neural DA, whereas specific behaviors involving, in particular, arousal and choice require normal levels of this neuromodulator.

neurotransmitters | locomotor activity | memory formation | choice behavior | feeding behavior

**A**n important challenge in neuroscience is to understand the roles of specific neurotransmitter systems on brain homeostasis and functioning. Dopamine (DA), a biogenic amine biosynthesized from tyrosine, is an essential neuromodulator in the mammalian central nervous system that is involved in attention, movement control, motivation, and cognition. Studies in *Drosophila melanogaster* indicate that DA also plays central regulatory roles in insects, specifically in the neural networks controlling locomotor activity and stereotypical behaviors (1–3), sleep and arousal (4–7), registration of salient stimuli (4, 8, 9), and associative olfactory learning (10–15). Some of these studies were based on genetic inactivation or overactivation of dopaminergic neurons. Dopaminergic neurons can corelease other neuroactive agents, such as neuropeptides, however. Therefore, one must ensure that the behavioral phenotypes observed specifically result from the lack of DA release to draw firm conclusions on brain DA function.

Nearly all neuropil regions of the insect CNS receive dense dopaminergic innervation. In particular, the *Drosophila* adult brain contains six paired clusters of dopaminergic neurons, some of which specifically project to higher brain centers, such as the central complex and the mushroom bodies (1, 10, 12, 13, 16–18). Tyrosine hydroxylase (TH) catalyzes the first and rate-limiting step in DA biosynthesis (Fig. S1A). Because DA is also required in *Drosophila* as a precursor substrate for cuticle sclerotization and melanization, inactivating mutations of the genomic *TH*, alias

*pale* (*ple*) locus, results in unpigmented cuticle and late embryonic lethality (19).

Alternative splicing of *Drosophila TH* (*DTH*) produces two enzyme isoforms, DTH1 and DTH2 (20, 21) (Fig. S1B). DTH1 is selectively expressed in DA neurons within the CNS, whereas DTH2 is expressed in peripheral nonnervous tissues, which include the hypodermal cells that secrete the cuticle matrix (20, 22). Here, we take advantage of this tissue-specific alternative splicing to construct a mutated transgene, *DTHg^{FS±}*, that only expresses an active TH enzyme in nonneural cells. Homozygous *ple* mutants are rescued by this transgene to adult stage, generating *Drosophila* essentially devoid of DA in the adult brain. We then studied the consequences of this neural-specific DA depletion on adult survival and behavior.

## Results

**Generation of Viable DA-Deficient *Drosophila*.** We first established the conditions for full rescue of *DTH* deficiency with the GAL4-upstream activation sequence (UAS) binary expression system by expressing genomic *DTH* (*DTHg*) driven by *TH-GAL4* and *Ddc-GAL4*, each of which contains regulatory sequences from genes involved in DA biosynthesis (Fig. S1A). The combined action of the *Ddc-GAL4* and *TH-GAL4* drivers is required for full rescue, with neither GAL4 driver alone being sufficient for full rescue to adult stage (Table S1).

We then used in vitro mutagenesis to introduce frameshift mutations in *DTHg* (Fig. S1 C and D and *SI Materials and Methods*). *DTHg^{FS+}* contains one additional base in a hypoderm-specific exon, and *DTHg^{FS±}* contains the same mutation plus another compensating mutation that removes one base in an adjacent common exon. When transcribed, *DTHg^{FS+}* only expresses active TH in neural tissues and, conversely, *DTHg^{FS±}* only expresses active TH in nonneural tissues. No *ple* rescue could be observed with *DTHg^{FS+}* (Table S2), confirming that an active DTH2 isoform producing cuticular DA is required for *Drosophila* development (22). In contrast, expression of *DTHg^{FS±}* yielded full rescue of *ple* to adulthood (Table S2), indicating that biosynthesis of neural DA is not essential for fly development and survival.

Immunohistochemistry confirmed that TH and DA are not detectable in adult brain of the *DTHg^{FS±}*-rescued *ple* mutants (Fig. 1A 3 and 6), whereas they are present in WT flies (Fig. 1A 1 and 4) or *ple* mutants rescued by the native *DTHg* construct (Fig. 1A 2
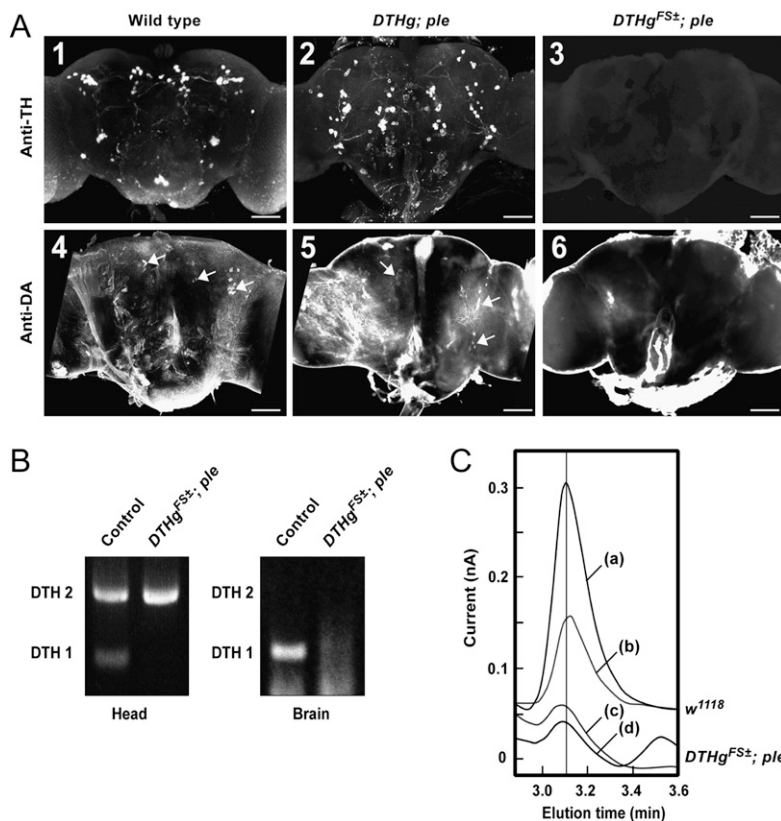
**Fig. 1.** Lack of TH expression and DA in the brain of $DTHg^{FS\pm}$-rescued *ple Drosophila*. (*A*) Anti-TH immunostaining on whole-mount adult brains of WT *Drosophila* (1) and *DTHg* (2) or $DTHg^{FS\pm}$-rescued (3) *ple* mutants. No TH immunoreactivity was detected in the brain of $DTHg^{FS\pm}$; *ple*. Anti-DA immunostaining. DA-positive cells can be observed in the brains of WT (4) and *DTHg*; *ple* (5) flies (arrows) but not in the brains of $DTHg^{FS\pm}$; *ple* flies (6). (*B*) Detection of the DTH1- and DTH2-specific spliced mRNAs by RT-PCR in heads (*Left*) and dissected brains (*Right*) of control or $DTHg^{FS\pm}$; *ple* flies. No *DTH* transcript is present in the brain of $DTHg^{FS\pm}$-rescued flies. (*C*) Representative graphs of DA assay by HPLC in brain extracts of control $w^{1118}$ (a and b) and $DTHg^{FS\pm}$; *ple* (c and d) detected with an optimal potential of +500 mV (a and c) or reduced to +420 mV (b and d). (d) Reduced potential did not decrease the amplitude of the residual "DA" peak of neural TH-deficient flies, indicating that this peak is not DA. The small additional peak on the right corresponds to a contaminant from eye pigments.

and 5). Accordingly, RT-PCR shows that the neural-specific *DTH1* mRNA is absent in head and brain extracts of $DTHg^{FS\pm}$; *ple* (Fig. 1*B*). Similarly, no TH is present in the larval CNS of $DTHg^{FS\pm}$; *ple*, whereas it is expressed at this stage in *DTHg*; *ple* (Fig. S2*A*). In contrast, DA is detected in larval neurons of both strains, although fewer positive cells could be seen in larval brain hemispheres of $DTHg^{FS\pm}$; *ple* versus *DTHg*; *ple* (Fig. S2 *A3* and *A4*). This suggests that systemic DA produced by peripheral TH can partially supply the mutant larval CNS with the missing biogenic amine.

DA was then assayed by HPLC to determine the absolute magnitude of reduction of DA level in adult brain (Fig. 1*C* and Tables S3 and S4). Brain extracts from control flies and *DTHg*; *ple* showed equivalent levels of DA per brain (52.0 and 48.9 pg per brain, respectively), indicating that expression of the *DTHg* transgene quantitatively restored brain DA. In contrast, $DTHg^{FS\pm}$; *ple* flies apparently had 7.5 pg of DA per brain, suggesting a strong (~85%) but incomplete reduction of DA level in these mutants. HPLC was then repeated using a decreased detector potential of +420 mV, near the half-maximal oxidation potential of DA. Whereas the DA peak in an extract from a control brain behaves as expected (relative DA peak area at +420 vs. 500 mV: 0.43), the residual peak in the $DTHg^{FS\pm}$; *ple* strain was unaffected by the reduced detector potential (relative "DA" peak area: 1.05) (Fig. 1*C* and Tables S3 and S4). We conclude that the residual peak in this strain is largely, if not totally, a non-DA contaminant and that brains of $DTHg^{FS\pm}$; *ple* flies lack detectable DA, with an estimated sensitivity limit of 2% WT levels.

Nevertheless, adult brain of DA-deficient flies contains differentiated dopa decarboxylase (Ddc)-positive neurons that do not express serotonin and so are most likely "dopaminergic" neurons devoid of TH and DA. These cells indeed distribute in clusters identical to the WT pattern of dopaminergic neurons (Fig. S2*B1*–3). Moreover, characteristic dopaminergic innervation of the mushroom bodies appears normal in the mutant (Fig. S2*B4*–6). Therefore, maintenance of the adult brain dopaminergic system does not seem to depend on the activity of TH or the presence of DA in *Drosophila*.

**DA Deficiency Reduces Activity and Arousal.** Unexpectedly, we observed that both *DTHg*; *ple* and $DTHg^{FS\pm}$; *ple* lines show similar and normal adult life expectancy (Fig. S3*A*), demonstrating that the lack of neural DA has no detrimental effect on *Drosophila* lifespan. This enabled us to compare the behaviors of these lines. First, we studied the effect of neural DA deficiency on walking behavior of young adult flies. $DTHg^{FS\pm}$; *ple* show reduced walking speed (median = 7.8 mm/s) compared with *DTHg*; *ple* (median = 10.8 mm/s) and WT (median = 15 mm/s) (Fig. 2*A*, *Left*). Similarly, the distance covered for 15 min is much shorter in $DTHg^{FS\pm}$; *ple* (median = 193 cm) than in *DTHg*; *ple* (median = 425 cm) and WT (median = 474 cm) (Fig. 2*A*, *Right*). Therefore, flies deficient in neural DA show markedly decreased locomotor behavior.

Startle-induced negative geotaxis monitors climbing ability and excitability in *Drosophila*, and DA modulates this behavior (1, 23). WT flies placed in a vial or a column respond to a gentle
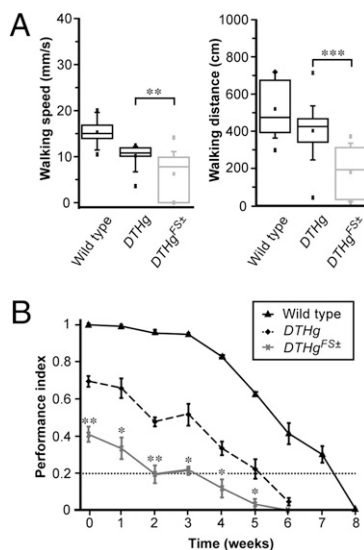
**Fig. 2.** Neural DA-deficient flies show locomotor deficits. (*A*) Box-and-whisker plots showing the distribution of walking speed (*Left*) and covered distance (*Right*) during spontaneous locomotor behavior of 5-d-old adult flies monitored in an open arena. The DA-deficient $DTHg^{FS\pm}$; *ple* flies show a statistically significant reduction compared with rescued $DTHg$; *ple* in both walking speed ($P = 0.015$) and covered distance ($P = 0.001$). $DTHg$; *ple* show a difference compared with WT flies for walking speed ($P = 0.0003$) but not walking distance ($P = 0.130$). Plots represent the median (horizontal line), mean (square), 25% and 75% quartiles (box), 10% and 90% quantiles (whiskers), and extreme values (crosses) (**$P < 0.02$ and ***$P < 0.005$). (*B*) Climbing abilities of WT flies, $DTHg$; *ple*, and $DTHg^{FS\pm}$; *ple* monitored by startle-induced negative geotaxis as a function of adult age. The robust negative geotactic behavior of young WT *Drosophila* declines steadily over time after 3 wk. $DTHg^{FS\pm}$; *ple* show from the eclosion a strong impairment in this behavior; after 2 wk, their mean PI was only 0.2 (horizontal dotted line). $DTHg^{FS\pm}$; *ple* values were compared for significance with $DTHg$; *ple* values (*$P < 0.05$ and **$P < 0.02$, Student's *t* test).

mechanical shock by fast climbing. This behavior is stable over the first 3 wk of adult life and then progressively declines with age, such that by day ~55, WT flies show no response (Fig. 2*B*). The $DTHg^{FS\pm}$; *ple* flies do not perform well in this test, although they are definitely able to climb. Just after eclosion, their performance index (PI) was ~0.4, and at 15–21 d of age, their PI was only ~0.2, comparable to the PI of 50-d-old WT flies (Fig. 2*B*, dotted line). $DTHg$; *ple* show PIs between those of WT and DA-deficient flies (Fig. 2*B*).

Monitoring activity over the light/dark cycle indicates that $DTHg^{FS\pm}$; *ple* are overall less active and possibly sleep more frequently than $DTHg$; *ple* during the day (Fig. S3*B*). Because both strains showed frequent periods of relative inactivity, we also monitored sleep by direct observation. The results demonstrate that $DTHg^{FS\pm}$; *ple* do sleep more than $DTHg$; *ple* during both day and night (Fig. S3*C*). Furthermore, we found that sleeping $DTHg^{FS\pm}$; *ple* flies respond less frequently to mild and moderate mechanical stimuli (Fig. S3*D*), whereas response to strong stimuli was comparable to that of $DTHg$; *ple* flies. Overall, these results argue for a decreased arousal state in the absence of brain DA.

Treatment with 3-iodotyrosine (3IY), a TH inhibitor, decreases activity and increases resting periods in WT flies (4), whereas caffeine has the opposite effects (24). We observed that the neural TH-deficient $DTHg^{FS\pm}$; *ple*, as expected, are not responsive to 3IY, whereas $DTHg$; *ple* flies reacted like WT (Fig. S5). In contrast, caffeine was found to decrease resting periods of both $DTHg$; *ple* and $DTHg^{FS\pm}$; *ple* (Fig. S4), sug-

gesting that caffeine does not act by modulating DA release in *Drosophila*.

**Impaired Aversive Olfactory Learning and Feeding Behavior.** We used classical olfactory conditioning to evaluate the ability of $DTHg^{FS\pm}$; *ple* flies to associate an aversive stimulus (electrical shock) with an odor. Either immediately ($t = 0$) or 2 h after training, these flies did not show any avoidance of the shock-associated odor (Fig. 3*A*). This is in contrast to the $DTHg$-rescued controls that demonstrate associative memory (PI = ~0.6) (Fig. 3*A*), similar to WT (25). Interestingly, the DA-deficient flies showed an inverse tendency to choose the shock-associated odor during testing, an effect that was significant 2 h after training (PI = $-0.13 \pm 0.05$, $n = 9$). We checked that shock avoidance (Fig. S5*A*) and odor perception (Fig. S5*B*) are preserved in DA-deficient as well as $DTHg$-rescued flies.

Pharmacological rescue experiments were performed in adult flies to distinguish between physiological or developmental effects of brain DA deficiency. Remarkably, treatment of adult $DTHg^{FS\pm}$; *ple* with the DA precursor L-DOPA significantly improved their negative geotaxis behavior (Fig. S6*A*). This indicates that progressive locomotor impairments directly result from DA deficiency and not from developmental abnormalities. Similarly, L-DOPA feeding of adult DA-deficient flies substantially increased their learning performance index towards a positive value (PI = $0.26 \pm 0.08$ with L-DOPA compared to $-0.11 \pm 0.07$ without L-DOPA at $t = 0$, $n = 6$) (Fig. S6*B*), confirming that the lack of neural DA induces an absolute defect in aversive olfactory memory.

Preparatory to appetitive conditioning trials, we tested the flies for sugar preference after a 21-h period of starvation. Whereas $DTHg$-rescued controls respond positively and comparably to WT, $DTHg^{FS\pm}$; *ple* flies showed no significant response to sucrose (Fig. 3*B*). The proboscis extension reflex appears normal in these mutants (Movie S1), however, indicating that sugar perception and reflex appetitive reactions are preserved. This suggests that neural DA is specifically required for behavioral attraction to sugar. Quantification of food intake revealed that $DTHg^{FS\pm}$; *ple* eat one-third as much as $DTHg$; *ple* (Fig. S6*C*), demonstrating that they are markedly hypophagic. Again, L-DOPA significantly improves this phenotype, whereas the drug has no effect on food intake of $DTHg$; *ple* flies. Interestingly, L-DOPA also has a positive effect on food intake of heterozygous *ple* flies (Fig. S6*C*), suggesting a dominant effect of *ple* on feeding behavior.

**Lack of Phototaxis but Preserved Visual Orientation and Spatial Memory.** Positive phototaxis is a characteristic behavior of *Drosophila* and many other insect species. In countercurrent assay, $DTHg^{FS\pm}$; *ple* flies were mainly recovered in the first tube of the apparatus, indicating that they are not attracted by light, whereas $DTHg$; *ple* showed partial rescue of phototactic behavior (Fig. 3*C*). The mutants have normal electroretinograms (Fig. S5*C*) and optomotor response (Fig. S5*D*), indicating that visual functions are preserved. We also performed individual assays to rule out the possibility that the lack of positive response in the countercurrent test was attributable to reduced activity. A single fly was placed at the entrance of a T-maze and allowed to choose freely between a dark tube and an illuminated tube until it reached one of the vials. Similar to WT, neurally rescued $DTHg$; *ple* show a significant preference toward the lighted tube. In contrast, $DTHg^{FS\pm}$; *ple* single flies show no preference, choosing either tube with equal probability (Fig. 3*D*).

We further checked the flies for visual tracking and orientation in the Buridan's paradigm, a cylindrical virtual reality arena in which two dark vertical stripes are presented opposite to each other. Under standard conditions, WT flies patrol between two given visual targets for a considerable length of time. Whole orientation curves indicate that $DTHg$; *ple* and $DTHg^{FS\pm}$; *ple* are comparably able to target the two stripes, with a fixation ability
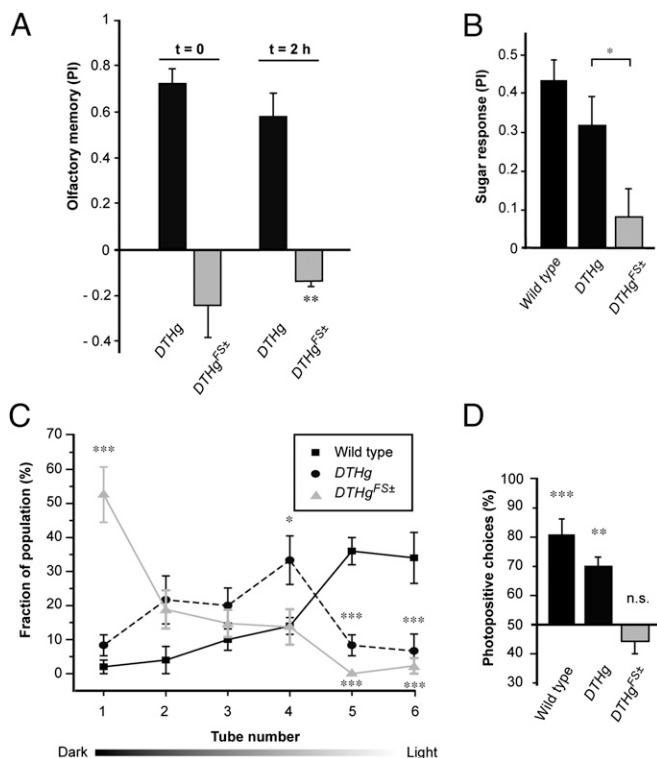
**Fig. 3.** Behaviors disrupted in neural DA-deficient *Drosophila*. (*A*) Aversive olfactory learning. The PI of flies tested either immediately (*t* = 0) or 2 h after electrical shock training is shown. Whereas *DTHg*; *ple* show normal conditioned avoidance of the aversive odor, the neural DA-deficient *DTHg^FS±*; *ple* do not learn. They rather show an abnormal preference for the shock-associated odor that is statistically significant 2 h after training (*P* = 0.312 at *t* = 0 and **P* = 0.012 at *t* = 2 h, Wilcoxon signed-rank test against 0 as random behavior). (*B*) Sugar preference. After a 21-h starvation period, flies were tested in a T-maze for their response to sucrose. WT and *DTHg*-rescued *Drosophila* show positive responses to sugar, whereas *DTHg^FS±*; *ple* flies appear not to be attracted (**P* < 0.05, Student's *t* test). The sugar response of the *DTHg^FS±*; *ple* flies was not different from 0 as random behavior (Wilcoxon signed-rank test). (*C*) Phototaxis assayed by countercurrent distribution. WT *Drosophila* display strong phototactic behavior and mostly distributes toward the light source in tubes 5 and 6. In contrast, most *DTHg^FS±*; *ple* flies remain in tube 1. This behavioral impairment is partially rescued in *DTHg*; *ple* flies [***P* < 0.0001, one-way ANOVA (Bonferroni-corrected) compared with WT values]. (*D*) Single-fly phototaxis assay. Flies were allowed to distribute freely between an illuminated tube and a dark tube. WT and *DTHg*-rescued *Drosophila* showed a strong preference for the illuminated tube. In contrast, the neural DA-deficient *DTHg^FS±*; *ple* distributed equally [***P* = 6.1 × 10^-5, **P* = 0.012, and *P* = 0.499 (not significant [n.s.]) for WT, *DTHg*; *ple*, and *DTHg^FS±*; *ple*, respectively, Wilcoxon signed-rank test; mean tested against reference constant (50% for equal distribution)].

different from random behavior, although less efficiently than WT flies (Fig. 4*A*). A closer look at the curves shows that both mutants differ in their orientation ability at small deviation angles (Fig. 4*B*), suggesting that the lack of DA makes visual orientation slightly less accurate. Therefore, in *Drosophila*, the presence of neural DA improves visual tracking but, nevertheless, is dispensable for this behavior.

Orientation in a changing environment requires the ability to retain and recall the position of a target. *Drosophila* possesses such a spatial orientation memory that can be tested by the detour paradigm. In this test, a fly walks toward a landmark that suddenly disappears while, simultaneously, a vertical distracter stripe appears laterally to the fly. After the fly approaches this second landmark, the distracter stripe also disappears within 1 s. In this
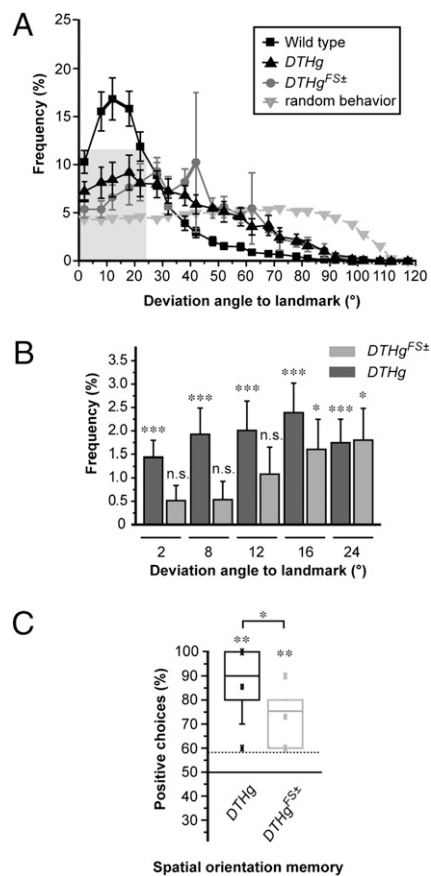


**Fig. 4.** Visual fixation and spatial memory are largely preserved in the absence of brain DA. (*A*) Whole orientation curves showing frequency of angle deviation between the fly trajectory and approached target. Mutant and rescue flies show a frequency distribution significantly different from the calculated random behavior (*DTHg*; *ple*: *P* = 0.007 and *DTHg^FS±*: *ple*: *P* = 0.005, *F* test), whereas both strains do not differ from each other (*P* = 0.455; mean of 13 flies of each genotype). This confirms that *DTHg*; *ple* and *DTHg^FS±*; *ple* flies are able to visualize and target the landmarks, although less consistently than WT flies (*DTHg*; *ple*: *P* = 0.028 and *DTHg^FS±*; *ple*: *P* = 0.036, *F* test). (*B*) Differences between angles to the approached target and calculated random behavior for small deviations (derived from shaded area of curves in *B* are shown. Trajectories of *DTHg^FS±*; *ple* target the landmarks but are not accurate, making angle frequency not significantly different from random behavior for small deviations [**P* < 0.05, not significant (n.s.)]. This defect is rescued in *DTHg*; *ple* flies that contain neural DA (***P* < 0.005). The Wilcoxon signed-rank test was used against 0 as random behavior. (*C*) Test of spatial orientation memory in the detour paradigm (details are provided in *Materials and Methods*). Ten flies per genotype and 10 tests per fly were recorded. The frequency of positive choices is represented by box-and-whisker plots showing the median (horizontal line), mean (square), 25% and 75% quartiles (box), 10% and 90% quantiles (whiskers), and extreme values (crosses). Random behavior would result in a reference value of 58% (horizontal dotted line). The *DTHg^FS±*; *ple* flies still retain spatial orientation memory (**P* = 0.0015, one-sample sign test against the random value), although their performance is reduced compared with the *DTHg*; *ple* flies (**P* = 0.034, Mann–Whitney *U* test).

situation, WT flies will turn back to their previous, still invisible, landmark, demonstrating a spatial orientation memory for their former target with a median frequency of ~80% (26). We observed that both DTHg; *ple* and *DTHg^FS±*; *ple* do recall the position of the first landmark with a median frequency of 90% and 73%, respectively (Fig. 4*C*). This demonstrates that the lack of brain DA has only a minor impact on spatial orientation memory in *Drosophila*.

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## Discussion

Here, we describe specific genetic tools to study DA as a signaling molecule in the *Drosophila* brain. We show that the embryonic lethality of *ple* is fully rescued by a combination of dopaminergic drivers (*Ddc-GAL4* and *TH-GAL4*) expressing a frameshift mutant of the TH gene (*DTHg^{FS±}*). The *DTHg^{FS±}; ple* mutants only express TH in nonneural tissues, principally the cuticle-producing cells of the hypoderm. This leads to the absence of DA in adult brain without apparent morphological disturbance of the dopaminergic system, as attested by L-DOPA rescue of defective behaviors. The resulting flies are fully viable with a normal lifespan and they show a number of distinctive phenotypes.

Mutants without neural DA show reduced locomotor activity and arousal, and they sleep significantly more than control flies during both day and night, consistent with previous reports on the role of DA in these behaviors (4–6). The lack of further effect of a TH inhibitor (3IY) demonstrates that spontaneous activity is not influenced by trace levels of neural DA or by cuticular DA in these flies. Other reports indicate that blockade of dopaminergic synaptic transmission increases activity, particularly startle-induced activity (1, 7, 27). This apparent discrepancy cannot be fully explained at present but might be related to the fact that only part of brain DA signaling is inhibited with the use of GAL4 drivers that do not express in the entire dopaminergic system.

The role of dopaminergic neurons in *Drosophila* aversive olfactory learning has been widely studied before (10–15). The present results show conclusively that DA is the critical signaling molecule released by these neurons that is required for this form of associative memory. Interestingly, although electrical shock avoidance is not altered, we observed that the DA-deficient flies have a striking tendency to choose the shock-associated odor 2 h after training, indicative of an inversion of the evaluation system. Such an apparently "masochistic" behavior could result from an inability of these flies to attribute a negative value to a reinforcing stimulus, leading to a bias toward an appetitive behavior. In a possibly related observation, Krashes et al. (12) recently reported that specific DA neurons can inhibit the expression of an appetitive memory performance.

Our study reveals the requirement for neural DA in sugar preference and normal food intake in *Drosophila*. The fact that serotonin is also involved in feeding in flies (28) suggests that these two biogenic amines directly interact in the control of this behavior. The lack of sugar preference, despite the intact proboscis extension reflex, may suggest that DA is required for reward and motivation in flies, as is the case in mammals, but this would need further work to be established. Remarkably, several of the deficits we observe in DA-deficient *Drosophila*, (e.g., hypoactivity and hypophagy readily rescued by L-DOPA) are quite similar to phenotypes observed in DA-deficient mice (29). The same is true for the preservation of dopaminergic neuron circuits. This argues for partial conservation of brain DA functions between flies and mammals.

The lack of phototaxis does not result from blindness because flies without neural DA have normal electroretinograms and optomotor response. Circadian entrainment to dim light also requires neural DA (30). We suspect that this is not related to the phototaxis defect, which was tested under high levels of illumination. An unexpected result of this study is that visual tracking and spatial orientation memory are largely retained in *Drosophila* lacking neural DA. Although neural DA improves visual fixation and orientation toward a landmark, possibly by increasing arousal level and attention (8), this neuromodulator appears to be dispensable for these behaviors. Our results, in agreement with previous findings (26, 31), suggest that the neural circuits involved in the formation of spatial memory do not depend on neural DA.

In conclusion, we find that activity, feeding, and certain choice behaviors are markedly altered or abolished in neural DA-deficient flies, whereas other complex behaviors are surprisingly well maintained and might be similar to WT without the decrease in arousal. This suggests that DA is implicated in many but not all aspects of brain functioning in *Drosophila*. The specific behaviors that absolutely require DA could be related to the primordial and evolutionarily conserved functions of this essential neuromodulator in primitive nervous systems.

## Materials and Methods

Further details and references of the procedures are provided in *SI Materials and Methods*.

**Drosophila Strains.** DTHg- and DTHg^{FS±}-rescued homozygous *ple Drosophila* were generated before each experiment 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. The Canton S strain was used as WT flies for comparison in behavior tests.

**Immunohistochemistry.** Adult brains were dissected in ice-cold *Drosophila* Ringer's solution and processed for whole-mount immunostaining by standard procedure. The following primary antibodies were used: rabbit anti-DA (1:100; ImmunoStar), rabbit anti-serotonin (1:500; Sigma-Aldrich), mouse monoclonal anti-TH (1:50; ImmunoStar), rat anti-Ddc (1:200; prepared in the laboratory of J.H.), and rabbit anti-GFP (1:500; Invitrogen Molecular Probes).

**DA Assay.** DA levels in *Drosophila* brain extracts were determined by HPLC coupled to electrochemical detection essentially using a mobile phase containing 4 mM decanesulfonic acid, 50 mM citrate/acetate (pH 4.5), and 20% (vol/vol) acetonitrile. Standard detection of DA was performed at a detector potential of +500 mV relative to an Ag/AgCl electrode. Electrochemical confirmation of the identity of the DA peaks was performed at a reduced potential of +420 mV.

**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 walking freely for 15 min in an open arena. Second, activity was monitored by recording infrared beam crossings in glass tubes (6.5-cm length, 3-mm inside diameter) using a *Drosophila* activity monitoring system (TriKinetics).

**Olfactory Conditioning.** To test for aversive olfactory learning, groups of about 35 flies were conditioned in a barrel-type machine by sequential exposure to two odors, octanol and methylcyclohexanol, for 60 s with 45-s rest intervals between presentation of odors (25). Exposure to the first odor was paired with electrical shocks.

**Phototaxis.** Phototactic behavior was tested by mass and single-fly assays. The mass assay was carried out in a dark room with the countercurrent procedure. 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 and a dark vial. 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.

**Visual Fixation and Orientation.** Orientation toward a landmark was analyzed in the Buridan's paradigm on freely walking 5-d-old *Drosophila* with shortened wings. The angular deviation between fly trajectory and the approached target was measured every 0.2 s for 15 min. Spatial orientation memory was tested in the detour paradigm (26). Ten consecutive trials of at least 10 flies per genotype were recorded. Data were analyzed with the Kruskal–Wallis ANOVA test.

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