Neuroscience

# Altered reactivity to threatening stimuli in *Drosophila* models of Parkinson's disease, revealed by a trial-based assay

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## Abstract

The fruit fly *Drosophila melanogaster* emerges as an affordable, genetically tractable model of behavior and brain diseases. However, despite the surprising level of evolutionary conservation from flies to humans, significant genetic and circuit-level differences hinder the interpretability of fruit fly models for human disease. Therefore, to facilitate fly-to-human translation with more direct behavior-level comparison, we surveyed the rarely-exploited, rich behavioral repertoire of fruit flies with genetic alterations relevant to Parkinson's disease (PD). Flies displayed variable behaviors, including freezing, slowing and running, in response to predator-mimicking passing shadows used as threatening stimuli in a single-animal trial-based assay. We found that the expression of human mutant Parkin in flies resulted in reduced walking speed and decreased reactivity to passing shadows. Flies with dopamine receptor mutations showed similar alterations, consistent with the motor and cognitive deficits typical in humans with PD. However, *Drosophila* overexpressing the human form of α-synuclein manifested in only moderate phenotypical alterations, suggesting that other fruit fly models may be favored in PD research. We also found age-dependent trends in behavioral choice across the fly lifespan, while dopamine receptor mutant flies maintained their decreased general reactivity throughout all age groups. Our data demonstrate that single-trial behavioral analysis can reveal subtle behavioral changes in mutant flies that can be used to further our understanding of disease pathomechanisms and help gauge the validity of genetic *Drosophila* models of neurodegeneration.

**eLife assessment**

The authors present **useful** findings on the use of a Drosophila behavioral paradigm for assessing different fly genetic models of neurodegeneration. The experimental design and analyses are **solid** and can be used for quick behavioral assessment in fly models of various neurodegenerative diseases, especially those having an impact on locomotion. The work will be of interest to Drosophila biologists using behavior as a readout for their studies.

## Introduction

Parkinson's disease (PD) affects over 6 million people worldwide, representing the second most common neurodegenerative disease. PD is slowly progressing and typically leads to years of aggravating disability, thereby placing a huge burden on families, health care systems and the society, measured in hundreds of billions of dollars annually (Olesen et al., 2012; Obeso et al., 2017; Przedborski, 2017; Bloem et al., 2021). Therefore, a hitherto elusive disease-modifying therapy is of prominent priority, envisioned to be fueled by research into basic mechanisms of the disease.

Patients with PD display a diverse set of motor and non-motor symptoms with an underlying progressive loss of midbrain dopaminergic neurons (Schapira, 2009; Schapira et al., 2017; Bloem et al., 2021), mechanisms of which are widely studied in animal models of the disease. Primate models offer the advantage of more direct translatability but are restricted to hard-to-handle neurotoxin approaches and do not promise fast progress. Unlike the genetic mouse models of Alzheimer's disease, most of the successful rodent models of PD are also toxin-based, making it difficult to exploit and further our understanding of the genetic bases of the disease (Cannon and Greenamyre, 2010; Bové and Perier, 2012; Breger and Fuzzati Armentero, 2019). This left a niche for the genetically tractable, affordable fruit flies as genetic models of PD (Feany and Bender, 2000; Guo, 2012; Hewitt and Whitworth, 2017), building on the homologies between the vertebrate basal ganglia and the fruit fly central complex (Strausfeld and Hirth, 2013). However, substantial genetic, anatomical, physiological, and behavioral discrepancies between insects and mammals call for better validation methods of fruit fly models for human disease. To address this, here we used a single-animal trial-based behavioral assay that facilitates fine-grained assessment of phenotypical behavioral changes in fruit flies with mutations relevant to understanding human PD.

Genes linked to familial forms of PD may serve as an ideal basis for genetic disease models. Notably, mutations in the *PARK2* gene, which encodes the Parkin protein involved in maintaining mitochondrial integrity, are associated with autosomal-recessive forms of PD (Guo, 2012). Parkin loss-of-function mutant flies were found to have advanced mitochondrial aging, structural mitochondrial damage and a consequential selective loss of dopaminergic neurons (Cackovic et al., 2018), leading to motor deficits assessed by a climbing assay (Chambers et al., 2013; Cackovic et al., 2018), as well as non-motor PD phenotypes including memory deficits (Julienne et al., 2017). Mutations in the *SNCA* gene of α-Synuclein (α-Syn) are also associated with familiar forms of PD (Polymeropoulos et al., 1997) and α-Syn has been shown to accumulate in Lewy-bodies and Lewy-neurites (Spillantini et al., 1997). α-Syn proteins have been found in the pre-synaptic terminals in humans and mice (Kahle et al., 2000) and are thought to be involved in dopamine (DA) synthesis under physiological conditions by reducing tyrosine hydroxylase activity (Perez et al., 2002). Expression of human α-Syn in flies has been proposed as a genetic model of PD, showing age-dependent loss of dopaminergic neurons and locomotor dysfunction in a

climbing assay (Feany and Bender, 2000; Haywood and Staveley, 2006). However, other studies found normal locomotion and dopaminergic cell counts in these flies, casting doubts on the validity of this model (Pesah et al., 2005; Nagoshi, 2018).

Despite the observed homology between mammalian and fruit fly DA systems in motor control and the establishment of *Drosophila* PD models based on human genetic information derived from familial PD patients, the role of *Drosophila* DA receptors in locomotor control is not well characterized. Nevertheless, it has been demonstrated that the D1-like DA receptor mediates ethanol-induced locomotion in the ellipsoid body (Kong et al., 2010). Furthermore, *Dop1R1* has been shown to be involved in turning behavior for goal-directed locomotion (Kottler et al., 2019) and startle-induced negative geotaxis (Sun et al., 2018). To shed light on the possible roles of DA receptors in threat-induced motor behaviors, we tested the behavioral responses of three dopamine receptor (*Dop1R1*, *Dop1R2* and *DopEcR*) insertion mutant lines to predator-mimicking passing shadows and compared them to established fruit fly PD models with partially known locomotor deficits.

We found that flies expressing the R275W mutant allele of human Parkin ('Parkin flies') showed slower average locomotion speed, which, in contrast, was not characteristic of flies expressing the A53T mutant allele of the human *SNCA* gene ('α-Syn flies'). Parkin flies also showed less frequent freezing and generally less behavioral reactivity to passing shadows compared to controls, whereas α-Syn flies showed increased durations of stopping after the stimuli. Dopamine receptor mutant flies showed reduced speed and less behavioral reactivity similar to Parkin flies. *Dop1R1* mutant flies exhibited more pronounced behavioral alterations than the other two receptor mutants in most of the parameters tested. These data demonstrate that mutations in DA receptor genes lead to specific patterns of behavioral deficits in *Drosophila*; hence, these dopamine receptor paralogs may have different functions in behavioral control. The modest phenotype of A53T α-Syn compared to Parkin flies suggests that the latter should be favored as a genetic model of human PD, at least with respect to the motor deficits examined in this study. We further propose that single-trial analyses such as those we present here help us gain a better understanding of the behavioral changes in fruit fly models of PD and are strong tools for validating *Drosophila* models of human diseases.

# Results

## A single-animal trial-based assay to test behavioral responses to predator-mimicking passing shadows

We designed a behavioral apparatus to examine the responses of individual flies to predator-mimicking passing shadows. To do this, we designed a transparent plexiglass arena (**Fig. 1a**), featuring 13 tunnels (53 mm x 5 mm) for simultaneous tracking of 13 individual flies. The height of the tunnels (1 mm) was designed to allow free walking in two dimensions but prevent jumps and flight. To simulate predatory threat, we created passing 'shadows' (**Fig. 1a**, right; see Methods) with a sliding red screen presented on a 10.1-inch display placed on the top of the arena. A high frame-rate camera was placed under the arena (**Fig. 1a**, left), allowing us to simultaneously record the movement of the animals and the shadows. All 13 tunnels housed a single fly in each session, enabling the collection of single-animal data from 13 flies in parallel. The locomotion of individual flies was tracked by custom software developed using the Bonsai visual programming environment (**Fig. S1**, see Methods). We recorded 40-minute-long sessions, consisting of 40 trials of 2-second-long shadow presentations separated by pseudorandom inter-trial-intervals to prevent the animals from learning temporal expectations of the shadow presentations (**Fig. 1b**).

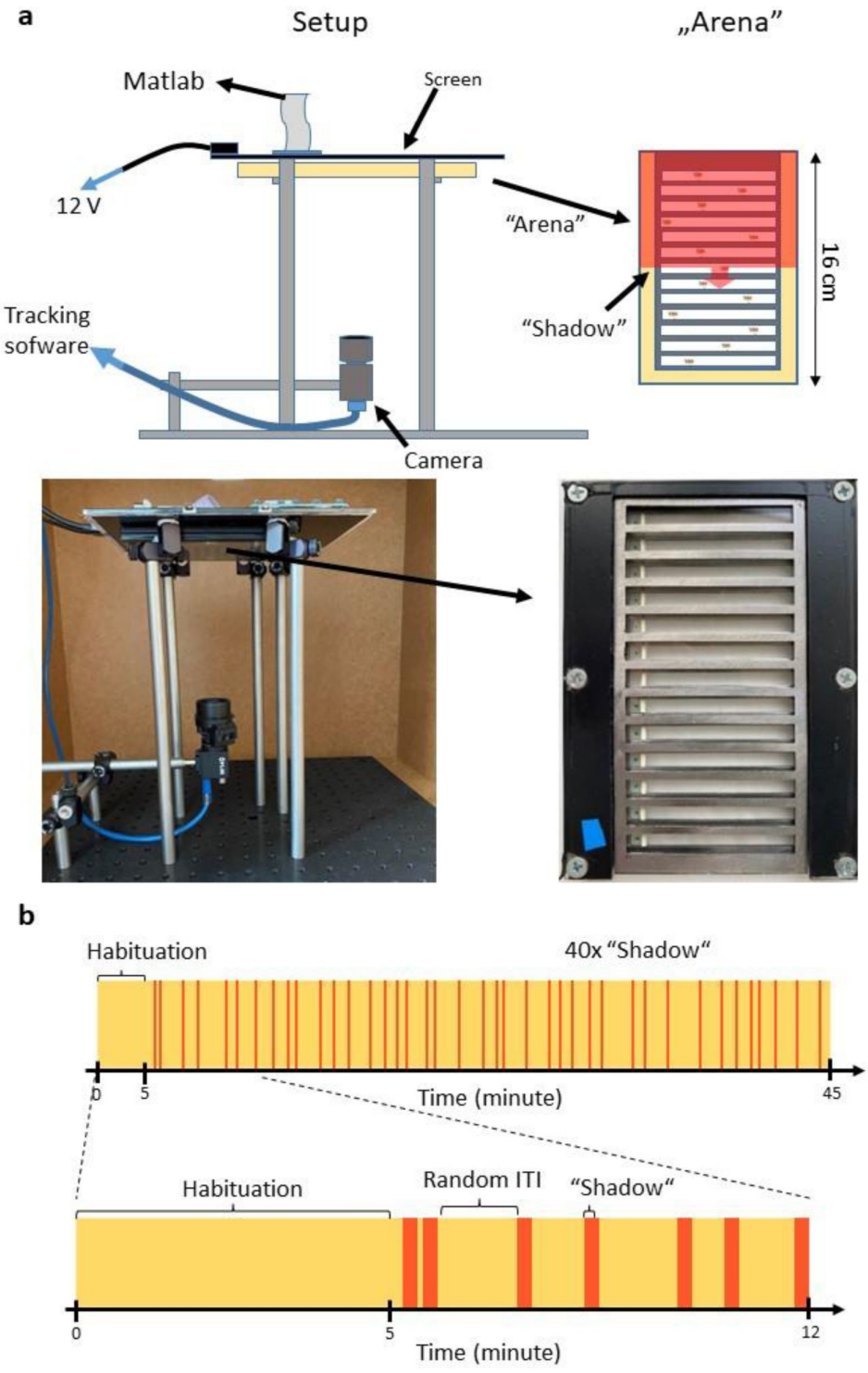


**Fig. 1.**

**Behavioral apparatus and experimental design.**

**a,** Top left, schematic of the experimental setup. Top right, schematic of the fly arena. Bottom left, photograph of the experimental setup. Bottom right, a photograph of the fly arena. **b,** Timeline of a session showing the shadow presentations in red over the yellow background.

Fruit flies exhibit a rich behavioral repertoire upon threatening stimuli, including freezing (or stopping) and various escape behaviors such as jumping, slow or fast take-off and running, modulated by walking speed at the time of the threatening stimulus (Zacarias et al., 2018). To study these behaviors, we calculated the speed and acceleration of fruit flies based on the tracked x-y position of their center of mass (**Fig. 2a**) and aligned these signals to the presentations of the shadow stimuli. To identify separate response types to the threating stimuli, we applied hierarchical clustering on these stimulus-aligned speed traces (see Methods). This analysis consistently revealed three main stereotypical behavioral responses across flies in addition to the trials where no significant response was evoked (**Fig. 2b**). Since jumping and flying was not possible in the arena, fruit flies were restricted to choose among freezing, slowing (also observed in (Zacarias et al., 2018)) and running. While this clustering approach was sufficient to reveal response types qualitatively, cluster boundaries were sensitive to genotype-specific differences across groups of flies (Király and Hangya, 2022). Therefore, for rigorous group comparisons, we determined exact definitions of each response type based on fly speed and acceleration (**Fig. 2c**). Reactions were considered robust if the absolute value of the acceleration reached 200 $mm/s^2$; otherwise, the trial was classified as a 'no reaction' trial. We considered the reaction as a 'stop' if the animal was moving before shadow presentation and its speed decreased to zero in the first second relative to the shadow presentation. If fly speed decreased to a non-zero value, the trial was defined as 'slow down', and if the fly accelerated after the shadow presentation, the trial was classified as 'speed up' (see also Methods).

## Parkinson's model and dopamine receptor mutant fruit flies showed reduced walking speed and decreased reactivity to threatening stimuli

To generate PD fly models, we expressed the mutant human Parkin (275W) and α-Syn (A53T) coding transgenes (*UAS-Parkin-275W* and *UAS-α-Syn-A53T*, respectively), applying the UAS-Gal4 system (Duffy, 2002). Transgenes were driven by *Ddc-Gal4* inducing the expression of the correspondent human mutant proteins in dopaminergic and serotoninergic fly neurons. Parkin and α-Syn flies were compared to control animals from the same genetic background without mutant transgenes (*Ddc-Gal4* animals were crossed with *iso* $w^{1118}$, the examined F1 generation flies are referred to as *iso* $w^{1118}$ **Fig. 3**) or mutants overexpressing GFP (**Fig. S2**). The same level of eye pigmentation and vision of the compared genotypes was achieved by the prior replacement of the *w** mutant first chromosome of the applied *Ddc-Gal4* stock for that of the wild-type. The dopamine receptor mutant groups were compared to their parental strains without the mutations ($y^1$ $w^{67c23}$ served as control for *Dop1R1* and *Dop1R2* and $w^{1118}$ for *DopEcR*; **Fig. 3**).

To test whether mutant flies showed differences in overall locomotion independent of the threatening stimuli, we analyzed average fly speed in the 200 ms time windows before stimulus presentation. We found that Parkin flies showed reduced mean speed compared to controls which expressed the Gal4 driver alone (24.96% reduction, $p = 6.55 \times 10^{-6}$, Mann-Whitney U-test; **Fig. 3a**, top), while α-Syn flies did not show significant reduction (p = 0.799, Mann–Whitney U-test). We observed similar changes in dopamine receptor mutant flies, where the *Dop1R1* and *DopEcR* defective lines showed a robust mean speed decrease compared to the control groups (*Dop1R1*, 19.68% decrease compared to $y^1$ $w^{67c2}$, p = 0.0016; *DopEcR*, 32.97% decrease compared to $w^{1118}$, p = 0.0034; **Fig. 3a**, bottom), while the *Dop1R2* mutant flies only showed a non-significant speed decrease (21.73% compared to $y^1$ $w^{67c23}$, p = 0.1009, Mann–Whitney U-test).

Next, we tested whether mutant flies showed a difference in their reaction to threatening stimuli. We found that PD model flies showed line-specific alterations in their freezing behavior. Parkin flies froze after stimuli less frequently, stopping in 37.72% of trials compared to the 43.48% observed in the *iso* $w^{1118}$ animals (p = 0.0043, Mann Whitney U-test; **Fig. 3b**, top). However, in the stop trials, they showed normal duration of pauses in locomotion (p = 0.8018 compared to *iso*

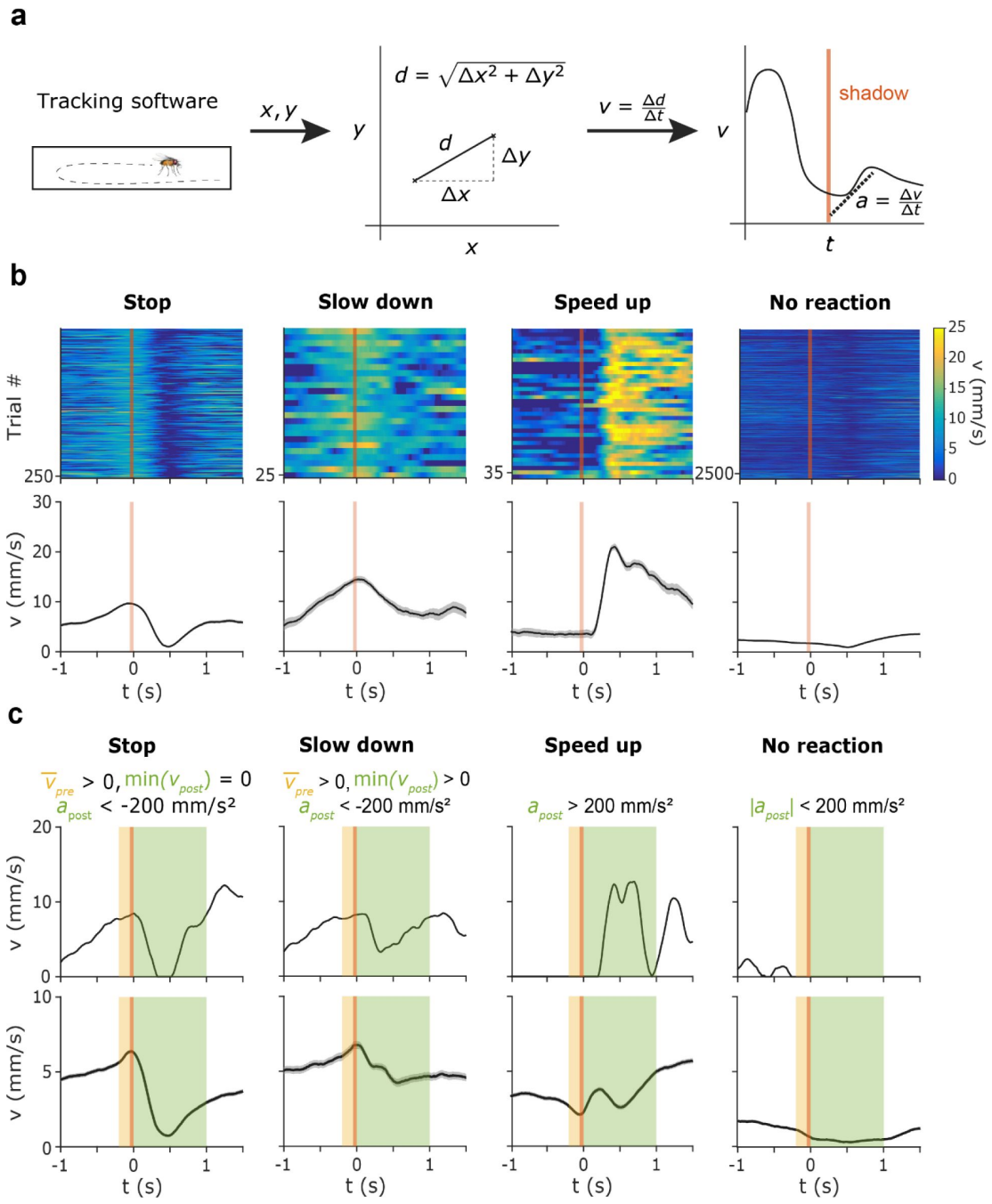


**Fig. 2.**

**Behavioral characterization of the escape behavior repertoire of individual fruit flies.**

**a,** Schematic for calculating speed and acceleration based on tracked position coordinates. **b,** Four characteristic escape behaviors categorized by PCA for an example session of a control fly (w1118; from left; stop, slow down, speed up, no reaction). Top, color-coded heatmaps indicating the walking speed of the fly (blue, low speed; yellow, high speed), aligned to shadow presentations (orange line). Bottom, average moving speed triggered on the shadow presentations (orange line). **c,** Threshold-based classification of behavioral responses (from left, stop, slow down, speed up, no reaction). Top, single-trial example for each response type. Time intervals for calculating the average speed before the shadow presentation (orange) as well as the speed and acceleration after the shadow presentation (green) are marked. Threshold values for each response type are displayed above the graphs. Bottom, average walking speed across the trials from all session of $w^{1118}$ flies, sorted by the type of behavioral response. Line and errorshade show mean ± SE.

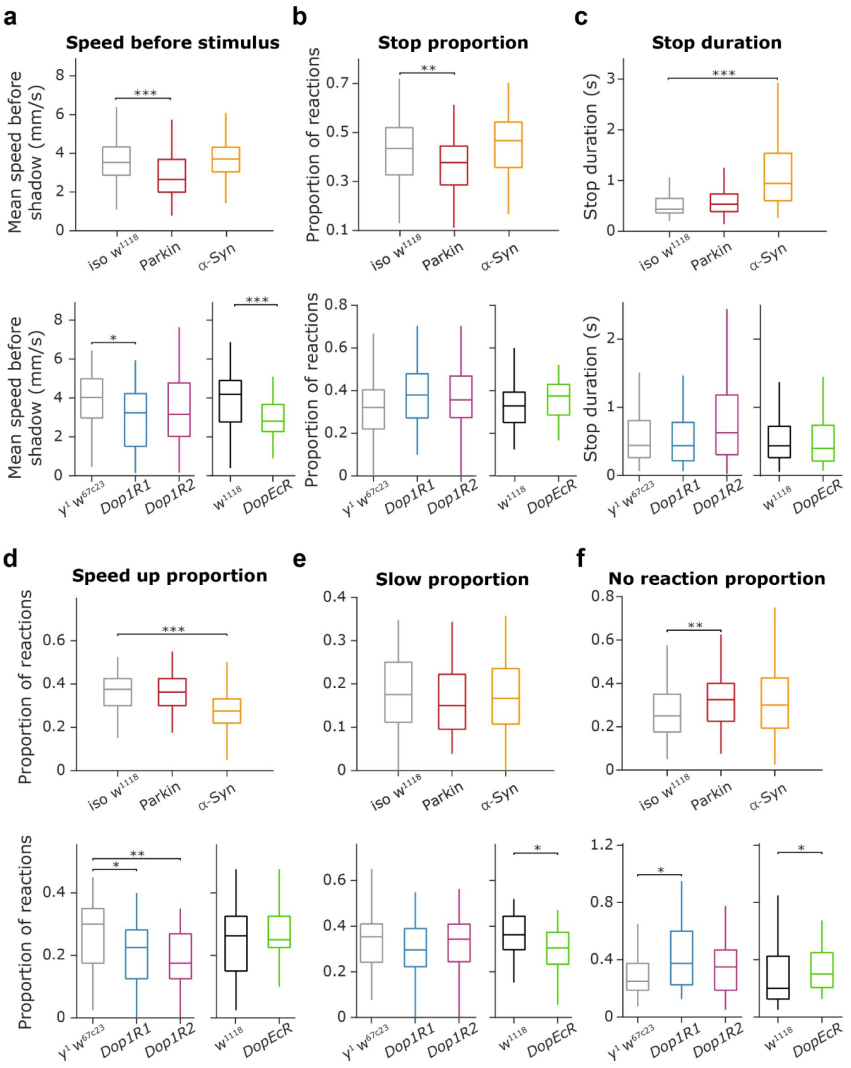


**Fig. 3.**

## Parkinson's model and dopamine receptor mutant fruit flies showed reduced walking speed and decreased reactivity to threatening stimuli.

**a**, Distribution of the mean speed measured in the time window [-0.2, 0] seconds relative to shadow presentation for the different mutant groups. Top, Parkin flies showed reduced mean speed compared to controls (Parkin vs. *iso* $w^{1118}$; p = 6.55 × $10^{-6}$, Mann–Whitney U-test). Bottom, *Dop1R1* and *DopEcR* mutant flies showed reduced mean speed compared to controls ($y^1w^{67c23}$and $w^{1118}$ respectively; p = 0.0016, p = 0.0034; Mann-Whitney U-test). **b,** Distribution of the proportion of stop trials in different mutant groups. Parkin flies showed a reduced tendency for stopping compared to *iso* $w^{1118}$ (p = 0.0043, Mann Whitney U test). **c,** Distribution of stop duration in different mutant groups. α-Syn flies showed increased stop durations compared to *iso* $w^{1118}$ (p = 2.18 × $10^{-11}$, Mann-Whitney U-test). **d,** Distribution of the proportion of speed up trials in different mutant groups. Top, α-Syn flies showed a reduced tendency to speed up compared to their controls (p = 8.4 × $10^{-6}$, Mann-Whitney U-test). Bottom, *Dop1R1* and *Dop1R2* mutant flies also showed a significantly reduced tendency to speed up compared to their controls (p = 0.0122 and p = 0.0024, respectively; Mann-Whitney U-test). **e,** Distribution of the proportion of slow down trials in different mutant groups. Bottom, *DopEcR* showed a 15.04% decrease compared to $w^{1118}$ (p = 0.020, Mann-Whitney U-test). **f,** Distribution of the proportion of 'no reaction' trials in different mutant groups. Top, Parkin mutants showed reduced reactivity compared to *iso* $w^{1118}$ controls (p = 0.0043, Mann-Whitney U-test). Bottom, *Dop1R1* and *DopEcR* mutants also showed reduced reactivity compared to $y^1w^{67c23}$ and $w^{1118}$ controls, respectively (p = 0.0173 and p = 0.0167, respectively; Mann-Whitney U-test). Box-whisker plots show median, interquartile range and non-outlier range. *, p < 0.05; **, p < 0.01; ***, p < 0.001. Exact genotypes: *iso* $w^{1118}$: *+; +; Ddc-Gal4/+.* Parkin: *+; +; Ddc-Gal4/UAS-Parkin-R275W.* α-Syn: *+; +; Ddc-Gal4/UAS-α-Syn-A53T*. $y^1$ $w^{67c23}$: $y^1$ $w^{67c23}$. *Dop1R*: $y^1$ *w*; Mi{MIC}Dop1R1*$^{MI04437}$. *Dop1R2*: $y^1$ *w*; Mi{MIC}Dop1R2*$^{MI08664}$. *DopEcR*: $w^{1118}$*; PBac{PB}DopEcR*$^{c02142}$*/TM6B, Tb*$^1$.

$w^{1118}$, Mann Whitney U-test; **Fig. 3c**, top). In contrast, α-Syn flies showed an unchanged stopping frequency when compared to controls (p = 0.0768 compared to *iso* $w^{1118}$, Mann-Whitney U-test), but their stop durations showed a large increase (116.98% increase compared to *iso* $w^{1118}$, $p = 2.18 \times 10^{-11}$, Mann-Whitney U-test). Interestingly, in contrast to PD model flies, dopamine receptor mutant flies did not show significant differences in their freezing behavior relative to controls (stop proportion: *Dop1R1*, p = 0.0631 compared to $y^1$ $w^{67c23}$; *Dop1R2*, p = 0.1838 compared to $y^1$ $w^{67c23}$; *DopEcR*, p = 0.1445 compared to $w^{1118}$; Mann-Whitney U-test; **Fig. 3b**, bottom; stop duration: *Dop1R1*, p = 0.893 compared to $y^1$ $w^{67c23}$; *Dop1R2*, p = 0.154 compared to $y^1$ $w^{67c23}$; *DopEcR*, p = 0.3576 compared to $w^{1118}$; Mann-Whitney U-test; **Fig. 3c**, bottom).

α-Syn flies showed a reduced aptitude to increase their speed, or 'run', upon encountering threatening stimuli (26.67% decrease compared to *iso* $w^{1118}$, $p = 8.4 \times 10^{-6}$, Mann-Whitney U-test; **Fig. 3d**, top). Similar results were found in *Dop1R1* (25% decrease compared to $y^1$ $w^{67c23}$, p = 0.0122; Mann-Whitney U-test; **Fig. 3d**, bottom) and *Dop1R2* (41.67% decrease compared to $y^1$ $w^{67c23}$, p = 0.0024, Mann-Whitney U-test), but not in *DopEcR* mutant flies (compared to $w^{1118}$, p = 0.6598, Mann-Whitney U-test). Frequency of slowing, i.e., reducing their speed without freezing in a full stop, was moderately decreased in Parkin (14.38% decrease compared to *iso* $w^{1118}$, p = 0.0723, Mann-Whitney U-test; **Fig. 3e**, top) and *DopEcR* mutant flies (15.28% decrease compared to $w^{1118}$, p = 0.020, Mann-Whitney U-test; **Fig. 3e**, bottom).

Overall, all mutations tested resulted in decreased reactivity to threatening stimuli, confirmed by a significant increase in the proportion of trials where no reactions were detected (**Fig. 3f**). This effect was significant for Parkin flies (30% increase compared to *iso* $w^{1118}$, p = 0.0043, Mann-Whitney U-test), as well as for *Dop1R1* (50% increase compared to $y^1$ $w^{67c23}$, p = 0.0173, Mann-Whitney U-test) and *DopEcR* mutant flies (50% increase compared to $w^{1118}$, p = 0.0167, Mann-Whitney U-test), but not for α-Syn (α-Syn vs. *iso* $w^{1118}$, p = 0.151, Mann-Whitney U-test) and *Dop1R2* mutant flies (*Dop1R2* vs. $y^1$ $w^{67c23}$, p = 0.1903, Mann-Whitney U-test).

## Reaction to threatening stimuli depends on fly walking speed

It has been shown that fruit flies may exhibit a different choice of escape behavior based on their momentary speed when encountering the threat (Zacarias et al., 2018). Therefore, we tested whether mutations in genes relevant to PD caused a change in this speed - behavioral response relationship. We calculated the probability of stopping, slowing, speeding up and no reaction as a function of speed for all mutants, as well as the ratio of each response type conditioned on walking speed at the time of shadow presentations (**Fig. 4a-b**). These analyses confirmed that 'running' and 'no reaction' was most likely at slow (< 5 mm/s) or zero walking speeds, while slowing down was more frequent as the walking speed increased. Freezing was most frequently observed in the 5-13 mm/s speed range. We did not observe significant difference between groups in their speed - behavioral response relationship for any of the response types (stops, p = 0.4226; slow down, p = 0.6025; speed up, p = 0.9074; no reaction p = 0.4692; two-way ANOVA genotype × speed interaction).

Since the distribution of the expression of various escape behaviors depended on walking speed, which was also different across the mutant lines tested (**Fig. 3**), we asked whether the difference in baseline speed could explain the observed differences in behavioral reactions. Therefore, we performed simulations where reaction probabilities were based on the baseline walking speed distribution of each mutant line to test whether behavioral responses could be predicted based on speed alone (**Fig. S3**). In most of the groups we found a significant difference between the predicted and the measured response type distributions, suggesting a role for other behavioral differences among the mutant lines beyond the baseline speed differences (Parkin, p = 0.0487; α-Syn, p < 0.000001; *Dop1R1*, p = 0.332; *Dop1R2*, p = 0.006157; *DopEcR*, p = 0.003901, chi-square test).

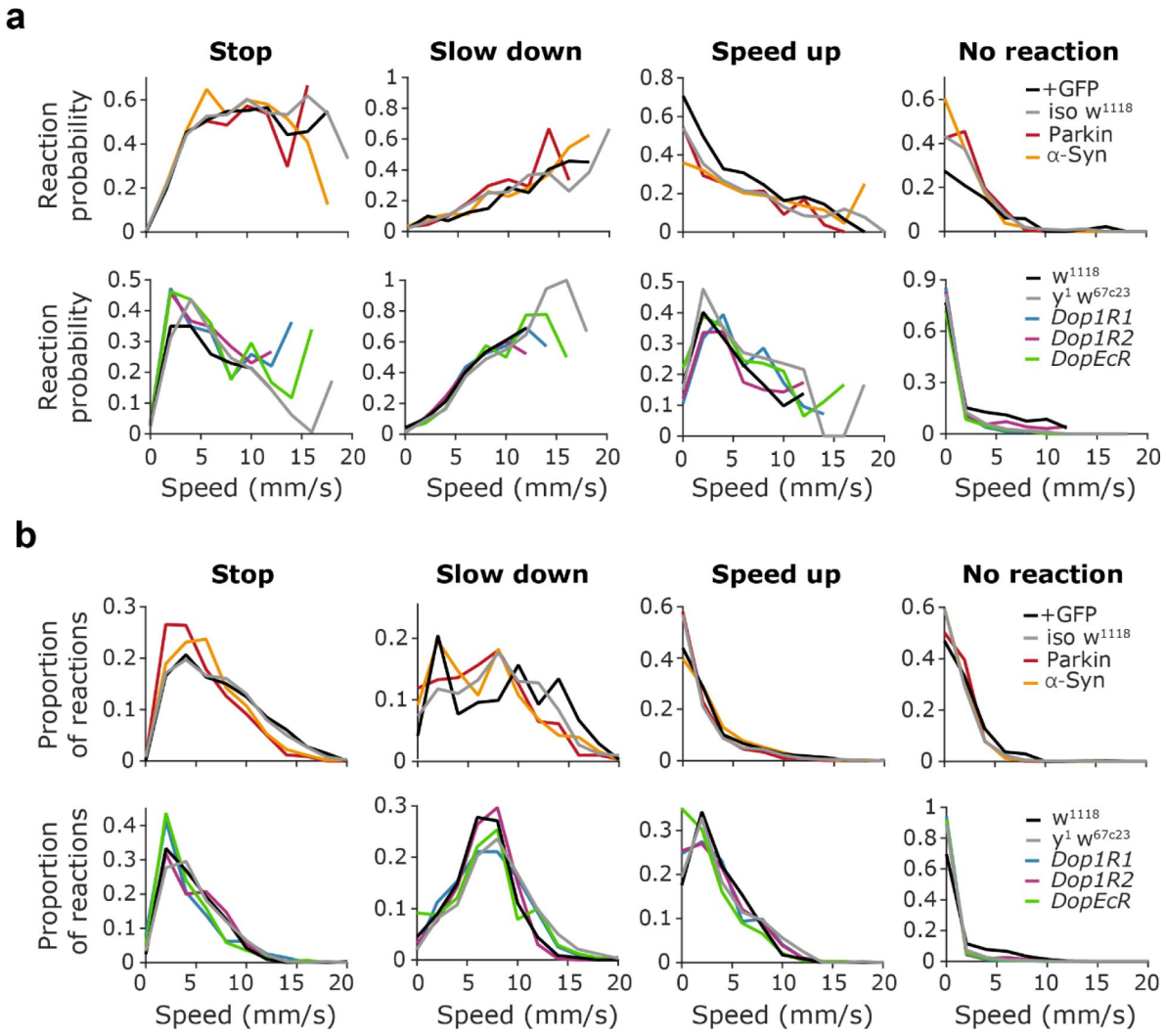


**Fig. 4.**

**Reaction to threatening stimuli depends on fly walking speed.**

**a,** Probability of a given response type as a function of average fly speed before the shadow presentation (200 ms pre-stimulus time window). From left, stop, slow down, speed up and no reaction trials are quantified. Top, Parkin and α-Syn flies. Bottom, *Dop1R1*, *Dop1R2* and *DopEcR* mutant flies. **b,** Proportion of a given response type as a function of average fly speed before the shadow presentation. Top, Parkin and α-Syn flies. Bottom, *Dop1R1*, *Dop1R2* and *DopEcR* mutant flies. Exact genotypes: *iso* $w^{1118}$: *+; +; Ddc-Gal4/+*. Parkin: *+; +; Ddc-Gal4/UAS-Parkin-R275W*. α-Syn: *+; +; Ddc-Gal4/UAS-α-Syn-A53T*. $y^1$ $w^{67c23}$: $y^1$ $w^{67c23}$. *Dop1R*: $y^1$ *w*; Mi{MIC}Dop1R1*$^{MI04437}$. *Dop1R2*: $y^1$ *w*; Mi{MIC}Dop1R2*$^{MI08664}$. *DopEcR*: $w^{1118}$; *PBac{PB}DopEcR*$^{c02142}$*/TM6B, Tb*$^1$.

### Changes in escape behavior from the first to the fourth week of life

The age of *Drosophilae* may have a significant influence on their responses to threatening stimuli. To test this, we examined control and dopamine receptor mutant flies ($w^{1118}$, $y^{1}$ $w^{67c23}$, *Dop1R1*, *Dop1R2*, *DopEcR*) in 5 age groups: from 1-day-old to 4-week-old (**Fig. 5**; **Fig. S4**). The animals were kept under conditions that accelerated their aging (29°C and 70% humidity). Our data showed that the behavior of 1-day-old flies was significantly different from all other age groups. One-day-old flies stopped significantly more often, and the frequency of stopping gradually decreased with age in all groups (two-way ANOVA, age, $p = 1.87 \times 10^{-20}$, $f = 26.11$; genotype, $p = 1.3 \times 10^{-29}$, $f = 38.42$; genotype × age, $p = 3.18 \times 10^{-6}$, $f = 3.54$; 1-day-old vs. 1-week-old, $p = 1.33 \times 10^{-5}$; 1-week-old vs. 2-week-old, $p = 0.017$, 2-week-old vs. 3-week-old, $p = 0.63$; 3-week-old vs. 4-week-old, $p = 0.098$, Tukey's test for post hoc analysis). In contrast, the probability of slowing down showed a substantial increase from 1-day-old to 1-week-old animals, and then remained unchanged until the 4th week (two-way ANOVA, age, $p = 9.11 \times 10^{-15}$, $f = 18.72$; genotype, $p = 1.4 \times 10^{-21}$, $f = 27.59$; genotype × age, $p = 0.0028$, $f = 2.291$; 1-day-old vs. 1-week-old, 1-week-old vs. 2-week-old, $p = 2.56 \times 10^{-8}$; $p = 1$; 2-week-old vs. 3-week-old, $p = 0.13$; 3-week-old vs. 4-week-old, $p = 0.093$; Tukey's test for post hoc analysis). The probability of speeding up showed a similar trend, except that the 4-week-old animals showed a significant decrease compared to 3-week-old flies (two-way ANOVA, age, $p = 2.48 \times 10^{-15}$, $f = 19.44$; genotype, $p = 2.28 \times 10^{-44}$, $f = 59.48$; genotype × age, $p = 1.1 \times 10^{-7}$, $f = 4.12$; 1-day-old vs 1-week-old, $p = 9.92 \times 10^{-9}$; 1-week-old vs. 2-week-old, $p = 0.79$; 2-week-old vs. 3-week-old, $p = 0.75$; 3-week-old vs. 4-week-old, $p = 0.024$; Tukey's test for post hoc analysis). Thus, different types of escape reactions showed similar trends across the fly lifespan, while dopamine receptor mutant flies maintained their decreased general reactivity throughout all age groups.

## Discussion

We tested *Drosophila* mutant strains relevant for Parkinson's disease in a single-trial single-animal behavioral assay. Our tests revealed strain-specific behavioral alterations in flies' reactions to predator-mimicking passing shadows, serving as proof of principle demonstration of the viability of single-trial approaches in *Drosophila*, a method widely used in mammalian studies. Specifically, we found reduced walking speed, decreased freezing frequency and decreased overall reactivity in Parkin flies. In contrast, α-Syn flies merely showed an increased freezing duration without a concomitant change in freezing frequency, suggesting that Parkin flies better recapitulate some behavioral features of human PD progression. *Dop1R* mutant flies resembled Parkin flies in their decreased walking speed and decreased reactivity to the threatening stimuli. The distribution of the behavioral response fruit flies chose to execute depended on their speed at the time of stimuli, and this speed - behavioral response relationship was robust across the tested genotypes. Nevertheless, differences in walking speed alone did not explain the strain-specific behavioral alterations. Age-dependence of the behavioral choice was also found to be stereotypic across the tested genotypes.

Recent studies increasingly recognize the importance of investigating subtle changes in fly behavior to better understand the manifestations of locomotor and other disorders (Geissmann et al., 2017; Zacarias et al., 2018; Seidenbecher et al., 2020). Along these lines, Aggarwal and colleagues introduced an automated climbing assay for fruit flies and revealed subtle motor deficits in different mutant strains (Aggarwal et al., 2019). We expanded the scope of these studies by examining a diverse behavioral repertoire of *Drosophila* in response to threatening stimuli. The diversity of freeze vs. flee responses of fruit flies, including different durations of stopping as well as running, were accurately described by Zacarias et al. (Zacarias et al., 2018), where they also identified DNp09 neurons as important controllers of running and freezing responses. We built on this behavioral characterization for our definitions of fly reactions to predator-mimicking stimuli.

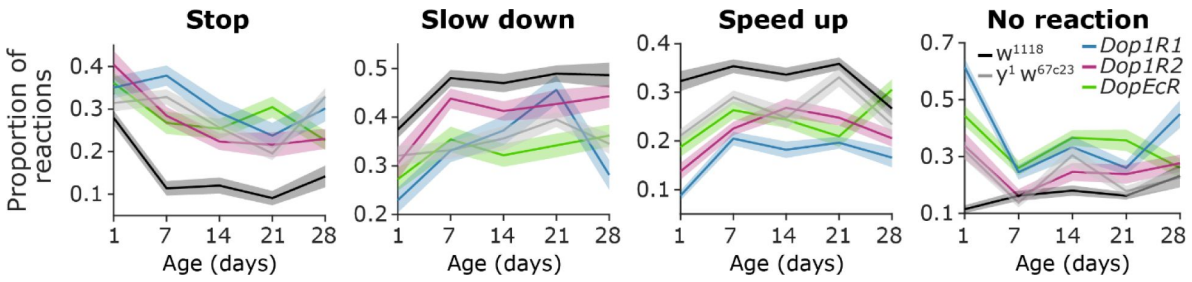


**Fig. 5.**

### Changes in escape behavior from the first to the fourth week of life

Proportion of responses as a function of age for different groups of mutants and controls. From left to right, stop, slow down, speed up and no reaction trials are quantified. Lines and errorshades show mean and standard error. Exact genotypes: *iso* $w^{1118}$: *+; +; Ddc-Gal4/+*. Parkin: *+; +; Ddc-Gal4/UAS-Parkin-R275W*. α-Syn: *+; +; Ddc-Gal4/UAS-α-Syn-A53T*. $y^1$ $w^{67c23}$: $y^1$ $w^{67c23}$. *Dop1R*: $y^1$ *w*; Mi{MIC}Dop1R1*$^{MI04437}$. *Dop1R2*: $y^1$ *w*; Mi{MIC}Dop1R2*$^{MI08664}$. *DopEcR*: $w^{1118}$; *PBac{PB}DopEcR*$^{c02142}$*/TM6B, Tb*$^1$.

Mutations in the *PARK2* gene lead to impaired ubiquitination and a consequential mitochondrial dysfunction (Guo, 2010, 2012), and early-onset PD in human patients. This led to the widespread use of Parkin flies as a genetic model pf PD (Guo, 2010; Chambers et al., 2013; Aggarwal et al., 2019). Of note, flies that lack Parkin display a significant degeneration of dopaminergic neurons (Whitworth et al., 2005). We found similarities between Parkin and *Dop1R* mutant flies in their altered responses to predator-mimicking passing shadows: reduced walking speed and decreased overall reactivity, suggesting that the lack of dopamine action through *Dop1R* may be one of the common pathways underlying motor deficits. This is in line with a recent study demonstrating impaired startle-induced geotaxis and locomotor reactivity in *Dop1R* mutant flies (Sun et al., 2018), similar to what had been shown for Parkin flies (Aggarwal et al., 2019), and a demonstration of dopaminergic control over walking speed (Marquis and Wilson, 2022). In contrast, flies expressing human α-syn showed moderate changes in motor behavior except for a marked prolongation of freezing duration upon threating stimuli. This is in accordance with studies suggesting that α-syn misexpression is not fully penetrant under some conditions (Pesah et al., 2005; Nagoshi, 2018), and calls for the use of other genetic *Drosophila* models, including Parkin flies, in studying the pathophysics of human PD.

The dopamine/ecdysteroid receptor *DopEcR* is a G-protein-coupled dual receptor for dopamine and the steroid hormone ecdysone. It has been proposed that the *DopEcR* may serve as an integrative hub for dopamine-mediated actions and stress responses in fruit flies (Petruccelli et al., 2020). We found that *DopEcR* mutant flies showed decreased mean walking speed, decreased probability of slowing down and decreased overall behavioral reactivity in response to predator-mimicking passing shadows. This pattern of alterations was largely similar to those observed in Parkin and *Dop1R* mutant flies, suggesting that the *DopEcR* may convey similar dopamine-mediated functions as *DopR1* at least in those motor aspects tested in the present study. A recent work demonstrated that serotonin also modulates both walking speed and startle response in flies, suggesting a complex neuromodulatory control over *Drosophila* motor behavior (Howard et al., 2019).

In humans, degeneration of midbrain dopaminergic neurons has been found as a direct cause of PD symptoms and DA supplementation with its precursor levodopa has been one of the most successful therapeutic approaches to date. The fruit fly and mammalian DA systems show a number of homologies which are thought to serve as a good basis for *Drosophila* PD models (Feany and Bender, 2000; Hewitt and Whitworth, 2017). Specifically, a deep-running evolutional conservation has been revealed between the arthropod central complex and the vertebrate basal ganglia, where GABAergic and dopaminergic neurons play key roles in motor control in both phylae (Strausfeld and Hirth, 2013). Clusters of fly dopaminergic neurons that project to the ellipsoid body, the fan-shaped body and the lateral accessory lobes are thought to share homologies with the striatum-projecting dopaminergic neurons of the substantia nigra in mammals. Both the fruit fly central complex and vertebrate basal ganglia mediate a range of functions from motor control and sensorimotor integration to action selection and decision making to motivation (Strauss, 2002; Claridge-Chang et al., 2009; Kong et al., 2010) and learning (McCurdy et al., 2021; Zolin et al., 2021; Fisher et al., 2022; Qiao et al., 2022; Taisz and Jefferis, 2022; Yamada et al., 2023). It has been found that fruit flies lacking the DA-synthetizing tyrosine hydroxylase enzyme in the central nervous system show reduced activity and locomotor deficit that worsen with age, and also exhibit marked impairments in associative learning based on both appetitive and aversive reinforcement (Riemensperger et al., 2011). This is in line with mammalian studies emphasizing the role of the dopaminergic nigrostriatal pathway in controlling goal-directed action (Eban-Rothschild et al., 2016), reinforcement learning (Schultz et al., 1997; Lak et al., 2014), and more recently, conveying aversive information (Menegas et al., 2015, 2018). Our findings are consistent with the apparent homologies across fruit fly and mammalian dopaminergic systems and suggest that a more in-depth investigation of specific behavioral changes and underlying dopamine-related dysfunctions may yield translatable results.

We also observed age-related changes in motor behavior in response to threatening stimuli, particularly in 4-week-old flies, which may parallel the age-dependent worsening of PD symptoms in humans.


## Acknowledgements

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## Data and code availability

All analysis functions are available at *https://github.com/hangyabalazs/Drosophila-behavior-analysis*. All data are available at *https://figshare.com/s/40539ee040a269bf05e5*.

## Methods

### Animals

We used 2-weeks-old *Drosophila melanogaster* (both males and females) raised at 24°C and 60% humidity in a natural light-dark cycle for experiments presented in **Figures 1**-**4**. For age-group comparisons (**Fig.5**.), fruit flies aged 1, 7, 14, 21 and 28 days were used. These flies were raised at 29°C and 70% humidity to accelerate aging.

Two sets of mutant groups were used for behavioral comparison. In the first set of animals we used *w*; UAS-Parkin-R275W* (created in the laboratory of Kah-Leong Lim, Neurodegeneration Research Laboratory, National Neuroscience Institute, Singapore)(Wang et al., 2007; Kovács et al., 2017) and *w*; UAS-alpha-Syn-A53T* (BDSC 8148) from the Eotvos Lorand University, with $y^1$, $v^1$*; UAS-GFP* (BDSC 35786) and *+; P{Ddc-GAL4.L}4.36* (modified version of BDSC 7009, the original first chromosome $w^{1118}$ has been replaced to $w^+$ in this study) as controls. For the second set of animals we used *Dop1R1*, *Dop1R2* and *DopEcR* mutant flies kindly donated by the Anne Von Phillipsborn lab, DANDRITE, Aarhus University and $y^1$ *w*; Mi{MIC}Dop1R1*$^{MI04437}$ (BDSC 43773), $y^1$ *w*; Mi{MIC}Dop1R2*$^{MI08664}$(BDSC 51098), and $w^{1118}$*; PBac{PB}DopEcR*$^{c02142}$*/TM6B, Tb*$^1$ (BDSC 10847) with $w^{1118}$ (BDSC 5905) $y^1$ and $y^1$ $w^{67c23}$ (BDSC 6599) as controls from the Bloomington stock centre. **Table 1**. shows the number of recorded flies for the age group comparisons, and **Table 2**. shows the number of recorded flies for comparing the mutant groups.

### Arena

We designed an arena made of four layers of plexiglass and metal that formed 13 tunnels (**Fig. 1a**). Each tunnel was 52mm x 5mm × 1mm, in which individual flies were able to move freely in two dimensions but could not fly. The bottom layer was a standard transparent plexiglass layer. The second layer contained the tunnel walls made of metal to prevent horizontal spread of light. The third layer was the top of the tunnels, made of transparent plexiglass, which could slide above the tunnel. It also contained a small hole on the side, where the flies could be inserted into the tunnel through a pipette. The fourth layer was a metal cover designed with cut-outs that matched the shape and position of the tunnels to prevent flies in the neighboring tunnels from detecting the shadow before it reached their position.

| | *Dop1R1* | *Dop1R2* | *DopEcR* | $w^{1118}$ | $y^{1}$ $w^{67c23}$ |
|---|---|---|---|---|---|
| 1-day-old | 35 | 36 | 36 | 35 | 41 |
| 1-week-old | 36 | 54 | 36 | 36 | 36 |
| 2-week-old | 36 | 36 | 36 | 36 | 30 |
| 3-week-old | 35 | 35 | 29 | 36 | 36 |
| 4-week-old | 30 | 36 | 34 | 30 | 30 |

**Table 1.**

**Number of recorded flies for the age group comparisons.**

| 2-week-old | No. of flies |
|---|---|
| *Dop1R1* | 45 |
| *Dop1R2* | 47 |
| *DopEcR* | 35 |
| $w^{1118}$ | 46 |
| $y^{1}$, $w^{67c23}$ | 40 |
| Parkin | 78 |
| α-Syn | 69 |
| *iso* $w^{1118}$ | 92 |
| *+GFP* | 65 |

**Table 2.**

**Number of recorded flies for comparing the mutant groups.**

## Movement tracking

Flies were tracked by using a FLIR Camera (FLIR Blackfly S USB3 FLIR Systems, Wilsonville, OR, US) placed under the arena (**Fig. 1a**). The frame rate was set to 100 frames/second to allow the detection of position with high temporal resolution. The recorded images were processed by Bonsai (2.5.2) tracking software (Lopes et al., 2015), using customized Bonsai code. The code extracts each tunnel areas separately from the image and detects flies by selecting the biggest and darkest pixel object in the tunnel area in real time. Fly position coordinates were calculated from the centroid. The software stored x-y position coordinates along with their timestamps in csv files, which were later processed in Matlab R2016a (Mathworks, Natick, MA, US).

## Predator-mimicking shadow stimuli

A 10.1-inch screen (HannStar HSD101PWW1-A00,) was placed on the top of the arena. The screen presented a yellow background. We implemented 'shadow' stimuli in red color, as these stimuli were suggested to be perceived by fruit flies as dark shadows and also enabled continuous tracking of the animals (Sharkey et al., 2020). The red color screen represented a large enough contrast change to evoke escape behaviors of flies, but also provided sufficient brightness to enable continuous motion tracking. A passing shadow stimulus was chosen, as it was affecting all tunnels simultaneously. To mimic an approaching shadow, a red screen slid in from the side, stayed on for 2 seconds, then slid out. The shadow stimuli were separated by pseudorandomized inter-trial intervals with an approximately exponential distribution with a mean of 52.2 seconds, preventing flies from anticipating the next shadow stimulus.

## Identification of behavioral response types to the threatening stimuli

Animal speed was calculated as a function of time in a -200 ms to 1000 ms window around each shadow stimulus in 10 ms time bins. These speed functions were normalized by subtracting the average speed in the 200 ms window before the shadow presentation to reveal stimulus induced absolute instantaneous speed changes. Principal component analysis (PCA) was used to reduce dimensionality of the normalized speed functions in the 1 s interval following shadow presentation in a way that the variance between trials is maximally preserved in the low-dimensional representation. Agglomerative hierarchical clustering was performed in the space spanned by the first three principal components to identify trials with distinct characteristic response types.

## Behavior detection

After identifying the four most frequently observed animal responses to shadow presentations, we algorithmically defined them based on the speed and acceleration thresholds. First, we examined the speed of the animal in the 200 ms time window before the shadow ($v_{pre}$); stopping/freezing or slowing down was only possible if the fly had been moving, defined by $v_{pre} > 0$ mm/s. Second, we examined the acceleration ($a_{post}$) in the 1 s interval following the shadow using a 100 ms moving average window to characterize the flies' response. Reactions were considered robust if the absolute value of the acceleration reached 200 mm/s$^2$. A trial was characterized as a 'stop' trial, if the average $v_{pre}$ was above 0 mm/s, the fly's first reaction was deceleration, and 0 mm/s speed was reached before accelerating again. If the first reaction was deceleration but 0 mm/s speed was not reached, the trial was classified as a 'slow down' trial. In 'speed up' trials, the first response following the shadow was acceleration. If the absolute value of $a_{post}$ did not reach the 200 mm/s$^2$ threshold during the 1 s interval following the shadow, the trial was labeled as a 'no reaction' trial.

### Data analysis and statistics

Data analysis was performed in Matlab R2016a (Mathworks, Natick, MA, US) using custom-written code. Statistical comparison between groups of flies was performed by two-sided Mann-Whitney U-test. Exact p values are reported in the Results section. The effect of movement speed on the distribution of behavioral response types was tested using Monte Carlo simulation (Fig.S3). First, we calculated the probability of each response type at different speed values from the grand average of all trails of every animal. Then we simulated behavior of virtual flies by drawing random velocity values from the velocity distribution of each genotype and then randomly selecting a reaction based on the reaction probabilities associated with the drawn velocity. Finally, we calculated reaction probabilities for the virtual flies and compared it with real data from animals of the same genotype. Differences were statistically tested by Chi-squared test.

## Supplementary Figure Legends

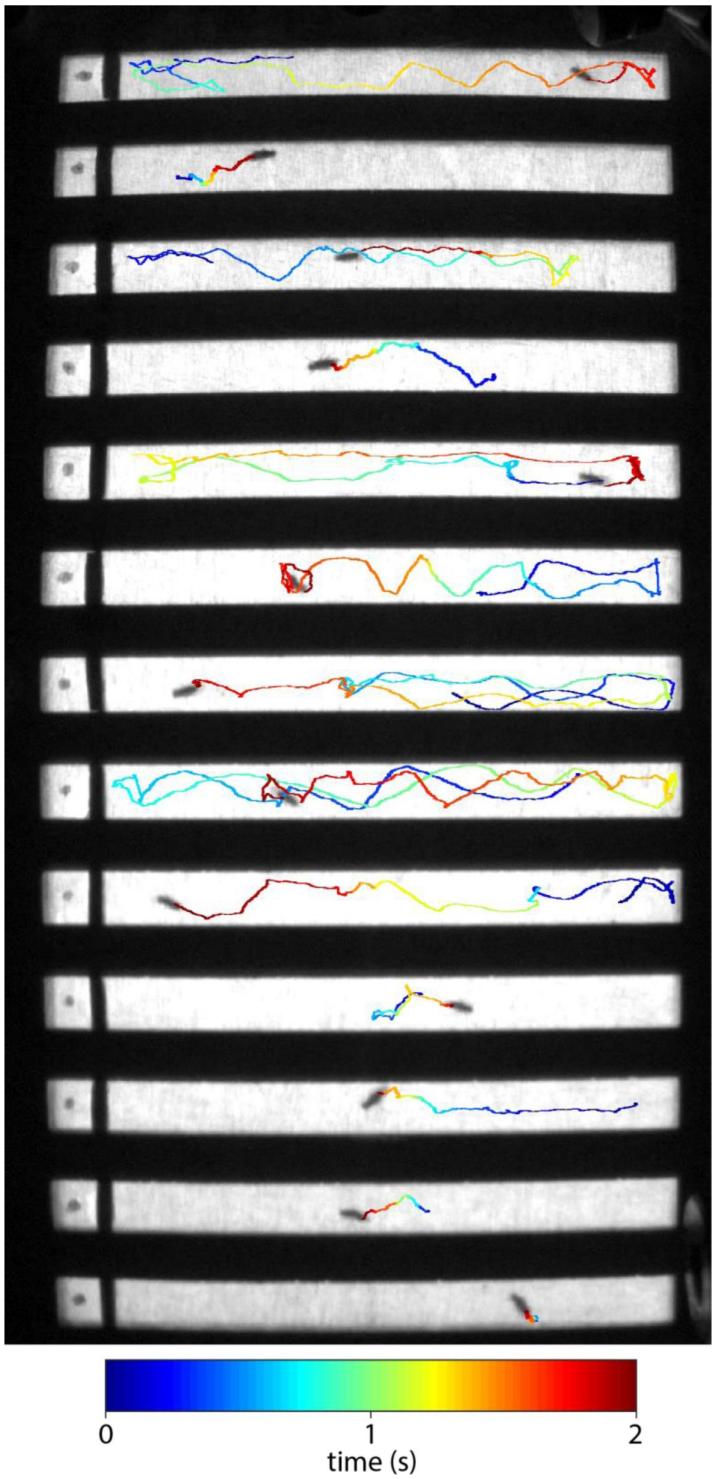


**Fig. S1.**

**Picture of the arena from the camera view**

The 13 tunnels of the arena from the camera view with the tracking lines showing that the animals can move in 2 dimnesions. The tracking line color indicates time, with dark blue and red marking the beginning and the end of the tracking, respectively.

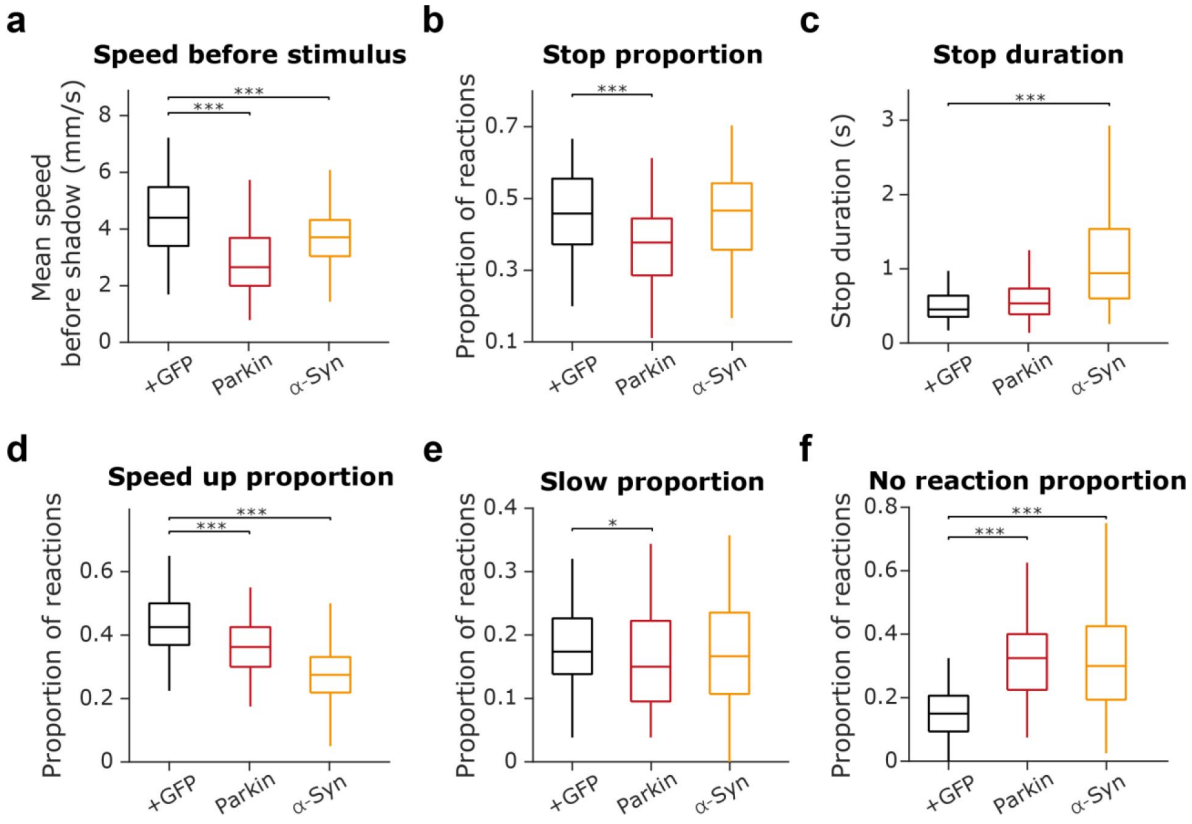


**Fig. S2.**

**Parkin and α-Syn fly group escape reaction compared to GFP mutant flies**

**a**, Distribution of the mean speed measured in the 0.2 second before shadow presentation for Parkin, α-Syn and +GFP mutant groups. Parkin and α-Syn flies showed reduced mean speed compared to +GFP controls (Parkin vs. +GFP, $p = 1.58 \times 10^{-11}$; α-Syn vs. +GFP, $p = 2.85 \times 10^{-4}$; Mann-Whitney U-test). **b,** Distribution of the proportion of stop trials in different mutant groups. Parkin flies showed reduced tendency for stopping compared to the +GFP group (Parkin vs. +GFP, $p = 7.1 \times 10^{-5}$; α-Syn vs. +GFP, p = 0.67; Mann-Whitney U-test). **c,** Distribution of stop duration in different mutant groups. α-Syn flies showed increased stop durations compared to GFP (Parkin vs. +GFP, p = 0.17; α-Syn vs. +GFP, $p = 3.98 \times 10^{-10}$; Mann-Whitney U-test). **d,** Distribution of the proportion of speed up trials in different mutant groups. Both Parkin and α-Syn flies showed a reduced tendency to speed up compared to +GFP controls (Parkin vs. +GFP, $p = 8.3 \times 10^{-5}$; α-Syn vs. +GFP, $p = 1.96 \times 10^{-12}$; Mann-Whitney U-test). **e**, Distribution of the proportion of slow down trials in different mutant groups. Parkin flies showed a reduced tendency to slow down compared to +GFP controls (Parkin vs. +GFP, p = 0.044; α-Syn vs. +GFP, p = 0.465; Mann-Whitney U-test). **f**, Distribution of the proportion of 'no reaction' trials in different mutant groups. Both Parkin and α-Syn flies showed reduced reactivity compared to +GFP controls (Parkin vs. +GFP, $p = 9.4 \times 10^{-13}$; α-Syn vs. +GFP, $p = 1,3 \times 10^{-8}$; Mann-Whitney U-test). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Exact genotypes: *iso* $w^{1118}$: *+; +; Ddc-Gal4/+*. Parkin: *+; +; Ddc-Gal4/UAS-Parkin-R275W*. α-Syn: *+; +; Ddc-Gal4/UAS-α-Syn-A53T*.

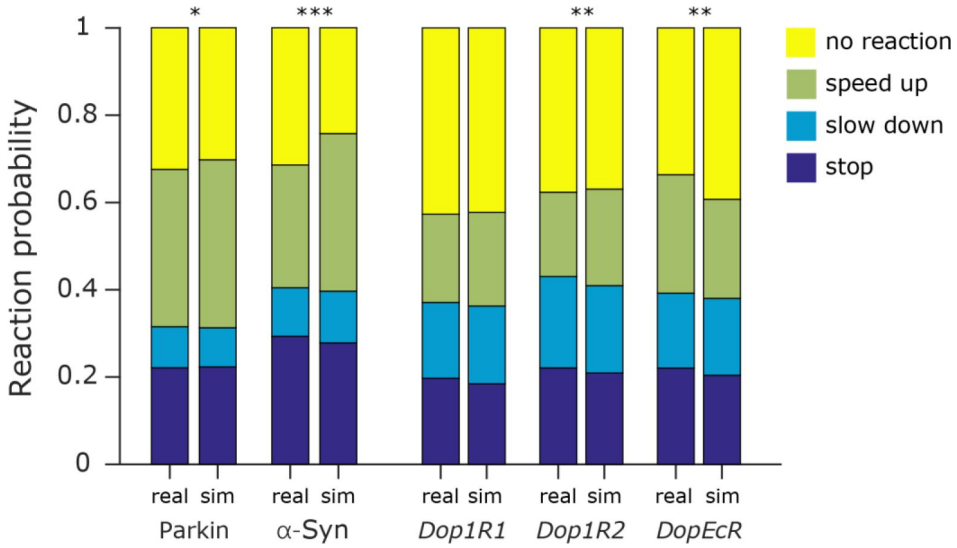


**Fig. S3.**

### Differences in walking speed do not explain different escape behaviors

Real and simulated proportion of response types are shown for the mutant groups tested. For the simulations, random velocity values were drawn from the velocity distribution of each genotype and then a response type was randomly selected based on the response type distributions associated with the drawn velocity. Bar plots show the proportion of response types color-coded. For each mutant group, the right bar shows the measured proportion of escape responses, while the left bar shows the simulated distribution. We found significant differences between the real and simulated distributions for the following groups: Parkin ($p = 0.0487$), α-Syn ($p < 0.000001$), *Dop1R2* ($p = 0.006157$) and *DopEcR* ($p = 0.003901$). We found no significant differences for the *Dop1R1* group ($p = 0.332$; Chi-square test for all the statistics). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Exact genotypes: *iso* $w^{1118}$: *+; +; Ddc-Gal4/+*. Parkin: *+; +; Ddc-Gal4/UAS-Parkin-R275W*. α-Syn: *+; +; Ddc-Gal4/UAS-α-Syn-A53T*. $y^1$ $w^{67c23}$: $y^1$ $w^{67c23}$. *Dop1R*: $y^1$ *w*; Mi{MIC}Dop1R1*$^{MI04437}$. *Dop1R2*: $y^1$ *w*; Mi{MIC}Dop1R2*$^{MI08664}$. *DopEcR*: $w^{1118}$; *PBac{PB}DopEcR*$^{c02142}$*/TM6B, Tb*$^1$.

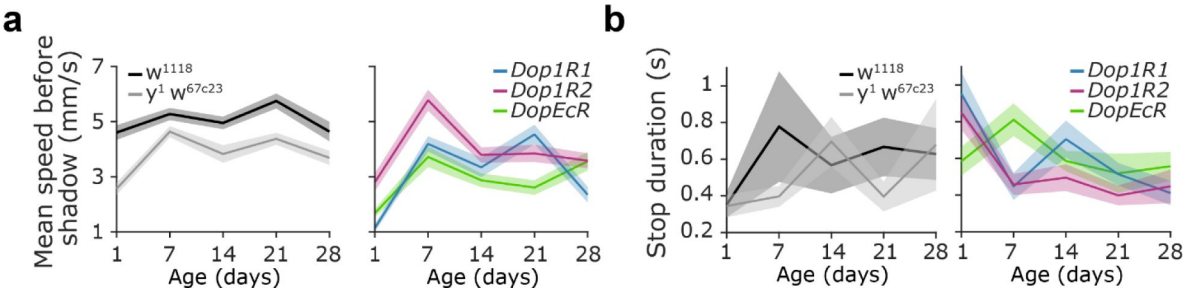


**Fig. S4.**

### Changes in escape behavior from the first to the fourth week of life

**a,** Average walking speed as a function of age for different groups of controls (left) and mutants (right). Lines and errorshades show mean and standard error. Mean speed showed significant differenceis among age groups (two-way ANOVA, age, $f = 41.38$, $p = 9.39 \times 10^{-32}$; genotype, $f = 42.19$, $p = 2.46 \times 10^{-32}$; genotype × age, $f = 4.36$, $p = 2.667 \times 10^{-8}$;Tukey's post hoc test: 1 day old vs. 1 week old, $p = 9.92 \times 10^{-9}$; 1 week old vs. 2 weeks old, $p = 7.34 \times 10^{-7}$; 2 weeks old vs. 3 weeks old, $p = 0.085$; 3 weeks old vs. 4 weeks old, $p = 0.0043$). **b,** Stop duration as a function of age for different groups of controls (left) and mutants (right). We found that age did not effect the duration of stops (two-way ANOVA, age, $f = 0.85$, $p = 0.49$; genotype, $f = 0.91$, $p = 0.46$; genotype × age, $f = 2.53$, $p = 0.0008$; Tukey's post hoc test: 1 day old vs. 1 week old, $p = 0.98$; 1 week old vs. 2 weeks old, $p = 0.992$; 2 weeks old vs. 3 weeks old, $p = 0.578$; 3 weeks old vs. 4 weeks old, $p = 0.9759$). Lines and errorshades show mean and standard error.

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**Reviewer #1 (Public Review):**

Summary: Translating discoveries from model organisms to humans is often challenging, especially in neuropsychiatric diseases, due to the vast gaps in the circuit complexities and cognitive capabilities. Kajtor et al. propose to bridge this gap in the fly models of Parkinson's disease (PD) by developing a new behavioral assay where flies respond to a moving shadow by modifying their locomotor activities. The authors believe the flies' response to the shadow approximates their escape response to an approaching predator. To validate this argument, they tested several PD-relevant transgenic fly lines and showed that some of them indeed have altered responses in their assay.

Strengths: This single-fly-based assay is easy and inexpensive to set up, scalable, and provides sensitive, quantitative estimates to probe flies' optomotor acuity. The behavioral data is

detailed, and the analysis parameters are well-explained.

Weaknesses: While the abstract promises to give us an assay to accelerate fly-to-human translation, the authors need to provide evidence to show that this is indeed the case. They have used PD lines extensively characterized by other groups, often with cheaper and easier-to-setup assays like negative geotaxis, and do not offer any new insights into them. The conceptual leap from a low-level behavioral phenotype, e.g. changes in walking speed, to recapitulating human PD progression is enormous, and the paper does not make any attempt to bridge it. It needs to be clarified how this assay provides a new understanding of the fly PD models, as the authors do not explore the cellular/circuit basis of the phenotypes. Similarly, they have assumed that the behavior they are looking at is an escape-from-predator response modulated by the central complex- is there any evidence to support these assumptions? Because of their rather superficial approach, the paper does not go beyond providing us with a collection of interesting but preliminary observations.

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**Reviewer #2 (Public Review):**

In this study, Kajtor et al investigated the use of a single-animal trial-based behavioral assay for the assessment of subtle changes in the locomotor behavior of different genetic models of Parkinson's disease of Drosophila. Different genotypes used in this study were Ddc-GAL4>UAS-Parkin-275W and UAS- α-Syn-A53T. The authors measured Drosophila's response to predator-mimicking passing shadow as a threatening stimulus. Along with these, various dopamine (DA) receptor mutants, Dop1R1, Dop1R2 and DopEcR were also tested.
The behavior was measured in a custom-designed apparatus that allows simultaneous testing of 13 individual flies in a plexiglass arena. The inter-trial intervals were randomized for 40 trials within 40 minutes duration and fly responses were defined into freezing, slowing down, and running by hierarchical clustering. Most of the mutant flies showed decreased reactivity to threatening stimuli, but the speed-response behavior was genotype invariant. These data nicely show that measuring responses to the predator-mimicking passing shadows could be used to assess the subtle differences in the locomotion parameters in various genetic models of Drosophila.

The understanding of the manifestation of various neuronal disorders is a topic of active research. Many of the neuronal disorders start by presenting subtle changes in neuronal circuits and quantification and measurement of these subtle behavior responses could help one delineate the mechanisms involved. The data from the present study nicely uses the behavioral response to predator-mimicking passing shadows to measure subtle changes in behavior. However, there are a few important points that would help establish the robustness of this study.

1. The visual threat stimulus for measuring response behavior in Drosophila is previously established for both single and multiple flies in an arena. A comparative analysis of data and the pros and cons of the previously established techniques (for example, Gibson et al., 2015) with the technique presented in this study would be important to establish the current assay as an important advancement.
2. Parkinson's disease mutants should be validated with other GAL-4 drivers along with Ddc-GAL4, such as NP6510-Gal4 (Riemensperger et al., 2013). This would be important to delineate the behavioral differences due to dopaminergic neurons and serotonergic neurons and establish the Parkinson's disease phenotype robustly.

3. The DopEcR mutant genotype used for behavior analysis is w1118; PBac{PB}DopEcRc02142/TM6B, Tb1. Balancer chromosomes, such as TM6B,Tb can have undesirable and uncharacterised behavioral effects. This could be addressed by removing the balancer and testing the DopEcR mutant in homozygous (if viable) or heterozygous conditions.
4. The height of the arena is restricted to 1mm. However, for the wild-type flies (Canton-S) and many other mutants, the height is usually more than 1mm. Also, a 1 mm height could restrict the fly movement. For example, it might not allow the flies to flip upside down in the arena easily. This could introduce some unwanted behavioral changes. A simple experiment with an arena of height at least 2.5mm could be used to verify the effect of 1mm height.
5. The detailed model for Monte Carlo simulation for speed-response simulation is not described. The simulation model and its hyperparameters need to be described in more depth and with proper justification.
6. The statistical analysis in different experiments needs revisiting. It wasn't clear to me if the authors checked if the data is normally distributed. A simple remedy to this would be to check the normality of data using the Shapiro-Wilk test or Kolmogorov-Smirnov test. Based on the normality check, data should be further analyzed using either parametric or non-parametric statistical tests. Further, the statistical test for the age-dependent behavior response needs revisiting as well. Using two-way ANOVA is not justified given the complexity of the experimental design. Again, after checking for the normality of data, a more rigorous statistical test, such as split-plot ANOVA or a generalized linear model could be used.
7. The dopamine receptor mutants used in this study are well characterized for learning and memory deficits. In the Parkinson's disease model of Drosophila, there is a loss of DA neurons in specific pockets in the central brain. Hence, it would be apt to use whole animal DA receptor mutants as general DA mutants rather than the Parkinson's disease model. The authors may want to rework the title to reflect the same.

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