

Effects of Aging, Potassium Channel, Stimulus Parameters on Visual Habituation in *Drosophila melanogaster* Using the Light off Jump Response

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Abstract

Habituation, one of the simplest forms of learning, is a basic adaptive process that enables organisms to filter repetitive, irrelevant stimuli. This project investigates the effects of stimulus parameters, aging and potassium channels mutations on visual habituation in white-eyed *cn bw* mutant *Drosophila melanogaster* using the jump response, a quantifiable escape behavior mediated by the Giant Fiber System (GFS). Using Generalized Linear Mixed Models (GLMM), we quantified habituation as a significant decline in response across 15 dimming pulses. We confirmed habituation through spontaneous recovery and dishabituation tests. The results produced three findings. Habituation was highly dependent on the interstimulus interval (delay), being strongest at 2 s and undetected at 5 s. Older (30-day-old) flies showed a slower habituation process than younger (10-day-old) flies, confirming that age-related decline affects neural plasticity. Since the basic neural mechanisms for learning to ignore stimuli are believed to be similar across species, the modification of this process in our aging flies helps explain why older humans often find it difficult to focus on a busy environment. Finally, flies carrying mutations in voltage-gated potassium channels, *eag Sh*; *cn bw* showed an increased in habituation process compared with *cn bw* alone. The increase may be attributed to faster short-term synaptic depression caused by hyperexcitability in these mutants.

Keywords

Habituation, Dishabituation, Neural Mechanisms, Spontaneous Recovery

1. Introduction

Habituation is a fundamental process that enables organisms to adapt to their environment, filtering out irrelevant stimuli to prioritize those critical for survival. Habituation, the simplest form of learning, is the decrease in response to a repeated stimulus over time [1]. This is a basic process of behavioral plasticity that must be carefully characterized to separate it from non-learning factors like sensory adaptation and motor fatigue [2]. While these cause a peripheral decline in response (receptor insensitivity or muscular exhaustion), habituation is generally attributed to synaptic plasticity that is mediated within the neural circuits of the central nervous system.

A perfect example of habituation is observed by people who live near a busy train station. They initially notice the train noises as disruptive, but over time, their brains adapt to the repeated, non-threatening stimulus, leading to a reduced response that allows them to focus on other tasks without distraction. The nematode *C. elegans* demonstrates a similar phenomenon when it experiences repeated harmless mechanical taps on its petri dish environment. The nematode displays an immediate backward movement as a defensive reaction when it detects any disturbance. The nervous system of *C. elegans* learns to handle its repeated harmless stimulus which results in decreased reversal responses that help the organism save energy for other activities [3].

Habituation is seen as a true learning process when it meets a series of parametric characteristics including spontaneous recovery and dishabituation [4]. Across evolution, habituation has been a critical survival mechanism, preventing energy waste from irrelevant reactions to predictable events including a fly ignoring repeated shadows. It is the brain's adaptive filter that reduces response to repetitive stimuli [1]. Deficits in habituation are highly important in clinical settings, as they serve as biomarkers for neurological dysfunction including autism [5], schizophrenia [6], and aging-related cognitive decline [7]. Beyond biology, habituation principles are being integrated into artificial intelligence and robotics to enable systems that filter noise and detect novelty [8] [9].

Physiologically, habituation is achieved through changes within the central nervous system. One of the neural mechanisms underlying habituation is the short-term synaptic depression [10]. Short-term synaptic depression is temporary and occurs at a synapse. It is caused by the rapid, repeated firing of the presynaptic neuron when exposed to a high frequency of stimuli. The persistent stimulus temporarily depletes synaptic vesicles that contain neurotransmitters. As synaptic vesicles deplete, less neurotransmitters are released into the synapse with each subsequent pulse, leading to weakening of the postsynaptic response and consequently to behavioral habituation. This response recovers during a period of rest (spontaneous recovery) because the presynaptic neuron has time to replenish its depleted synaptic vesicles [10]. Another neural mechanism is the dual process theory [11]. This model assumes both habituation process and sensitization process compete to determine the net response or final response magnitude. Whereas the habitua-

tion process is caused by short-term synaptic depression, sensitization is the general arousal process that causes response to increase. This mechanism is essential for understanding dishabituation, where a new stimulus activates the sensitization process, reinstating the original response and demonstrating that the response decrement was temporary but not permanent.

In the laboratory, habituation is typically evoked by administering a series of repeated stimuli that are uniform in both strength (light intensity in the case of this work) and frequency or interstimulus interval. The strength of habituation is profoundly influenced by the stimulus train, specifically the frequency of stimulation, the total number of stimuli delivered and the light intensity. Habituation is usually a decrease in response amplitude but can also be a decline in frequency of an all-or-none response (the binary jump outcome) as observed in our study.

Aging, a biological process, profoundly impacts cognitive functions including learning and memory. Despite their short lifespan, mostly around 40 to 60 days under optimal conditions, fruit flies can live longer depending on environmental variables such as temperature. Fruit flies are thought to experience many of the same basic aging mechanisms seen in other animals including humans [12]. Aging is linked to a reduction in neural plasticity, sensory sensitivity and molecular signaling pathways [13] which are vital for habituation. To begin examining aging in the nervous system of fruit flies, we selected the giant fiber startle response, a behavior that is easily elicited and quantified, mediated by a neural pathway that is accessible to physiological study. Habituation of this escape behavior has been previously demonstrated using visual stimulation and direct electrical stimulation of the brain in tethered flies [14]. In this study, we hypothesized, based on the established principles that aging reduces neural plasticity, a shared biological hallmark across species, that 30-day old flies would show slower habituation compared to 10-day old flies [13].

The startle response in the fruit fly is a rapid, putatively defensive behavior mediated by the Giant Fiber System (GFS) pathway. While the sensory input that triggers the GFS can vary (looming shadow, wind or light-off), the resulting motor output follows a quick jump and flight initiation. The type of stimulus determines the sensory input that activates the GFS. The looming shadow stimulus type creates shadow pattern project onto the fly that mimics an approaching predator or object. This is highly effective especially when combined with air movement [15] and triggers GFS in flies with normal eye coloration.

For this study, we utilized a simple light-off stimulus with *cinnabar brown* (*cn bw*) mutant flies. In these flies, which lack the brown and red screening pigments of the eye and therefore have white eyes, a simple reduction in light intensity is sufficient to trigger the giant fiber system and evoke the escape response. The *cn* and *bw* mutations in *Drosophila melanogaster* combine to produce white eyes by disrupting eye pigmentation and pigment transport pathways in the compound eyes [16].

Potassium channels are specialized proteins found within neuronal cell mem-

branes that control the flow of potassium ions in and out of cell. These channels, especially the voltage-gated potassium channel types like *ether-à-go-go* (*eag*) and *Shaker* (*Sh*) open in response to membrane depolarization, allowing potassium ions to exit the neuron, thereby rapidly repolarizing the membrane to prevent excessive firing and calcium influx [17]. The *ether-à-go-go* (*eag*) and *Shaker* (*Sh*) genes encode voltage-gated potassium channel proteins that regulate neuronal excitability by controlling potassium ion currents. The *cn bw* mutant compares the baseline *cn bw* against the mutant *eag Sh*; *cn bw* which includes mutations affecting potassium channels.

We defined habituation as a progressive, statistically significant decline in jump probability across 15 repeated 20 millisecond dimming pulses. This definition is validated by assessing the two fundamental criteria for habituation [2] [11]: dishabituation, where a novel mechanical stimulus actively restored the jump response, and spontaneous recovery, where the response naturally returned after a rest period without any external input.

This project aimed to establish a robust habituation protocol for a behavior that is presumably important to fitness and to explore how it is affected by age and by ion channel mutations that alter neural functioning.

Objectives

1) Confirm habituation in visual response: To demonstrate that *Drosophila melanogaster* shows a progressive reduction in jump probability across repeated dimming pulses, indicative of habituation, and to distinguish the strength of habituation by the slope of response decline tested with Generalized Linear Mixed Models (GLMMs).

2) Investigate stimulus parameter effects: To examine how interstimulus interval or delay (ISI of 2 s, 3 s or 5s) and light intensity (levels 3 or 4 in arbitrary units) modulate habituation rates.

3) Assess aging's impact on habituation: To determine whether aging impairs habituation, we tested the expectation that 30-day-old flies maintained at 25°C would show a slower habituation rate compared to 10-day-old flies, reflecting age-related declines in neural plasticity.

4) Evaluate potassium channel effects on habituation:

- To test the hypothesis that *eag* and *Sh* mutations increase habituation rates compared to *cn bw* controls due to hyperexcitability from disrupted voltage-gated potassium channels, potentially resulting in a steeper response decrement.

2. Methods

2.1. Fly Strains

Two mutant stocks of *Drosophila melanogaster* were used. The first was *cinnabar brown* (*cn bw*), which had white eyes due to the lack of screening pigments in their eyes. This makes them sensitive to a simple light-off stimulus, which triggers their escape jump. The *cn bw* stock was obtained from Chun-Fang Wu.

The second strain, *ether-à-go-go Shaker; cinnabar brown* (*eag Sh; cn bw*) adds potassium channel mutations (*eag* and *Sh*) to this background, allowing us to test how hyperexcitability affects habituation in the same visual circuit. The *eag Sh; cn bw* mutant stock was made by crossing virgin females of *eag Sh* and males of *cinnabar brown* (*cn bw*). Males of the F1 offspring were crossed with fresh stocks of virgin females of *eag Sh*. F2 offspring were all homozygous for *eag* and *Sh* alleles with white-eyes. White-eyed F2 were selected and crossed to establish the *eag Sh; cn bw* stock.

2.2. Rearing Conditions

Flies were kept in standard vials containing a cornmeal-based food medium (Jazz-mix *Drosophila* food, Fisher Scientific, Hanover Park, Illinois, USA) prepared at a ratio of 347 g per liter of water. Newly emerged flies were collected every 1 to 5 days and kept at densities of up to 20 - 30 flies per vial to prevent overcrowding. Flies were transferred to fresh food vials at 6 to 10-day intervals to ensure optimal health and prevent progeny from appearing. All stocks were maintained in a 25°C incubator.

2.3. Age Cohorts

Food vials containing a high density of late-stage, mature (dark) pupae were selected. All existing adult flies were removed and discarded, ensuring only pupae remained. The cleared vials were maintained under standard conditions for 5 days to allow adult eclosion, generating a cohort of flies aged 0 - 5 days. These flies were then transferred to fresh food vials and aged for an additional 5 days, resulting in the 10-day-old experimental cohort with a final age range of 5 - 10 days post-eclosion. Using the same initial 0 - 5-day cohort, the 30-day-old group was generated through sequential transfers and extended aging for a further 25 days, resulting in a final age range of 25 - 30 days post-eclosion.

2.4. Jump Tests

Using an aspirator, a fly was transported into a spherical glass flask of diameter 5 cm with a foam plugged in the truncated neck of the flask. This glass chamber was transported into a testing enclosure to test the startle response. The chamber was inverted (neck down), and placed in an enclosure with dimensions of 57 × 53 cm. The enclosure was covered with heavy black plastic to block out ambient light, that could be opened in the front to allow access and observation. A steady background light was supplied by a classroom-type microscope illuminator (Bausch and Lomb) microscope illuminator positioned 35cm directly above the chamber, shining straight down onto it.

The stimulus light was provided by a fiber-optic lamp (PL900-A, Dolan-Jenner Industries, Inc., Lawrence, Massachusetts, USA). The two ends of the Y-shaped fiber-optic light guide (with detachable lenses removed) were positioned horizontally 5.5 cm from opposite sides of the testing chamber. The lamp's dimmer knob has six factory-marked indicator dots, where 0 indicates fully off, 3 indicates approximately 50% of maximum output, and 6 indicates 100% output. For the pur-

poses of this experiment, these dial positions are referred to as “3 units” and “4 units” (with “4 units” obtained by rotating the knob approximately two-thirds of the way between the 3 and 6 marks). These intensity levels are arbitrary and instrument-specific; we did not measure absolute light output in lux or irradiance. However, the relative difference between levels is consistent across experiments, and the full lamp configuration (model, distance, and chamber geometry) is provided here to allow approximate replication.

The stimulus consisted of a brief darkening of the fiber illuminator light, achieved by closing a shutter (VMM-T1, Vincent Associates, Rochester, NY, USA) placed between the lamp and the entrance of the fiber optic light guide. Since a constant level of background light was maintained by the microscope illuminator above the test chamber, this reduction in light was referred to as a “dimming pulse.” The shutter control allowed precise adjustment of both the stimulus duration and the time between stimuli (interstimulus interval). The pulse duration of each dimming pulse was set to 20 milliseconds. Responses were observed and recorded using a smartphone video camera (iPhone). Videos were analyzed manually to score jumps as binary outcomes (1 = jump, 0 = no jump).

To assess jump probability, flies were given at least 2 minutes to acclimate to the chamber before a series of stimuli was administered. Each stimulus train included 15 dimming pulses, each lasting 20 milliseconds with varying baseline light intensity and interstimulus interval. To assess how changes in light intensity and interstimulus interval (stimulus frequency) affect habituation results, light intensity was maintained at 3 units and 4 units while interstimulus interval was changed from 2 s, 3 s to 5 s.

For some trials, we quantified two criteria for habituation: spontaneous recovery (the passive, time dependent return of a habituated response after the repetitive stimulus has stopped) and dishabituation (the active restoration of a habituated response by presenting a novel, strong stimulus different from the habituating one). For one set of trials, after the 15-pulse habituation train, the same fly was subjected first to dishabituation, then another habituation trial, then to spontaneous recovery, then to a final habituation trial. Immediately after pulse 15, the fly was subjected to a mechanical disturbance by placing its spherical glass flask in a vortexer for 30 seconds at medium speed setting. This vortexing provided a novel mechanical stimulus. Following vortexing, the flies were immediately returned to the testing chamber and subjected to a second 15-pulse train to assess the magnitude of response restoration. Following this, to test for spontaneous recovery, the same flies were allowed to rest undisturbed for one minute before receiving a final 15-pulse habituation train.

To prevent potential confounding between the dishabituation stimulus and the measurement of spontaneous recovery, the experiment was replicated with an independent-measures design. In this refined protocol, different cohorts of flies were used for each restoration test. One cohort was subjected to a 1-minute dishabituating stimulus (vortexing) followed by re-testing. A separate, time-matched control cohort experienced a 1-minute rest period in the chamber prior to re-test-

ing. This allowed the processes of stimulus-induced recovery and spontaneous recovery to be quantified independently.

2.5. Sample Characteristics and Exclusions

For each combination of genotype (*cn bw* or *eag Sh; cn bw*), age (10-day or 30-day), interstimulus interval (2 s, 3 s, or 5 s), and light intensity (3 or 4 arbitrary units), a separate cohort of 20 flies was tested ($n = 20$ per condition). Sex was not recorded, but both males and females were present in each cohort. No flies were excluded from the analysis, and every fly included in the study responded at least once during the 15-pulse train.

Flies were never reused across different stimulus conditions. Each individual contributed to only one combination of ISI, intensity, and age. For dishabituation and spontaneous recovery experiments that used an independent-measures design, separate cohorts of $n = 10$ flies per genotype were used, as indicated in the corresponding figure legends.

2.6. Analysis

Jump probability was assessed by scoring whether a fly jumped (1) or did not jump (0) for each of the 15 dimming pulses in a stimulus train. Statistical analysis was performed using a Generalized Linear Mixed Model (GLMM) implemented with the `glmer()` function from the `lme4` package in R to quantify the rate of habituation (slope) across the 15 stimulus pulses. The model specification was $\text{Response} \sim \text{Age} * \text{as.numeric(Pulse)} + (1|\text{FlyID})$, where the binary response (jump = 1, no jump = 0) was modeled with a binomial distribution and logit link function. The fixed effects were Age (a categorical factor with two levels: 10-day and 30-day) and Pulse number (1 - 15), with their interaction term to test for differences in habituation rates. For trials with K⁺ channel mutants, genotype replaced age as a fixed effect (all flies were 10 days old in those experiments). A random intercept for individual flies (FlyID) was included to account for within-subject correlations across the 15 repeated trials.

To account for repeated measurements within individual flies, we compared several mixed-effect model structures using the `lme4` package in R. The candidate models included: (1) a null model with random intercept only (1|FlyID); (2) a model with random intercept and random slope for Pulse (Pulse|FlyID); and (3) a model with random intercept only but including an autocorrelation structure. All models were fitted with the same fixed effects (Age * as.numeric(Pulse) for age experiments; Genotype * as.numeric(Pulse) for genotype experiments). Models were compared using AICc (corrected for small sample size), BIC, and log-likelihood ratios, as well as model weights derived from AICc. The random-intercept-only model consistently yielded the lowest AICc and BIC values and the highest model weight (AICc weight > 0.95 in all cases). Models with random slopes either failed to converge or produced singular fits with higher AICc values. Therefore, the simpler random-intercept-only structure was retained for all final analyses.

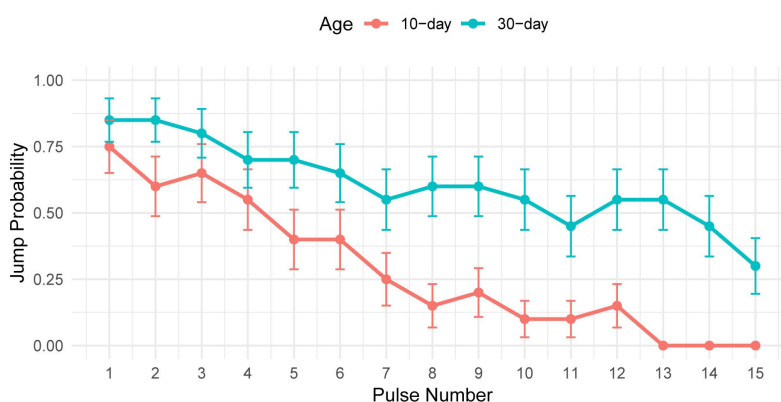
3. Results

All experiments were conducted with $n = 20$ flies per experimental condition (genotype $\times$ age $\times$ ISI $\times$ intensity) unless otherwise noted. No flies were excluded. Each fly was tested in only one stimulus condition, and different cohorts were used for dishabituation and spontaneous recovery tests. The following figures show the probability of the light-off jump response across 15 repeated dimming pulses, with statistical details from Generalized Linear Mixed Models (GLMM) provided below each plot.

For simplicity, flies in the 5 - 10 day range are referred to as “10-day-old”, and flies in the 25 - 30 day range as “30-day-old” throughout the Results and figures.

The first set of experiments shows how interstimulus interval, light intensity and age modulate the fly’s habituation process (**Figures 1-3**).

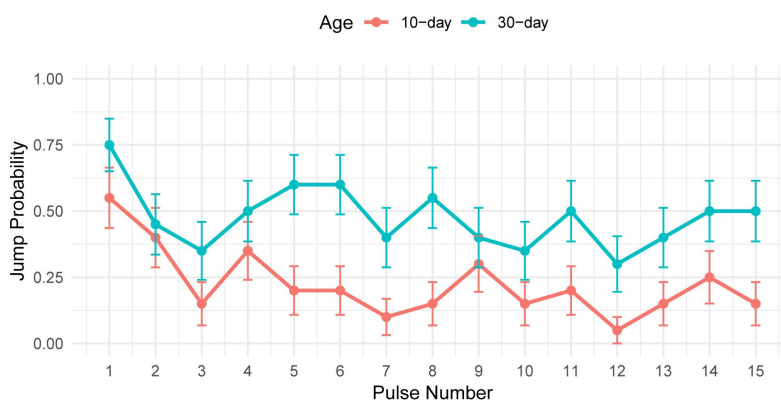
Habituation in cn bw flies (ISI= 2s, intensity = 3)



Fixed effects	Estimate	Std. Error	z value	Pr (> z)
(Intercept)	1.46379	0.29984	4.882	1.05e-06***
Age 30 day	0.33835	0.41883	0.808	0.419179
as.numeric (Pulse)	-0.34777	0.04460	-7.798	6.27e-15***
Age 30 day: as.numeric (Pulse)	0.19408	0.05397	3.596	0.000323***

(a)

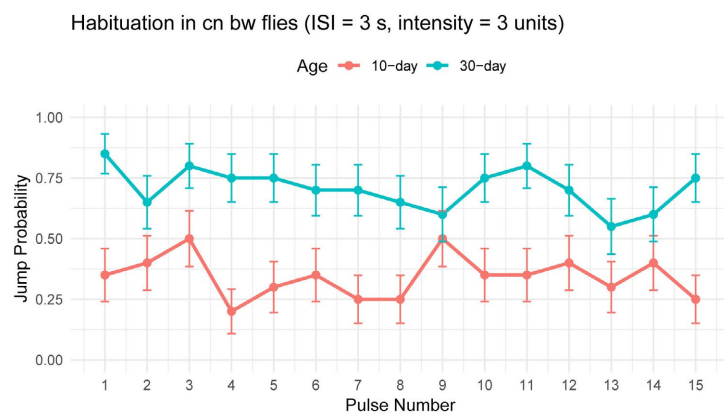
Habituation in cn bw flies (ISI = 2 s, intensity = 4 units)



Fixed effects	Estimate	Std. Error	z value	Pr (> z)
(Intercept)	-0.48377	0.27366	-1.768	0.07709
Age 30 day	0.71184	0.36663	1.942	0.05219
as.numeric (Pulse)	-0.10197	0.03364	-3.031	0.00243**
Age 30 day: as.numeric (Pulse)	0.06169	0.04309	1.432	0.15218

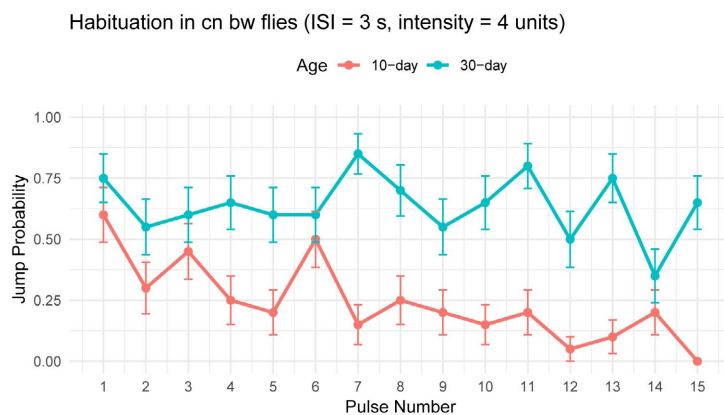
(b)

Figure 1. (a) Habituation rate of 10-day-old (red) and 30-day (blue) old *cinnabar brown* flies tested under the optimal stimulus condition of Interstimulus interval (ISI) = 2 seconds and Light Intensity = 3 units. The probability of the light-off jump response (y-axis) is plotted against the stimulus number (x-axis, Pulse). Fixed effects are interpreted as described in results. **(b)** Habituation rate of 10-day-old (red) and 30-day (blue) old *cinnabar brown* flies tested under the optimal stimulus condition of Interstimulus interval (ISI) = 2 seconds and Light Intensity = 4 units. The probability of the light-off jump response (y-axis) is plotted against the stimulus number (x-axis, Pulse). Fixed effects are interpreted as described in results.



Fixed effects	Estimate	Std. Error	z value	Pr (> z)
(Intercept)	-0.607010	0.274761	-2.209	0.0272*
Age 30 day	1.854126	0.383787	4.831	1.36e-06***
as.numeric (Pulse)	-0.008205	0.028660	-0.286	0.7747
Age 30 day: as.numeric (Pulse)	-0.032872	0.041504	-0.792	0.4283

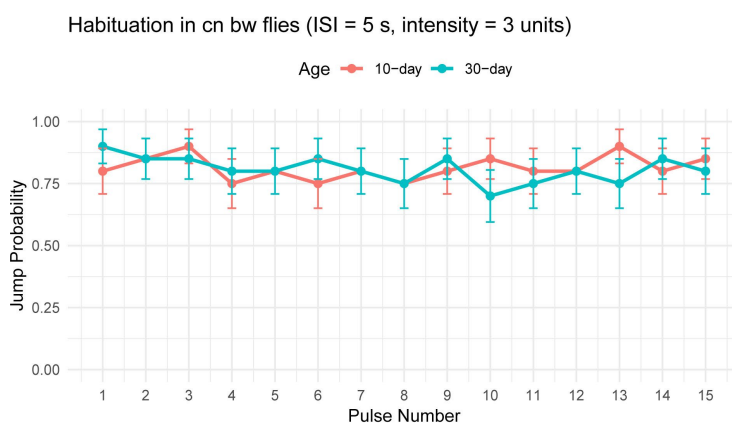
(a)



Fixed effects	Estimate	Std. Error	z value	Pr (> z)
(Intercept)	0.07093	0.27014	0.263	0.792889
Age 30 day	0.66039	0.37147	1.778	0.075443
as.numeric (Pulse)	-0.17035	0.03546	-4.804	1.55e-06***
Age 30 day: as.numeric (Pulse)	0.14942	0.04510	3.313	0.000922***

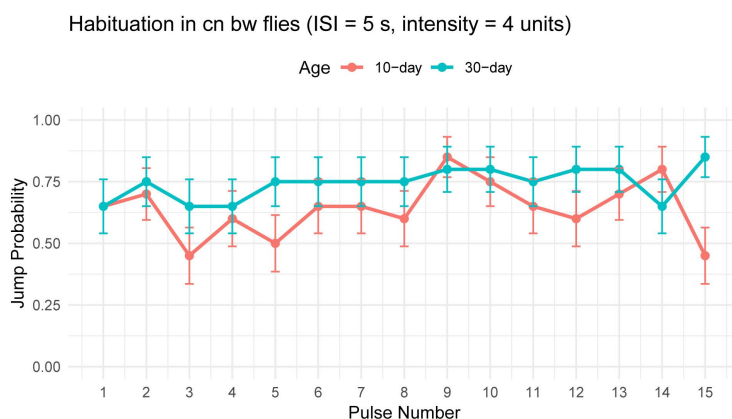
(b)

Figure 2. (a) Habituation rate of 10-day-old (red) and 30-day (blue) old *cinnabar brown* flies tested under the optimal stimulus condition of Interstimulus interval (ISI) = 3 seconds and Light Intensity = 3 units. The probability of the light-off jump response (y-axis) is plotted against the stimulus number (x-axis, Pulse). Fixed effects are interpreted as described in results. **(b)** Habituation rate of 10-day-old (red) and 30-day (blue) old *cinnabar brown* flies tested under the optimal stimulus condition of Interstimulus interval (ISI) = 3 seconds and Light Intensity = 4 units. The probability of the light-off jump response (y-axis) is plotted against the stimulus number (x-axis, Pulse). Fixed effects are interpreted as described in results.



Fixed effects	Estimate	Std. Error	z value	Pr (> z)
(Intercept)	1.38776	0.30823	4.502	6.72e-06***
Age 30 day	0.34285	0.44573	0.769	0.442
as.numeric (Pulse)	0.01059	0.03431	0.309	0.758
Age 30 day: as.numeric (Pulse)	-0.04739	0.04834	-0.980	0.327

(a)



Fixed effects	Estimate	Std. Error	z value	Pr (> z)
(Intercept)	0.41503	0.25093	1.654	0.0981
Age 30 day	0.31046	0.36832	0.843	0.3993
as.numeric (Pulse)	0.02017	0.02789	0.723	0.4694
Age 30 day: as.numeric (Pulse)	0.02312	0.04159	0.556	0.5782

(b)

Figure 3. (a) Habituation rate of 10-day-old (red) and 30-day (blue) old *cinnabar brown* flies tested under the optimal stimulus condition of Interstimulus interval (ISI) = 5 seconds and Light Intensity = 3 units. The probability of the light-off jump response (y-axis) is plotted against the stimulus number (x-axis, Pulse). Fixed effects are interpreted as described in results. **(b)** Habituation rate of 10-day-old (red) and 30-day (blue) old *cinnabar brown* flies tested under the optimal stimulus condition of Interstimulus interval (ISI) = 5 seconds and Light Intensity = 3 units. The probability of the light-off jump response (y-axis) is plotted against the stimulus number (x-axis, Pulse). Fixed effects are interpreted as described in results.

To show how the results were analyzed, we will focus on the first trial presented, **Figure 1(a)**, comparing 10-day-old and 30-day-old flies when interstimulus interval (ISI) is 2 s and light intensity level is at 3 units. **Figure 1(a)** shows a plot where the x-axis represents the stimulus pulse number (1 to 15), and the y-axis represents the jump probability ranging from 0 to 1, where 0 indicates no jump and 1 indicates a jump by all 20 flies tested. Note the analysis variables below the plot in **Figure 1(a)**. I will now explain how these variables are to be interpreted.

GLMM is essentially doing linear regression on logit transformed data where $n = \ln\left(\frac{p}{1-p}\right)$ where p is the jump probability and n is the logit transformed value of p. The reverse of the logit transformation is $p = e^n / (e^n + 1)$. GLMM yields a best-fit linear equation for the logit-transformed values from the baseline treatment group (10-day-old flies in this case) where *intercept* is the y intercept and *as.numeric (pulse)* is the slope, which gives the linear equation $y = \text{intercept} + \text{as.numeric (pulse)} * x$, where x is the pulse number from 1 to 15 and y is the logit-transformed response probability predicted by the equation. In any regression model, the *intercept* is the predicted value of the dependent variable when all independent variables in the model are set at zero which is achieved in this case by setting the pulse number to 0 (this y intercept is an extrapolation, because the first actual pulse is 1). At pulse 0, the model estimates that the 10-day-old flies have a high baseline probability of jumping (estimate = 1.46, which after reverse logit transformation gives a probability of 0.81). The p value for the *intercept* tests whether the log-odds of jumping for the young flies (10-day old) at the *intercept* is different from 0 which corresponds to a probability of 0.5 (since 0 is the logit value of 0.5).

Age 30-day = 0.34 is the difference in the *intercept* between 30-day-old flies and 10-day-old flies at extrapolated pulse 0. At pulse 0, the 30-day-old flies have log-odds that are 0.34 higher than the young flies, suggesting the old flies started with

a slightly higher initial jump probability (estimate of $1.46 + 0.34 = 1.80$ which reverse-transforms to a real probability of 0.86). The p value for the Age 30-day tests whether there is a difference in the initial jump probability at extrapolated pulse 0 between 30-day-old and 10-day-old flies. In **Figure 1(a)**, the difference is not statistically significant because the p value is 0.42 (p value > 0.05). The two age groups are not different at the start of the trial.

As.numeric (pulse) = -0.35 is the rate of habituation (slope of the logit transformed data) for the 10-day-old flies. It shows how much the log-odds of jumping change over 15 pulses. For the young flies, with each additional pulse, their log-odds of jumping decline by 0.35. The p value for the *as.numeric (pulse)* tests whether there is a relationship between pulse number and jump probability for the 10-day-old flies with the null hypothesis that the slope is zero. The p value is statistically significant (p value < 0.001) indicating the young flies are habituating.

Age 30 day: *as.numeric (pulse)* = 0.19. This is the interaction term, and it shows how the habituation rate(slope) for the 30-day-old flies differs from the habituation rate of the 10-day-old flies. The slope of logit-transformed data for the 30-day-old flies is $-0.35 + 0.19 = -0.15$. This implies that 30-day-old flies do habituate (negative estimate) but they do so at a significantly slower rate compared to the young flies. The p value for the Age 30 day: *as.numeric (pulse)* tests whether the rate of habituation (slope) for the older flies (30-day-old) flies is different from that of the young (10-day-old) flies. The significant p value (p value < 0.001) confirms that there is difference in habituation rate (slope) between the two groups of flies.

To summarize the tests associated with these four parameters, the *intercept* tests whether the baseline group's intercept is different from 0.5. *Age30-day* tests whether the two treatment groups have different intercepts. *as.numeric (pulse)* tests whether the baseline group's slope is different from 0. *Age30day:as.numeric (pulse)* tests whether the two treatment groups have different slopes.

3.1. Stimulus Parameters

Habituation is frequency dependent, showing that the time between stimuli is a critical factor determining whether habituation can occur. Habituation is stronger at higher stimulus frequency (**Table 1**). This can be observed in **Figure 1(a)** when interstimulus interval (ISI) is 2 s (0.5Hz), resulting in steep decline with transformed slope -0.347 and $p < 0.001$ in young flies. Conversely, at longer interstimulus intervals (lower frequency), such as 5 s (0.2 Hz), the long intervals allow spontaneous recovery for neural circuits to reset, preventing habituation altogether as observed in **Figure 3(a)** and **Figure 3(b)**. The medium ISI of 3 seconds (0.33 Hz) falls in an intermediate range, where the balance between stimulation frequency and recovery time creates a threshold effect, making habituation dependent on the light intensity.

Light intensity worked as a complex modulator, demonstrating interaction with the frequency of stimulation (**Table 1**). At the medium ISI of 3 s, habituation was absent at light intensity at 3 units (**Figure 2(a)**), indicating insufficient stimulus

strength to overcome the recovery period and induce cumulative synaptic depression. In contrast, at light intensity at level 4, a significant decline emerged as seen in **Figure 2(b)**, suggesting that higher intensity enhances the neural activation, driving a stronger response to habituation.

Table 1. Summary of habituation success.

Interstimulus interval (Frequency)	Light intensity (arbitrary units)	
	3	4
2 s (0.5 Hz)	Strong habituation	Weak habituation
	Weak habituation	Weak habituation
3 s (0.33 Hz)	No habituation	Strong habituation
	No habituation	No habituation
5 s (0.2 Hz)	No habituation	No habituation
	No habituation	No habituation

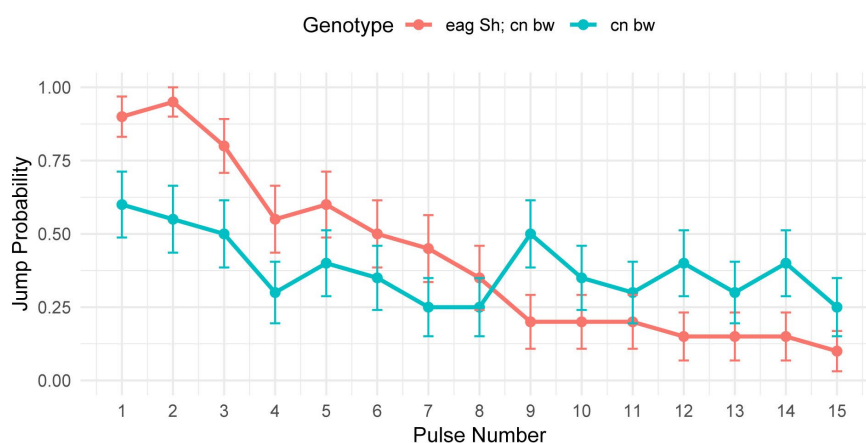
Keys: Red = 10 days old flies; Blue = 30 days old flies.

3.2. Dishabituation and Spontaneous Recovery

There was a significant increase in jump probability after performing dishabituation process in the habituated response (**Table 2**). There was a substantial restoration of jump probability in *eag sh; cn bw* and *cn bw* (**Figure 4**). In addition, there was a significant increase in jump response in both *eag sh; cn bw* and *cn bw* in the spontaneous recovery test from 0 and 0 at the end of habituation following dishabituation to 0.85 and 0.75 respectively. To avoid potential confounding effect between dishabituation influencing the later spontaneous recovery test we implemented an independent assessment design (**Figures 5 and 6**). Separate cohorts of 10 habituated flies each were used to assess dishabituation (through mechanical vortex) and spontaneous recovery (rest). In **Figure 5**, there was a significant increase in jump probability after performing dishabituation process in the habituated response from 0 and 0.2 to 1 and 0.8 respectively. Likewise, in **Figure 6**, there was a significant increase in jump probability after performing spontaneous recovery process in the habituated response from 0 and 0.1 to 0.7 and 0.5 respectively.

Table 2. Summary of potassium ion channel genotype effect on habituation.

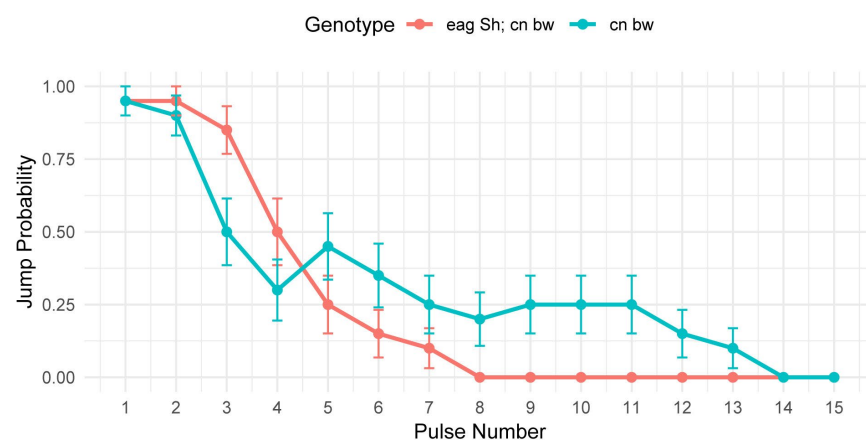
Observation in	Initial habituation	Dishabituation	Spontaneous recovery
<i>eag Sh; cn bw</i>	Rapid, steep decrement (strong habituation)	Strong reversal of habituation Extremely rapid re-habituation (more than <i>cn bw</i>)	Strong reversal of habituation Extremely rapid re-habituation but weaker than after dishabituation
<i>Cn bw</i>	Slower, gradual decrement (weak habituation)	Strong reversal of habituation Rapid re-habituation	Strong reversal of habituation Strong re-habituation but weaker than after dishabituation

Habituation in *Drosophila* (ISI = 3 s, intensity = 4 units)

Fixed effects:	Estimate	Std. Error	z value	Pr (> z)
(Intercept)	2.07969	0.30918	6.727	1.74e-11***
Genotypecn bw	-2.08738	0.39584	-5.273	1.34e-07***
as.numeric (Pulse)	-0.32029	0.03851	-8.318	<2e-16***
Genotypecn bw:as.numeric (Pulse)	0.25901	0.04759	5.442	5.27e-08***

(a)

Dishabituation Following Vortex (ISI = 3 s, intensity = 4)



Fixed effects:	Estimate	Std. Error	z value	Pr (> z)
(Intercept)	4.6416	0.7006	6.626	3.46e-11***
Genotypecn bw	-3.1284	0.7570	-4.133	3.58e-05***
as.numeric (Pulse)	-1.0959	0.1530	-7.162	7.97e-13***
Genotypecn bw:as.numeric (Pulse)	0.7793	0.1578	4.940	7.80e-07***

(b)

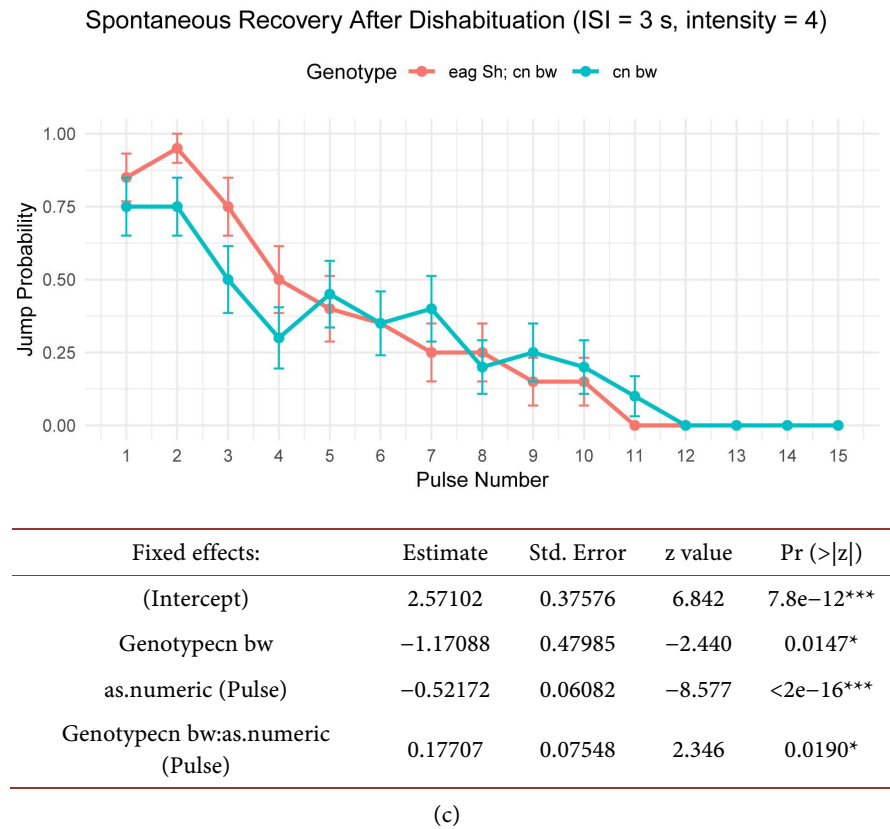
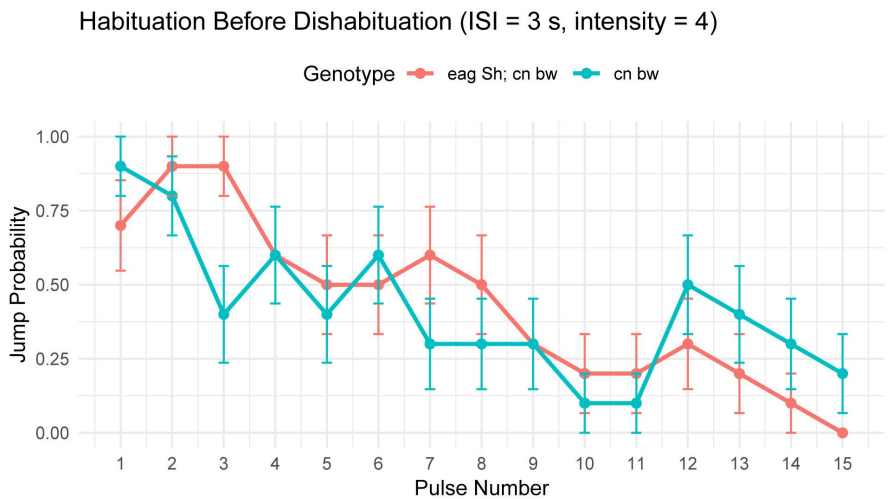


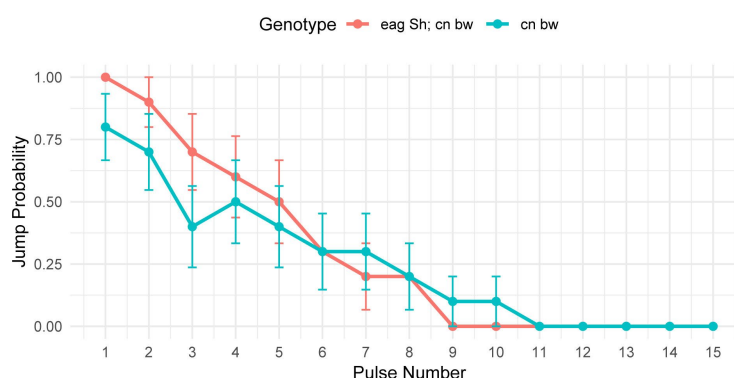
Figure 4. (a) Habituation rate for *eag Sh; cn bw* and *cn bw* tested under the stimulus condition of Interstimulus interval (ISI) = 3 seconds and Light Intensity = 4 units. The probability of the light-off jump response (y-axis) is plotted against the stimulus number (x-axis, Pulse). Fixed effects are interpreted as described in results. **(b)** Dishabituation in *eag sh; cn bw* and *cn bw* under the stimulus condition of Interstimulus interval (ISI) = 3 seconds and Light Intensity = 4 units. The probability of the light-off jump response (y-axis) is plotted against the stimulus number (x-axis, Pulse). Fixed effects are interpreted as described in results. **(c)** Spontaneous recovery in *eag Sh; cn bw* and *cn bw* tested under the stimulus condition of Interstimulus interval (ISI) = 3 seconds and Light Intensity = 3 units. The probability of the light-off jump response (y-axis) is plotted against the stimulus number (x-axis, Pulse). Fixed effects are interpreted as described in results.



Fixed effects:	Estimate	Std. Error	z value	Pr (> z)
(Intercept)	1.91642	0.42128	4.549	5.39e-06***
Genotypecn bw	-1.02075	0.55674	-1.833	0.0667
as.numeric (Pulse)	-0.28485	0.05085	-5.602	2.12e-08***
Genotypecn bw:as.numeric(Pulse)	0.12401	0.06629	1.871	0.0614

(a)

Dishabituation Following Vortex (Independent Measures)

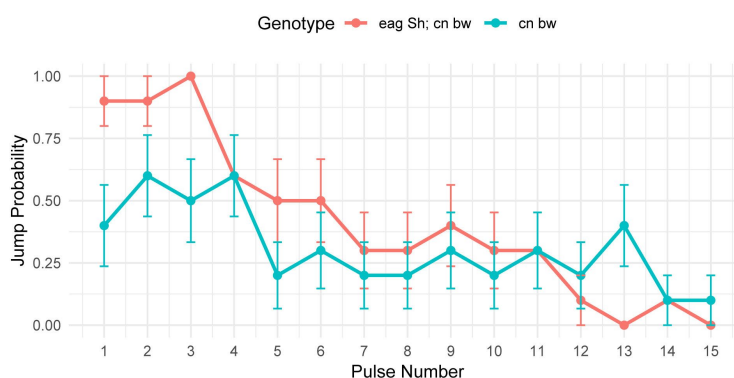


Fixed effects:	Estimate	Std. Error	z value	Pr (> z)
(Intercept)	3.60319	0.49828	7.231	4.78e-13***
Genotypecn bw	-1.94891	0.59550	-3.273	0.00107**
as.numeric (Pulse)	-0.74301	0.09034	-8.224	<2e-16***
Genotypecn bw:as.numeric (Pulse)	0.31051	0.10577	2.936	0.00333**

(b)

Figure 5. (a) Habituation rate before dishabituation (independent measures) in *eag Sh; cn bw* and *cn bw* tested under the stimulus condition of Interstimulus interval (ISI) = 3 seconds and Light Intensity = 3 units. The probability of the light-off jump response (y-axis) is plotted against the stimulus number (x-axis, Pulse). Fixed effects are interpreted as described in results. (b) Dishabituation rate following vortex stimuli (independent measures) in *eag Sh; cn bw* and *cn bw* tested under the stimulus condition of Interstimulus interval (ISI) = 3 seconds and Light Intensity = 3 units. The probability of the light-off jump response (y-axis) is plotted against the stimulus number (x-axis, Pulse). Fixed effects are interpreted as described in results.

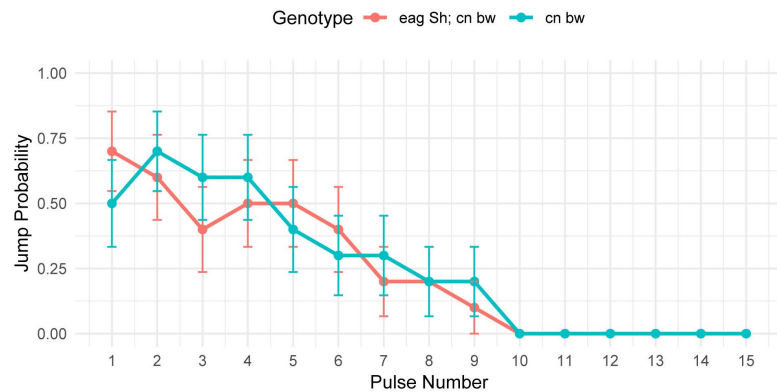
Habituation Before Spontaneous Recovery



Fixed effects:	Estimate	Std. Error	z value	Pr (> z)
(Intercept)	2.46705	0.47439	5.200	1.99e-07***
Genotypecn bw	-2.35491	0.59628	-3.949	7.84e-05***
as.numeric (Pulse)	-0.37777	0.06066	-6.228	4.72e-10***
Genotypecn bw:as.numeric (Pulse)	0.25510	0.07475	3.413	0.000643***

(a)

Spontaneous Recovery Following Rest (Independent Measures)



Fixed effects:	Estimate	Std. Error	z value	Pr (> z)
(Intercept)	1.51108	0.32285	4.680	2.86e-06***
Genotypecn bw	-0.03300	0.45060	-0.073	0.942
as.numeric (Pulse)	-0.42563	0.05543	-7.678	1.61e-14***
Genotypecn bw:as.numeric (Pulse)	0.02713	0.07570	0.358	0.720

(b)

Figure 6. (a) Habituation rate before spontaneous recovery (independent measures) in *eag Sh; cn bw* and *cn bw* tested under the stimulus condition of Interstimulus interval (ISI) = 3 seconds and Light Intensity = 3 units. The probability of the light-off jump response (y-axis) is plotted against the stimulus number (x-axis, Pulse). Fixed effects are interpreted as described in results. (b) Spontaneous recovery following rest (independent measures) in *eag Sh; cn bw* and *cn bw* tested under the stimulus condition of Interstimulus interval (ISI) = 3 seconds and Light Intensity = 3 units. The probability of the light-off jump response (y-axis) is plotted against the stimulus number (x-axis, Pulse). Fixed effects are interpreted as described in results.

3.3. Age

Aging significantly impaired the habituation process, but only under conditions that produced robust habituation, **Table 1**. Under optimal habituation conditions, interstimulus interval of 2 s and light intensity at level 3 units, 30-day-old flies showed a significantly shallower habituation slope than 10-day-old flies (*Age30day:as.numeric*) (*Pulse* = 0.194, $p < 0.001$; **Figure 1(a)**). The older flies maintained higher response probabilities throughout the pulse train. Under mod-

erate habituation conditions (ISI 3s, Intensity 4), there was also a significant age-related impairment with old flies habituating more slowly than the young flies (interaction = 0.149, $p < 0.001$; **Figure 2(b)**). When young flies showed weak or no habituation, there were no significant age differences in slope or habituation process as seen in **Figure 1(b)**, ISI 2s Intensity 4: $p = 0.152$; and **Figure 3(a)** and **Figure 3(b)** ISI 5s: $p > 0.32$ and $p > 0.57$.

3.4. Potassium Ion Channel Mutation

The results presented in **Figure 4** detail the comparison of habituation process between hyperexcitable mutant *eag Sh; cn bw* and *cn bw*. Conceptually, *cn bw* (the genotype used throughout these experiments) is the control, but to analyze the habituation of *eag sh; cn bw*, that genotype was taken as the baseline for analyzing these experiments.

All comparisons were performed under the interstimulus interval of 3 seconds and light intensity of 4 units. Mutations in voltage-gated potassium channels significantly accelerated the habituation process, **Table 2**. The *eag Sh; cn bw* mutants showed a significantly steeper negative slope than *cn bw* controls as seen **Figure 4(a)** and **Figure 4(b)**. Disruption of potassium channels through *eag Sh* mutations increases neuronal excitability, which in turn may accelerate short-term synaptic depression, leading to faster behavioral habituation. This provides a direct relationship between specific ion channel function, synaptic plasticity mechanisms, and learning behavior.

4. Discussion

This study utilized the visual light-off jump response in *Drosophila melanogaster* to systematically characterize habituation, a fundamental form of non-associative learning, and to define the influence of extrinsic factors including interstimulus interval and light intensity as well as intrinsic factors such as age and neural excitability. We demonstrated that aging impairs plasticity, while hyperexcitability caused by potassium channel mutations accelerates it. Habituation is a measurable behavioral indicator of neural plasticity and may provide a robust assay for investigating the synaptic mechanisms underlying cognitive aging.

4.1. Validation and Optimization of Habituation Protocol

Our systematic approach confirms that the *Drosophila* light-off jump response is a valid model for studying short-term, non-associative learning, while also defining the stimulus parameters that optimize its manifestation. It is important to carefully characterize habituation to distinguish the observed reduction in jump probability from non-learning phenomena including sensory adaptation and motor fatigue. We confirmed habituation by demonstrating a statistically significant decline in jump probability across 15 sequential 20 millisecond dimming pulses, quantified using generalized linear mixed models (GLMMs).

To confirm the behavioral decrement was genuine learning, we rigorously as-

sessed response recovery through spontaneous recovery and dishabituation [2]. Recognizing the fundamental risk that the stimulus required for dishabituation affects the measurement of spontaneous recovery, we implemented an independent measures design. The dishabituation test, using mechanical vortexing, resulted in a recovery of the jump response that was significantly higher than the recovery observed in the time-matched spontaneous recovery control group. The spontaneous recovery tests the return of the response after a rest period confirmed that the habituation was reversible. This differential recovery suggests that dishabituation actively recruits a separate, arousal-driven system to override the habituation-induced inhibition.

4.2. Influence of Stimulus Parameters

The strength of habituation was profoundly shaped by the characteristics of the stimulus train. Our findings show a specific effective range of stimulation defined by the interaction of stimulus timing and light intensity.

The interstimulus interval (ISI), the time delay between successive dimming pulses, emerged as the primary determinant of habituation success. This parameter influences the frequency of synaptic depression underlying habituation. Longer interstimulus interval allowed recovery between pulses, preventing depression from accumulating [18]. The requirement for interstimulus interval to induce habituation aligns with short-term synaptic depression, the primary neural mechanism for non-associative learning. High interstimulus interval of 2 s leads to depletion of the readily releasable vesicle pool, causing a steep behavioral decline (habituation). At a long interstimulus interval, synaptic vesicles replenishment between pulses prevents any accumulating depression.

Light intensity proved to be a critical modulator of stimulus efficacy. This was clearly demonstrated at the intermediate interstimulus interval of 3 s. At lower intensity (3 units), habituation failed to occur in both age treatment groups. The light intensity strength was insufficient to overcome the recovery period provided by the 3 s interstimulus interval. At high intensity (level 4), the stimulus strength increased. The increased stimulus strength was sufficient to drive the circuit into habituation in both groups. It was only when stimulus strength was adequate that the age-related impairment became apparent with older flies habituating significantly more slowly.

4.3. Age-Related Effect

Aging significantly impaired the capacity for habituation, supporting the hypothesis that aging reduces neural plasticity in the *Drosophila* visual-motor circuit. At shorter ISIs (2 s) and lower intensity (3 units), 30-day-old flies exhibited a shallower habituation slope compared to 10-day-old flies, indicating slower adaptation to repetitive stimuli. Similarly, at ISI 3 s and higher intensity (4), older flies showed reduced habituation. These findings suggest age-related declines in neural plasticity, potentially due to reduced synaptic efficacy, or altered gene expression

in learning pathways [13]. In contrast, no significant age effects occurred at longer ISIs (5 s), where habituation was absent overall. Our finding implies that aging reduces habituation, reflecting cognitive declines in higher organisms, including humans, where habituation deficits are link to disorders like Parkinson's disease [7]. The difficulty of older (30-days) flies to ignore repetitive stimuli mirrors the behavioral difficulties faced by elderly individuals who experience reduced or diminished sensory filtering, leading to increased distractibility and struggle processing information amongst background noise.

4.4. Potassium Channel on Habituation

The *ether-à-go-go (eag) Shaker (Sh)* strain showed a significantly accelerated habituation compared to the *cn bw* control, providing a direct connection between potassium ion channel function, and neuronal excitability.

The accelerated habituation observed in *eag Sh; cn bw* mutants supports the predicted effects of reduced potassium currents. The *ether-à-go-go (eag)* and *Shaker (Sh)* genes encode voltage-gated potassium channels responsible for neuronal repolarization [19]. Their combined mutation produces a hyperexcitable process characterized by an increased action potential [10]. Broader action potentials prolong depolarization, leading to excessive calcium influx per stimulus at synaptic release sites. This heightened calcium load drives accelerated depletion of the neurotransmitters of synaptic vesicles, resulting in the steeper behavioral decline we quantified. Our results directly confirm and extend earlier neurogenetic findings in the *Drosophila* giant fiber escape pathway [20], demonstrating that mutations in genes encoding specific potassium channels significantly affect habituation.

4.5. Implications and Applications

The findings from this study go beyond the simple confirmation of habituation in *Drosophila*. They establish a robust, quantifiable model for understanding the interaction between intrinsic (genetic, age) and extrinsic (stimulus parameters) factors in neural plasticity. This work provides a foundational framework for understanding the specific genetic and circuit-level mechanisms underlying short-term memory decay and restoration in a simple model organism.

By systematically varying interstimulus interval and light intensity, we demonstrated that habituation is not a fixed genetic trait, but a dynamic process shaped by sensory input parameters.

The results provide a clear demonstration in *Drosophila* that aging impairs habituation in a circuit-specific manner. This positions the fly as a powerful model for aging neuroscience, allowing rapid genetic screens for interventions that restore neural plasticity in older animals.

The slower habituation in 30-day-old flies mirrors the sensory filtering deficits seen in human aging, cognitive impairment, and Parkinson's disease [12]. This assay could be used to test drugs or genetic interventions aimed at preserving or restoring

ing habituation-like plasticity.

The accelerated habituation in *eag Sh* mutants' models conditions of neuronal hyperexcitability, such as some forms of epilepsy, autism spectrum disorder, where sensory habituation is often altered [5]. This provides a genetically tractable system to explore how ion channel mutations lead to altered information filtering at the circuit level.

5. Limitations of the Present Study

Several limitations should be considered when interpreting our findings. First, all experiments were conducted using white-eyed *cn bw*-derived lines. While the white-eye background eliminates confounding from screening pigments and allows a simple light-off stimulus, it may alter baseline visual sensitivity or neural processing compared to wild-type pigmented flies. Second, we relied on a single behavioral assay, the Giant Fiber System mediated light-off jump response. Habituation in other sensory modalities such as olfactory, gustatory or in different motor output pathways may follow different rules or show different age and genotype dependent effects. We did not examine intermediate ages or flies older than 30 days, therefore, the trajectory of age-related decline in habituation beyond this range remains unknown. Future studies using a broader age continuum and additional genetic backgrounds will be needed to generalize these conclusions.

Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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