HELLENIC REPUBLIC

National and Kapodistrian University of Athens

Department of Biology

Athens International Master's Programme in Neurosciences

Biomedical Sciences Research Center "Alexander Fleming",

34 Fleming Street, 16672 Vari, Greece

RESEARCH THESIS PROJECT

BEHAVIORAL AND ELECTROPHYSIOLOGICAL CHARACTERIZATION OF GENES GOVERNING SHOCK HABITUATION LATENCY IN DROSOPHILA MELANOGASTER

Eleni Giannopoulou

Student ID: 111807

2021

Contents

Title

Behavioral and electrophysiological characterization of genes governing shock habituation latency in *Drosophila melanogaster*

Authors and Affiliations

Eleni Giannopoulou[1,2]

[1]*Biomedical Sciences Research Center "Alexander Fleming", Vari, Greece,* [2]*International Masters in Neurosciences, University of Athens, Greece*

Corresponding author: Eleni Giannopoulou (elenigian@biol.uoa.gr)

Summary

Habituation is a fundamental process for all living organisms to decrease responsiveness to repetitive or prolonged non-reinforced stimuli. In humans, impaired habituation has been correlated with a variety of neuropsychiatric disorders. Premature habituation, or shortened habituation latency is a defective phenotype that does not allow proper discrimination between novel and pre-experienced stimuli and attribute the appropriate importance. *14-3-3ε*, *ptp61F* and *corkscrew* are three of the genes governing shock habituation latency in *Drosophila melanogaster*, whose molecular mechanism is still unknown, and is implicated in protection from premature habituation. The aim of this study was to confirm previous results in mutants and determine the role of the three genes in habituation latency. Therefore, we performed a series of behavioral and electrophysiological experiments using RNA interference to silence each gene in specific neuronal populations and circuits. Special focus was given at the protein expression or elimination in the mushroom bodies and the brain as a total. Some of the previous behavioral results were confirmed, while others were not, due to specific difficulties. Finally, we tried to study habituation and simulate our behavioral results with electrophysiology in a different well-identified neuronal pathway, the giant fiber system.

Highlights

Premature habituation to electroshock does not allow the organism to discriminate between novel and pre-experienced stimuli and react accordingly. *14-3-3ε, ptp61F* and *corkscrew* genes have been shown to govern both shock habituation latency to footshock and habituation of the long latency response of the giant fiber system.

Keywords

Drosophila, premature habituation, electric stimulation, behavior, electrophysiology, 14-3-3ε, ptpt61F, corkscrew

Introduction

Organisms constantly receive stimuli from their environment, novel or pre-experienced, which they need to evaluate correctly so that they save energy and focus on those with high importance for their survival and reproductive success (Giles and Rankin, 2009).

Habituation is the core gating mechanism to decrease responsiveness to repetitive or prolonged non-reinforced stimuli with little predictive value and importance (Thompson and Spencer, 1966; Rankin, Gannon and Wicks, 2000; Rose and Rankin, 2001; Acevedo *et al.*, 2007). It is a process present in most circuits and modalities of the nervous system and highly conserved across animal species, yet mechanistically complicated (McDiarmid, Yu and Rankin, 2019). It works as a highly adaptive form of selective attention (Giles and Rankin, 2009) and despite being considered the simplest form of non-associative learning (Lieberman, 2000), the plethora of different underlying mechanisms function in orientation to survival, reproduction, higher brain functions and behavioral flexibility (McDiarmid, Bernardos and Rankin, 2017; McDiarmid, Yu and Rankin, 2019). It cannot be misinterpreted as either sensitization or fatigue since it is stimulus specific, there is spontaneous recovery from it after a given time and a dishabituator can completely disrupt it (Solokov, 1963). The characteristics of habituation were firstly described by Thompson and Spencer in 1966 and revised in 2009 by Rankin et al.

In humans, habituation deficits have been associated with learning disabilities (Gillberg, 2003; Slaats-Willemse *et al.*, 2003), schizophrenia (Meincke *et al.*, 2004; Hammer *et al.*, 2011; Williams *et al.*, 2013), attention-deficit/hyperactivity disorder (Slaats-Willemse *et al.*, 2003; Massa and O'Desky, 2012; Morello *et al.*, 2020) and migraines (Coppola, Pierelli and Schoenen, 2009; Kalita, Bhoi and Misra, 2014), among other conditions. It has been suggested that it may relate to disease phenotype either as a cause, symptom or therapy (Acevedo *et al.*, 2007; McDiarmid, Bernardos and Rankin, 2017). Since habituation may underlie selective attention (Groves and Thompson, 1970; Gillberg, 2003), reduced, null or premature habituation to a stimulus would not permit discrimination between novel and pre-experienced stimuli essential in mediating appropriate responses (Acevedo *et al.*, 2007).

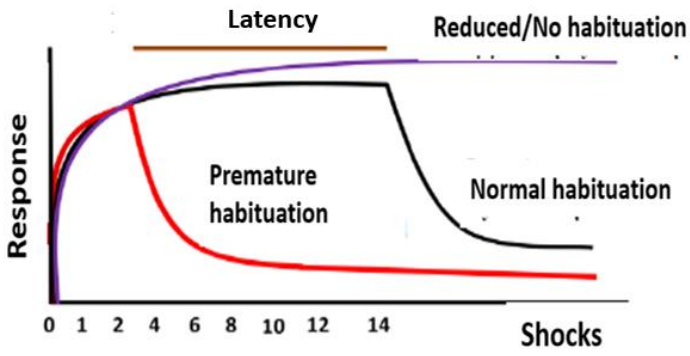


Image 1. **Habituation of response to repetitive electric shocks.** Normal habituation (black): diminished response after 15 shocks. Premature habituation (red): diminished response after 2-4 shocks. Reduced/No habituation (purple): no diminishing response. *(Adapted from R.Christodoulou, bachelor thesis, 2018).*

A diminished response to the stimulus earlier than normally expected is characterized as premature habituation, and it needs to be prevented since it does not permit the organism to properly discriminate and process the information they receive since the information content does not stay available for long enough (Semelidou, Acevedo and Skoulakis, 2018).

Drosophila's advanced molecular and classical genetics have established it as a powerful model for studying habituation of various sensory modalities, like mechanosensory (Acevedo *et al.*, 2007) and escape responses (Engel and Wu, 2009).

Genetic screening habituation experiments in the lab have shown that *14-3-3ε, ptp61F* and *corkscrew* genes are implicated in protection from premature habituation to electroshock (Acevedo, 2004; Kadas, 2011; Christodoulou, 2018).

D14-3-3ε is a small acidic molecule that belongs to the big 14-3-3 protein family and is by 82% identical to the mammalian 14-3-3ε isoform. It is ubiquitously expressed in all tissues, stages of development and cells examined (Philip, Acevedo and Skoulakis, 2001), with a particular abundance in the CNS (Roseboom *et al.*, 1994). The homozygous null mutants for D14-3-3ε are semi- lethal, immunocompromised and infertile (Acevedo, 2004). The protein is required for the timing of mitosis and regulation of the cell cycle (Su *et al.*, 2001; Tsigkari, Acevedo and Skoulakis, 2012). It is implicated in photoreceptor differentiation via the Ras/Raf signaling pathway (Chang and Rubin, 1997) and the A-P polarity in the oocytes (Benton, Palacios and Johnston, 2002). 14-3-3s are generally a positive regulator of synaptic plasticity as well as learning and memory in mammals (Qiao *et al.*, 2014). Presynaptic inhibition of 14-3-3s may contribute to impairments in LTP and cognitive functions.

To investigate CNS-mediated habituation and the anatomical sites that are involved, we used a previously described behavioral paradigm of habituation to electroshock which was developed in the lab (Acevedo *et al.*, 2007) and studied how specific Drosophila lines would habituate to repetitive 45V-electroshocks, which can be

categorized in the general family of aversive stimuli. Although the neuronal pathway implicated is still unknown, the mushroom bodies (MBs) have been shown to be essential for evaluation and response to repetitive stimulation with electric shock. Processes especially within the α/β lobes seem to be essential to maintain latency and therefore protect from premature habituation (Acevedo *et al.*, 2007).

Previous work in the lab, showed that mutations in the *14-3-3ε* gene lead to a robust premature habituation phenotype in the behavioral electroshock habituation paradigm (Acevedo *et al.*, 2007) after training of the flies with 1, 2 or 4 electric shocks.

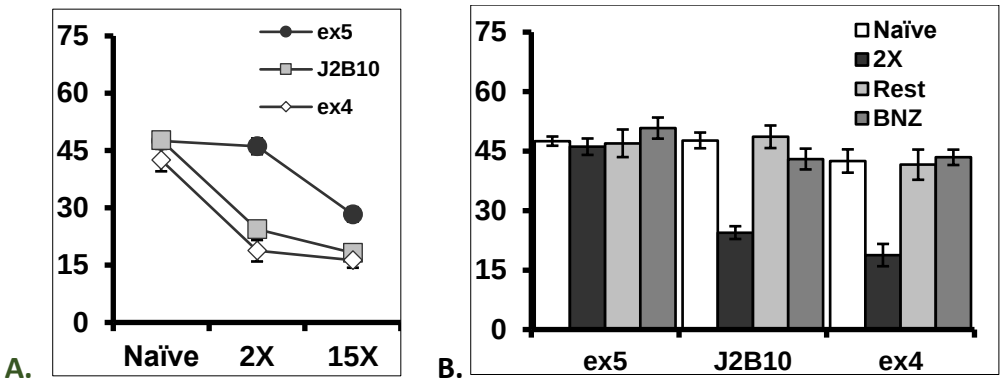
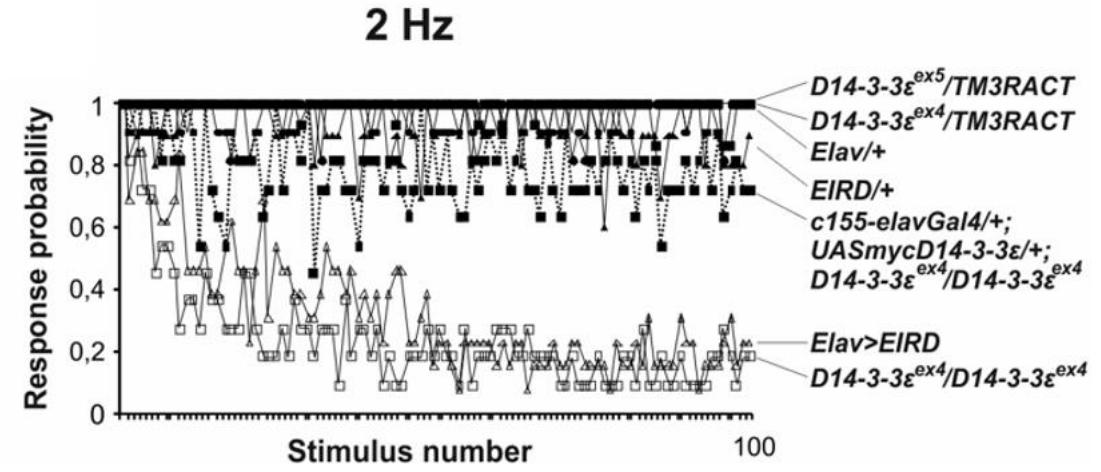


Image 2. **Habituation to 45V-electroshock in ex4, ex5 and j2b10 14-3-3ε mutants.** A/B. ex4 and j2b10 mutants habituate normally after training with 15 shocks and prematurely after training with 2 shocks. (*Adapted from S.F.Acevedo, PhD dissertation, 2004*)

Similar results were obtained studying habituation of the same mutant strains with electrophysiology in a different, yet well-identified neuronal pathway, the giant fiber system (GFS) (Kadas, 2011).



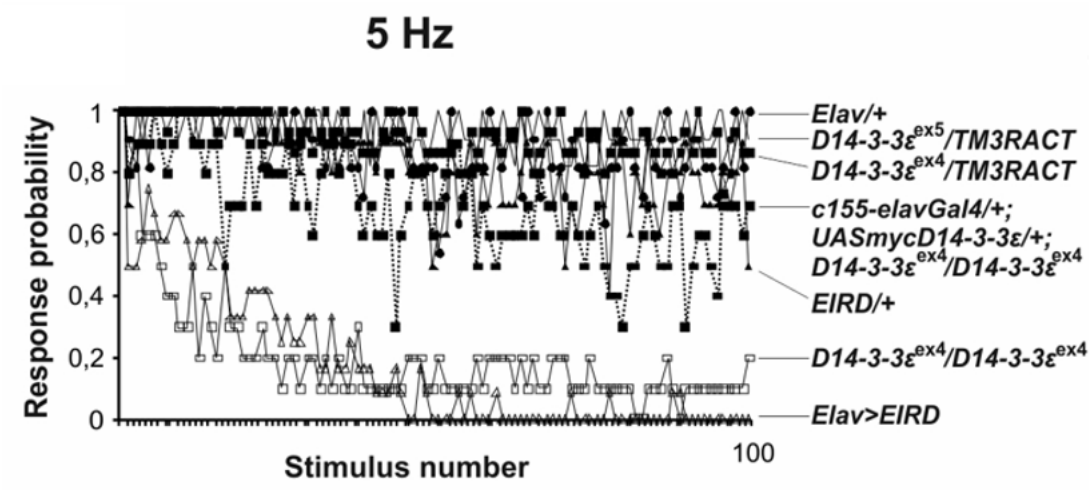


Image 3. **Homozygous mutations ex4 and pan-neuronal abrogation of 14-3-3ε with EIRD RNAi leads to premature habituation of the long latency response at 2Hz and 5Hz stimulation.** *(Adapted form D.Kadas, PhD dissertation, 2011)*

To briefly describe it, a visual stimulus activates a series of cholinergic neurons presynaptic to the ColA interneurons, which output to the two giant fiber (GF) interneurons (Gilbert and Strausfeld, 1991). The GFs output to the tergotrochanteral motoneurons (TTMns) that innervate the tergotrochanteral muscle (TTM, jump muscle) and to the peripherally synapsing interneuron (PSI), which subsequently outputs to the dorsolongitudinal muscle motoneurons (DLMns), which innervate the dorsal longitudinal muscles (DLMs, wing depressors) (Koenig and Ikeda, 2005).

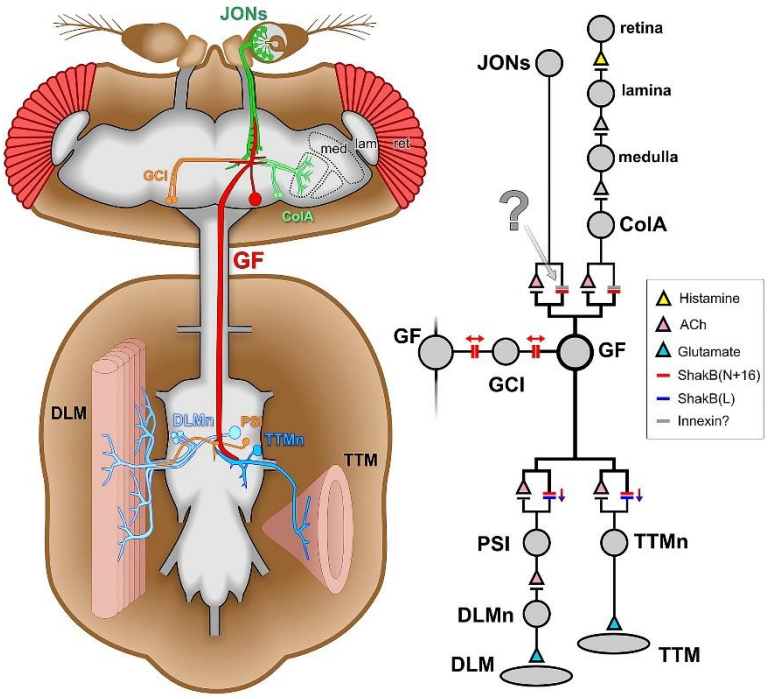


Image 3. **The giant fiber system in Drosophila.** (*Adapted from* Pézier *et al.*, 2016)

The "jump and flight" escape response presents plasticity and habituation. Although its locus has not been identified yet, research suggests that it lies within the pathway afferent to the GFs (Engel and Wu, 1996, 1998; Engel *et al.*, 2000). When electrical stimulation is strong enough, it can initiate action potentials right in the GFs bypassing the afferent pathways (short latency response), and recordings from the efferent muscles can be used to study the GF action potentials (Kadas *et al.*, 2012). However, the long latency response is the one that undergoes habituation (Engel and Wu, 1996).

The electrophysiological premature habituation phenotype of the mutant D14-3-3ε fly strains was confirmed using RNA interference in an *elav,Gal4>RNAi-for-14-3-3ε* cross line where the protein's concentration gets reduced pan-neuronally.

Protein-tyrosine phosphatase 61F (Ptp61F) belongs to the cytosolic protein Tyrosine phosphatases and the non-receptor class I subfamily. It is implicated in several cellular functions: cellular response to hypoxia (Lee, Feldman and O'Farrell, 2008), axon guidance (Clemens *et al.*, 1996), mitotic cell cycle (Chen *et al.*, 2007), negative regulation of cell proliferation and MAPK and Ras pathway (Tchankouo-Nguetcheu *et al.*, 2014), of ERK1 and ERK2 cascade (Willoughby *et al.*, 2017) and of IFR pathway (Wu *et al.*, 2011; Tchankouo-Nguetcheu *et al.*, 2014), regulation and stability of proteins (Huang *et al.*, 2007), etc. Its human orthologues PTPN1 and PTPN2 are related to diabetes mellitus, non-insulin-dependent (omim).

Mutations in the *ptp61F* gene led to a premature habituation phenotype in the electroshock habituation paradigm as well, after exposure to 2 and 4 electric shocks during training (Christodoulou, 2018). These results were confirmed with RNA interference to reduce the expression of the phosphatase in the entire brain only during adulthood, by crossing *elav;Gal80^{ts}* female flies with male flies of the RNAi for ptp61F strain. Induction of RNAi expression 48 hours before the experiment led the induced group of flies to prematurely habituate to footshock. MBs were shown to not be implicated in ptp-61F-mediated protection from premature habituation, as *leo,Gal80^{ts}>ptp61F-RNAi* induced-flies with eliminated protein concentration only in the MBs did not habituate prematurely.

Similar results were obtained by R.Christodoulou (2018) experimenting on flies which carried mutations in the *csw* gene that also led to a premature habituation phenotype in the electroshock habituation paradigm.

The protein-tyrosine phosphatase corkscrew (csw) belongs to the cytosolic protein Tyrosine phosphatases and the non-receptor class I subfamily as well. It has been shown that csw is necessary in all signaling pathways of Tyrosine receptors and functions as a negative regulator (Oishi *et al.*, 2009). It is important for normal CNS development and the Egfr pathway (Perkins *et al.*, 1996) and seems to be implicated in the mitotic cell cycle (Chen *et al.*, 2007), it is also a negative regulator of apoptosis (Smith *et al.*, 2002). Its human orthologues PTPN11 and PTPN6 are related to *juvenile*

myelomonocytic leukemia, metachondromatosis-PTPN11-related and *Nooman syndrome 1.*

Results from electroshock habituation experiments with *leo,Gal80^{ts}>csw-RNAi* flies showed that elimination of the protein within the MBs does not lead to the same phenotype after training with 4 shocks, which indicates that these structures are not implicated in protection from premature habituation for the csw protein either.

The aim of this study was to confirm previous results in mutants (Acevedo, 2004; Christodoulou, 2018) and further investigate the role of these 3 genes in habituation latency/protection from premature habituation to repetitive electric shock. We performed a series of behavioral experiments using RNA interference to silence each one of the genes in specific neuronal circuits at specific developmental stages. Finally, we tried to simulate the behavioral habituation phenotypes of each gene's disruption with electrophysiology in the GFS, keeping in mind that the neuronal systems implicated are distinct to each other.

Materials and Methods

Drosophila culture and strains

Drosophila melanogaster stocks were cultured in standard wheat-flour-sugar food containing the following ingredients for 8L of food; 8.5L of distilled water, 140g semolina, 180g whole wheat flour, 180g brown sugar, 72g fructose, 15g soy flour, 6g $CaCl_2$, 210g dry yeast, 45g agar, 15g nipagen, and 25g propionic acid. The flies were raised at 22°-25°C, except the ones carrying the *Gal80^{ts}* target system that were raised at 18°C until hatching and then placed at 30°C for 48 hours before testing in order to induce the expression of the transgene of interest, always in a stable 12h light/dark cycle. All experiments were performed in 2-5 days old flies.

We used the following drivers to selectively express each transgene of interest in different neuronal sets; *elavGal4* for expression in the entire CNS (previously described by Gouzi et al., 2011), *elav;Gal80^{ts}* for whole-CNS expression during adulthood and *elav;247Gal80,Gal80^{ts}* for whole-CNS expression except for the MBs (both constructed in the lab), *739,Gal80^{ts}* for expression in the α/β lobes of the MBs (kind gift from Gregg Roman, University of Houston, USA).

The MiMIC lines and RNAi lines with the following codes were obtained from the Bloomington Drosophila Stock Center (University of Indiana, USA);

Ptp61F mutant #37014 (y[1] w[*]; Mi{y[+mDint2]=MIC}Ptp61F[MI03132]), CSW mutant #41405 (y[1] Mi{y[+mDint2]=MIC}csw[MI05169]w[*]), Ptp61F-RNAi #56510 (w[*]; P{w[+mC]=UAS-Ptp61F.RNAi}i2-5), CSW-RNAis #33619 (y[1]v[1];P{y[+t7.7] v[+t1.8]=TRiP.HMS00012}attP2) and #60448 (y[1]v[1];P{y[+t7.7]

v[+t1.8]=TRiP.HMJ22804}attP40), D14-3-3ε-RNAi #34884 (y[1]sc[*]v[1]sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01229}attP2) and #35441.

GENOTYPE		SOURCE	EXPRESSION PATTERN
Drivers			
elavGal4		BDSC	Pan-neuronal expression
elav;Gal80^{ts}		Constructed in lab	Pan-neuronal expression in adulthood
elav;247Gal80,Gal80^{ts}		Constructed in lab	Pan-neuronal expression out of MBs
739,Gal80^{ts}		Gregg Roman	Pan-neuronal expression out of MB α/β lobes
MiMICs			
Ptp61F		BDSC #37014	-
Corkscrew		BDSC #41405	-
RNAi			
Ptp61F		BDSC #56510	-
Corkscrew		BDSC #33619	-
Corkscrew		BDSC #60448	-
D14-3-3ε		BDSC #34884	-
D14-3-3ε		BDSC #35441	-

Western blotting

Total protein levels were determined in 1 adult female head homogenate in 1x Laemmli buffer (50 mM Tris pH 6.8, 5% 2-mercaptoethanol, 2% SDS, 10% glycerol, and 0.01% bromophenol blue). Protein electrophoresis was performed in 12,5% SDS-PAGE gels in Tris–glycine buffer. The proteins were transferred onto nitrocellulose membranes and immunoblot analysis was performed using specific polyclonal antibodies against D14-3-3ε protein (73b, 1:2000) and Syntaxin (8C3, 1:4000). Subsequently, membranes were probed with horseradish peroxidase-conjugated secondary antibodies applied at 1:5000 concentration, visualized with Clarity Max ECL Substrate (BIO-RAD), and exposed to Super RX film (Fuji Film). The intensity of each immunoreactive band was estimated by densitometric quantification using ImageLab software. Differences in protein expression levels were revealed after normalization of all values using the loading control anti-Syntaxin (mAb 8C3, Developmental Studies Hybridoma Studies, 1:4000).

Behavioral assays

All behavioral experiments were carried out in a balanced design, where all genotypes involved in an experiment were tested per day. The experimenter was blind to the genotype and the order of genotypes submitted to training and testing was randomized. Behavioral experiments were replicated at least 3 independent times and

at least once with flies from different crosses and a different time period (biological replicates). Data are shown as mean±SEM and all bars are shown as avoidance followed by premature habituation at 4 or 6 shocks followed by normal habituation at 15 shocks.

All flies were tested 2-5 days after emergence. They were collected after brief anesthesia with CO_2 at least 2 days before testing and kept in 3mL-food vials in groups of 50-70 flies. To maintain endo-genotype consistency and avoid developmental effects, most of the drivers used contained the $Gal80^{ts}$ temporal transgene. All these crosses were raised at 18°C (uninduced state) for the control condition, unless stated otherwise, so that the transgene does not get expressed, until transfer to 30°C 48 hours before the experiment, so that the RNAi gets expressed supposing at the maximum (induced state). All flies were transferred to fresh 3mL-food vials 1-1,5 hour before the experiment and kept in a dark box for the whole time so that they get used to no-light conditions and the only stimulus driving the tested behavior is the experimental one. All experiments were performed under dim red light at 24°-25°C and 65%-70% humidity.

Electroshock avoidance test

Experiments were performed at 24°C and 67% humidity. Approximately 50 naive flies were placed at the choice point of a T-maze and left to choose between an electrified and an otherwise identical inert standard copper grid for 90 seconds. In the electrified grid, there were delivered 1.2sec-duration stimuli of 45V intensity every 4 sec. The airflow in all arms of the maze was kept constant at 500mL/min at all times. The performance index (PI) was calculated as the fraction of flies avoiding the electrified grid minus the fraction of flies that did not.

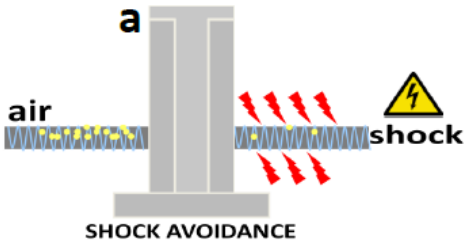


***Image 4*. Avoidance of the electric footshock.** Naive flies prefer to move in the unshocked arm.

Electroshock habituation assay

Experiments was performed as previously described by Acevedo et al. (2007), at 24°C and 67% humidity. During the training stage, a set of around 50 flies was trained in the upper arm of a standard T-maze lined with an electrifiable grid, by receiving 4 or 6 (in premature habituation training) or 15 (in normal habituation training) x1.2-sec electroshocks of 45V each. Right afterwards, the flies were left to rest for 30 seconds before placed in the lower choice point of the maze and left to choose between an

electrified and an otherwise identical inert standard copper grid for 90 seconds as above. At the end of this period, the flies in each arm were trapped and counted, and the performance index was calculated as previously described.

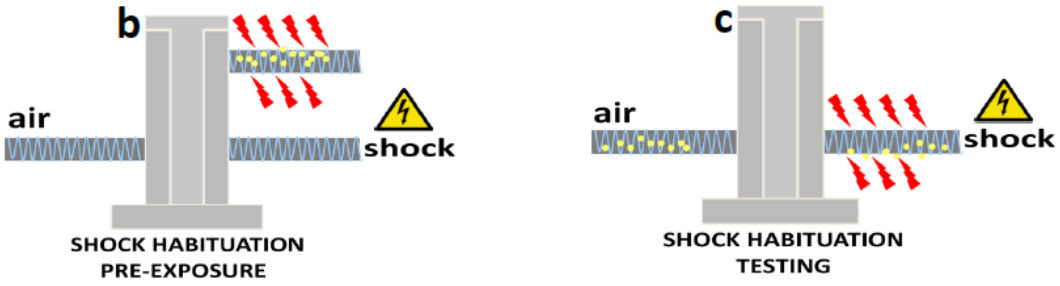


***Image 5*. Shock habituation training and testing.** b: Pre-exposure/training to electric footshock. c: testing of habituation.

Odor discrimination test

Experiments were performed at 25°C and 65% humidity under dim red light. Approximately 50 naïve flies were placed at the choice point of a T-maze and left for 90 sec to freely choose between two aversive odorants; 0.05% benzaldehyde (BNZ) and 50% 3-octanol (OCT), both diluted in iso-propyl myristate solution. The airflow in both arms of was kept constant at 500mL/min. Avoidance to both odors was tested simultaneously for each strain and all strains were tested in a given session. The flies of each arm were then trapped and counted to calculate the performance index (PI) as the fraction of flies avoiding the one odorant over the other.

Learning assay

To assess learning efficiency, the flies were subjected to the negatively reinforced paradigm described by Gouzi *et al.* (2011), coupling two aversive odors as conditioned stimuli (CS+ and CS-) with the electric shock as the unconditioned stimulus (US). The two aversive odorants used were 0.05% benzaldehyde (BNZ) and 50% 3-octanol (OCT), both diluted in iso-propyl myristate solution. Approximately 50 flies were placed in the upper arm of a standard T-maze into a tube lined with an electrifiable grid and presented with air (500 mL/min) for 15sec before initiation of training with the appliance of 6 electroshocks of 90V intensity every 5sec, for a 30-sec-period during which the air current carries the shock-associated odor (CS+, CS/US pairing). Two groups of animals of the same genotype and induction state were trained simultaneously, one to avoid BNZ and the other OCT, while the complementary odorant was used as the respective control. After the CS+ presentation, the flies were left to rest for 30sec before the CS- presentation of the air current carrying plain the odor for another 30sec period. Flies were then left to rest for 30sec again before getting transferred to the lower choice point of the T-maze. At this point, they were left for 90 sec to freely choose between air current carrying the CS+ odor and air current carrying the CS- odor. The half-learning performance index was calculated as

the fraction of flies that avoided the shock-associated CS+ odor minus the fraction that avoided the control odor. One performance index (PI) was calculated as the average of the half-learning indexes for each of the two groups of animals trained to complementary conditioning stimuli and ranges from 100 (perfect learning) to 0.

Electrophysiology

All electrophysiological experiments were carried out in a balanced design, where all genotypes involved in an experiment were tested per day. The order of genotypes submitted to testing was in the consecutive order of 1 control to 1 experimental. The experiments were replicated at least 3 independent times and were performed on at least 8-10 animals of each genotype so that the data size and distribution permit statistical analysis. They still need to be replicated on flies from different crosses and a different time period (biological replicates). Data are shown as mean ±SEM.

Preparation for recording

The flies get briefly anesthetized with CO_2. Each fly is glued to a thin metal wire on the back of their neck using super glue, let to dry for a few seconds and then the wire with the fly is placed in a humid chamber (petri dish with a wet piece of paper inside) (Engel and Wu, 1996) and left to recover for at least 1h at either 21-24^{o}C or 30^{o}C if the flies are induced carrying the *Gal80^{ts}* temporal expression gene. The fly is placed on the electrophysiology stage, with the legs touching the ground so that it can step on a solid surface during the whole procedure and noise from excessive movement gets diminished. Uninsulated tungsten electrodes are used to penetrate the tissues (Kadas *et al.*, 2012). The electrodes are placed as follow: 1 stimulation electrode in each one of the eyes, 1 recording electrode in the upper part of the back and close to the middle trying to target the 5th-6th DLM fibers and finally 1 reference electrode in the abdomen (right below the end of the back) (Engel and Wu, 1992). Delivery of a low-Voltage stimulus confirms if all electrodes are placed correctly, corrected when needed.

Electrophysiological recordings

There was need to set up and optimize the conditions and the electrophysiological setup for studying habituation so that the data are in line with literature. During this procedure, wild-type Canton-S flies were studied. Final habituation protocol performed is presented right below.

The fly is left to relax for 10min with the electrodes penetrating the tissues. Single (0.1ms) pulses are applied, with 30sec interstimulus interval to avoid habituation of the response with a Grass S88 stimulator (Grass Technologies), starting with low voltages (4V) and subsequently increasing by 1-2V the intensity of the next stimulus delivered after 30sec until a clear short latency response appears. Data are digitized with an analog-to-digital converter (Digidata 1200; Molecular Devices) without filtering and are analyzed and displayed with SignalExpress LabView 2009 software (National Instruments). The recording helps determine the response thresholds for short-latency and long-latency. The fly is left to rest for 10min before applying the

habituation protocol. Humidity in the room during habituation needs to be around 40-60%.

1000 stimuli are applied with an intensity near the top of the long-latency stimulus range (0.5-1V below the upper threshold) with a frequency of 2Hz, 3Hz or 5Hz. When finished, the fly is removed from the stage. The “yes” or “no” response of the fly after each given stimulus/if the fly responded or not to a specific stimulus are counted and the probability of response per stimulus and the failure criterion for each genotype tested are addressed. The probability of response is defined as the percentage of animals that respond to a certain stimulus. The failure criterion involves the average number of stimuli at which the animals fail to respond for 1, 2, 3, 4 and 5 consecutive times. Flies that failed to respond to either or both the first two stimuli, were excluded from the analysis.

Statistical analysis

Raw data derived from the behavioral assays were analyzed parametrically with the JMP7.1 statistical software (SAS Institute Inc.) as previously described by Gouzi *et al.* (2011). The means and SEMs were compared following an initial, significant differences-indicating ANOVA (positive ANOVA), using Least Square Means Contrast analyses relative to the designated control. Similarly, learning performance indices were calculated for each genotype as indicated above and following positive ANOVA, means and SEMs were compared to that of their genetic or treatment controls using Dunnett’s tests (Keramidis *et al.*, 2020).

Raw data from all electrophysiological experiments were analyzed in Microsoft Office Excel, by counting the number of “yes” or “no” responses after a given stimulus and addressing the probability of response per stimulus and the failure criterion for each genotype tested. The probability of response is defined as the percentage of animals that responded at a certain stimulus. The failure criterion involves the average number of stimuli at which the animals failed to respond for 1, 2, 3, 4 and 5 consecutive times. Hypothesis testing was performed through two-tailed t-tests between the groups. Flies that failed to respond to either or both the first two stimuli, were excluded from the analysis.

Western blot results were analyzed with ImageLab software and Microsoft Office Excel by calculating densitometry and the ratio of the D14-3-3ε relative to that of the control Syntaxin. The ratio of the control genotype was set to 1 and all experimental ratios were reported as relative to that.

The level of significance in all cases was set at p-value=0.05.

Results

1. 14-3-3ε

1.1 #34884 RNAi is the most potent for the reduction of 14-3-3ε

An RNAi screening was initially performed through Western blot analysis to determine which of the available RNAi lines is the most potent for the reduction of the 14-3-3ε protein in the CNS. Female *elav,Gal4* flies were crossed with males from 4 different RNAi strains (#34884, #31497, #35441, EPS117) and raised at 25°C. Three female flies of the F1 generation of each cross were transferred to 30°C in order to induce the expression of the RNAi 24 hours before extraction of their head proteins to be used as samples in the Western blot. As positive control there were used heads of 3 females that belonged to the F1 generation of *elavGal4>W^{1118}(w)* crossed flies.

The Western blot was performed according to the protocol described at the Materials and Methods' section and antibodies against the 14-3-3ε protein of interest permitted the visualization of its expression in the different samples and the determination of any differences between them compared to the housekeeping protein's (syntaxin) expression. Every sample was loaded in triplicate.

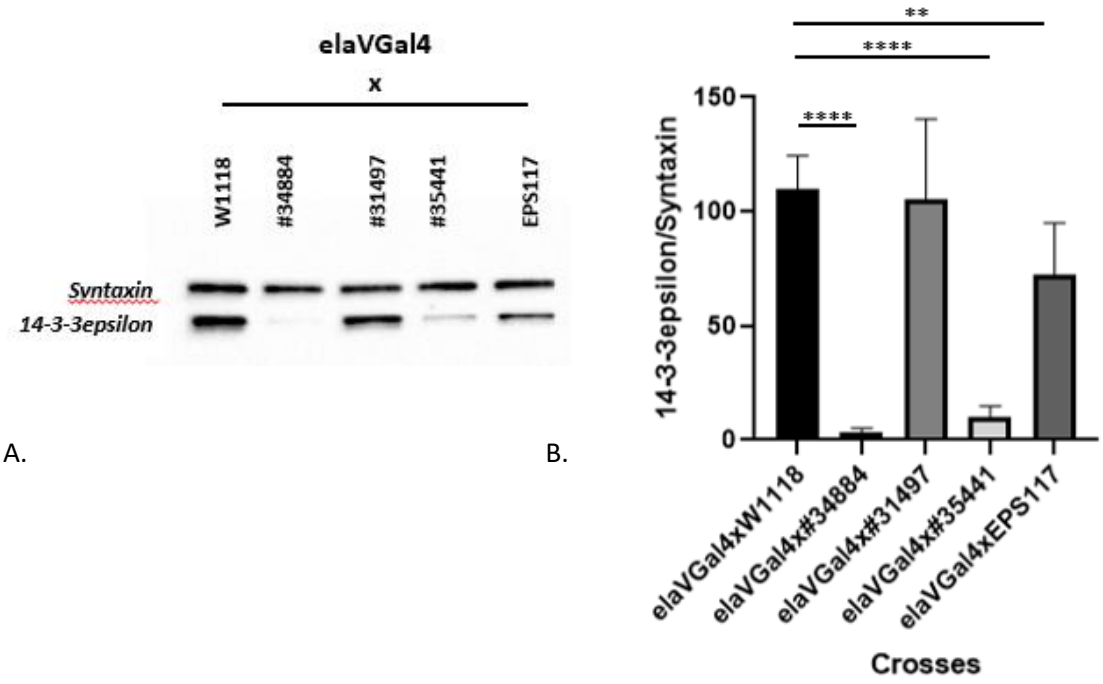


Figure 1. RNAi screening for 14-3-3ε using Western blot analysis. (A) Western blot results for 14-3-3ε's expression in the different RNAis crossed with *elav,Gal4* flies compared to control *elav,Gal4>W^{1118}(w)*. (B) Quantification of RNAis for 14-3-3ε compared to the control *elav,Gal4>W^{1118}(w)*.

Data are presented as mean±SEM.

As shown in Figure 1A, crossed flies with #34884 (*y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01229}attP2*) and #35441 (*y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.GL00366}attP2*) RNAis show significantly less 14-3-3ε protein expression compared to the control line, with the first one being more potent than the second by leading to an almost 100% reduction of the protein's expression. The quantification of the protein's expression in each genotype was the average of a triplicate analysis.

In order to confirm the results from the Western blot, male flies from the *#34884 RNAi* strain were crossed with *yw; P[Tub-G4]/TM3-Sb* female flies that ubiquitously express Gal4 (tubulin, Gal4), which pointed out that the homozygous state of F1 is 100% lethal. Consequently, the #34884 RNAi line was selected for the behavioral experiments, as the most prominent for the elimination of the 14-3-3ε protein.

1.2 Electroshock habituation paradigm

1.2.1 Elimination of 14-3-3ε throughout development does not lead to premature habituation

In order to explore if the premature habituation phenotype of the mutants is confirmed by RNA interference, the RNAi was expressed to reduce the protein's concentration in the entire CNS throughout development.

elavGal4 female flies were crossed with males from the *#34884 RNAi* strain and raised at 18°C in food with ampicillin. A group of 1-3days old flies of the F1 generation were transferred to 30°C 48 hours before the performance of the behavioral experiment in order to induce the RNAi's expression. To maintain endo-genotype consistency, the fly group of the same young age that was constantly kept at the standard 18°C conditions was used as the negative control group. All these flies were subjected to the electroshock habituation assay as described in the Materials and Methods' section.

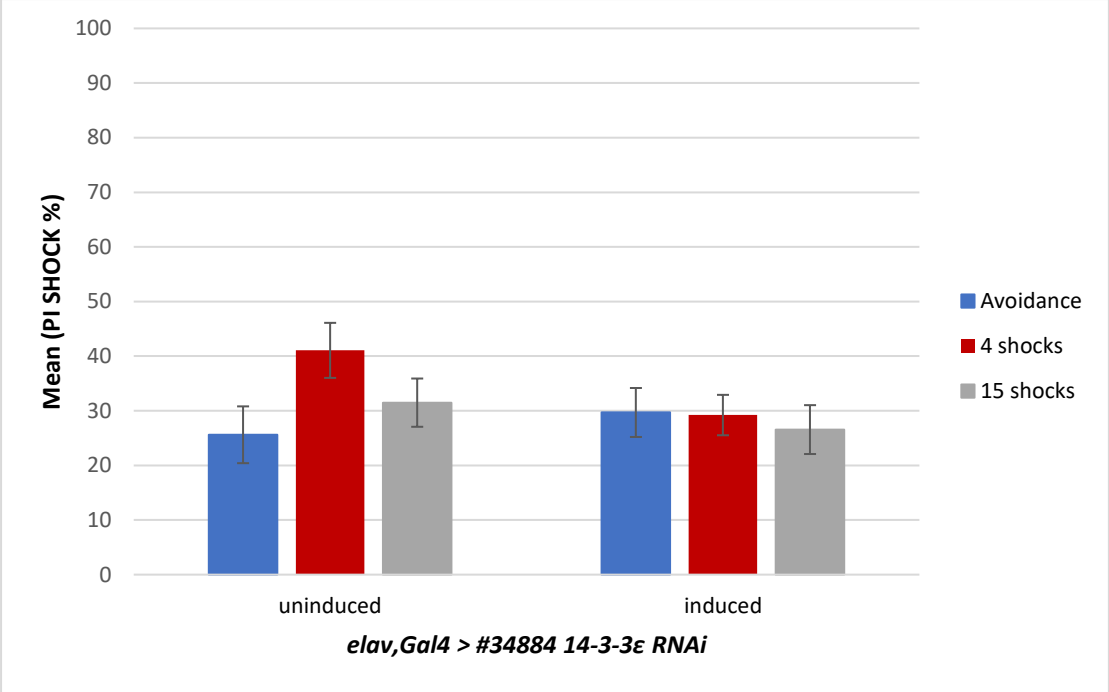


Figure 2. Electroshock habituation of *elavGal4 > #34884 14-3-3ε RNAi*. Both uninduced and induced flies do not habituate neither prematurely after training with 4 shocks (p-values 0.0174 and 0.9365 respectively) nor normally after training with 15 shocks (p-values 0.3980 and 0.6520 respectively).

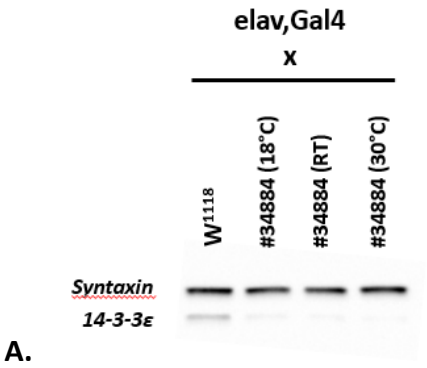
Data are presented as mean±SEM.

Uninduced flies showed no habituation to repetitive footshock after training either with 4 shocks (p-value=0.0174) or with 15 shocks (p-value=0.3980), compared to the avoidance performance index (PI) of the same group. Induced flies did not habituate either, neither after training with 4 shocks (p-value=0.9365) nor training with 15 shocks (p-value=0.6520).

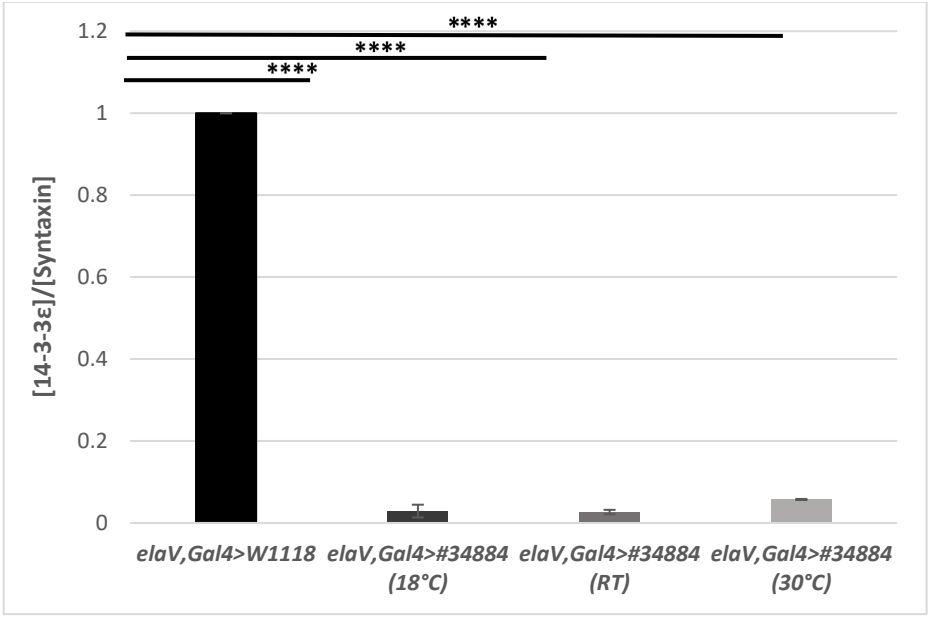
Therefore, the defective phenotype of the mutants was not uncovered (Acevedo, 2004).

Interestingly, the avoidance PIs in both conditional groups were really low in value compared to the PIs of the majority of normal controls from experiments in the lab, a fact which makes the comparison with the experimental groups difficult increasing the possibility for a defective phenotype to remain hidden.

In order to check the 14-3-3ε protein's expression between the induced (30°C) and uninduced (18°C) state, a Western blot analysis of samples from these 2 conditions and an independent control line was performed.



A.



B.

Figure 3. Western blot analysis of induction of #34884 14-3-3 RNAi's expression at different temperatures for 48 hours. There is extremely significant reduction in the expression of 14-3-3ε at all temperatures compared to the control (p-value<0.0001), but no significant difference between the different induction states. (A) Western blot. (B) Quantification for relative protein expression.

Data are presented as mean±SEM.

The blot showed that both states show very low concentration of the protein of interest compared to a positive control (p-value<0.0001), with no significant difference between them (p-value=0.0842). This result explains the phenotypic similarities between the different groups.

1.2.2 Elimination of 14-3-3ε in adulthood does not lead to premature habituation

In order to explore if the premature habituation phenotype of the mutants could be confirmed with RNA interference, trying at the same time to avoid any developmental effect that would complicate the situation (as above), the RNAi was expressed in the whole CNS only in the adult stage.

elav;Gal80^{ts} female flies were crossed with males form the *#34884 RNAi* strain and raised at 18°C in food with ampicillin, in order for the RNAi to not be expressed during development. A group of 1-3 days old flies of the F1 generation were transferred to 30°C 48 hours before the performance of the behavioral experiment in order to induce the RNAi's expression. Flies of the same young age that were constantly kept at the standard 18°C conditions were used as negative controls. All flies were subjected to the electroshock habituation assay as described at the Materials and Methods' section.

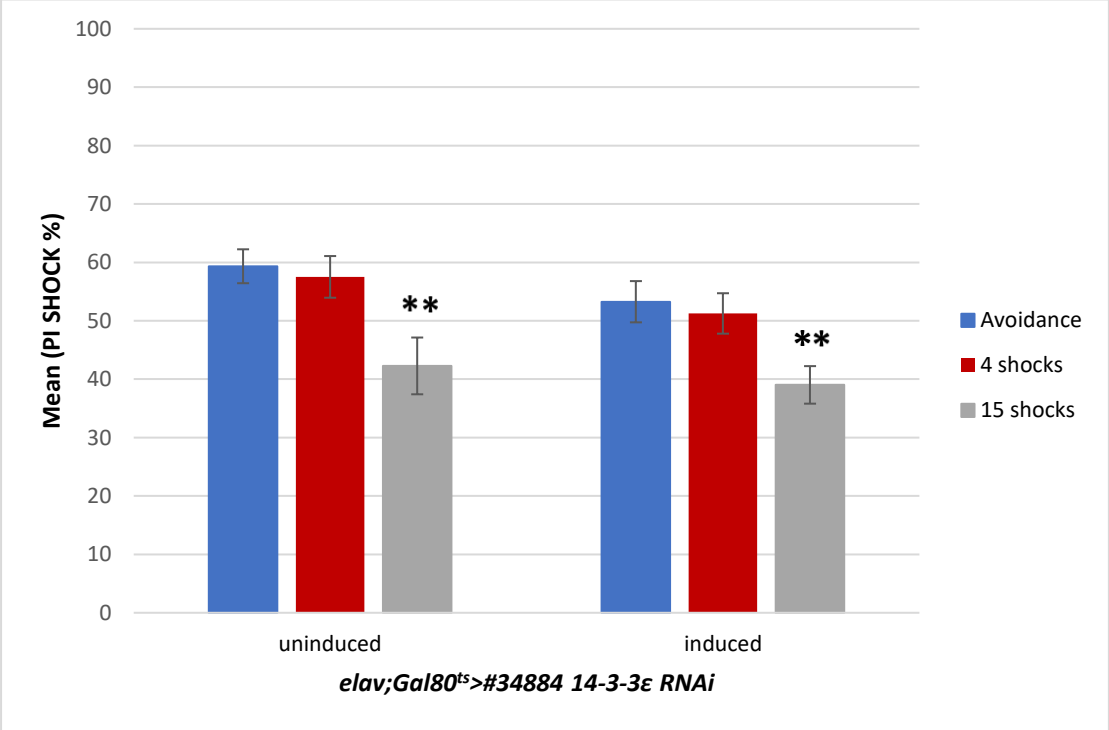


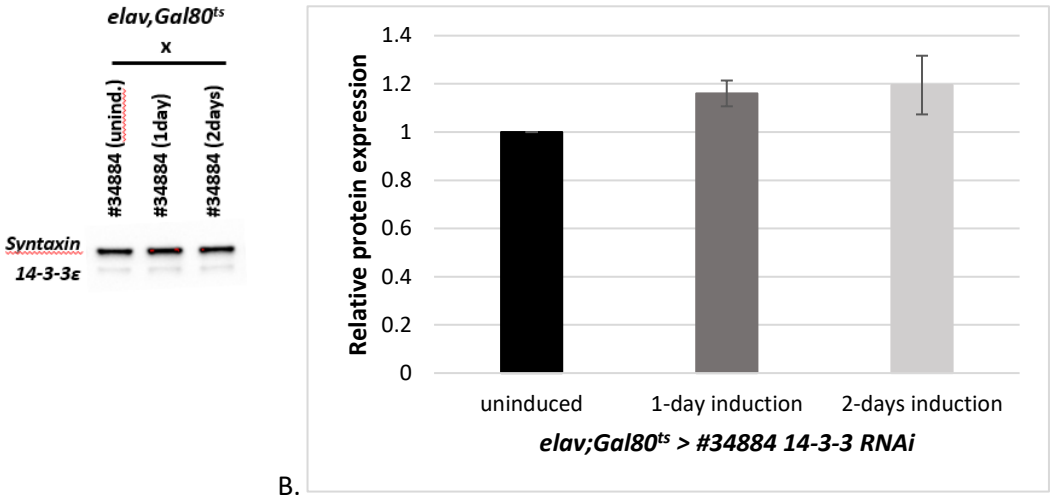
Figure 4. Electroshock habituation for *elav;Gal80^{ts}>#34884 14-3-3ε RNAi*. Both uninduced and induced flies do not prematurely habituate after training with 4 shocks (p-values 0.7195 and 0.7080 respectively), while they normally habituate after training with 15 shocks (p-values 0.0010 and 0.0090 respectively).

Data are presented as mean±SEM.

Both uninduced and induced flies habituated normally after training with 15 shocks, with p-values 0.0010 and 0.0090 respectively. The uninduced flies did not prematurely habituate after training with 4 shocks (p-value=0.7195), as expected from the results of the mutants. However, the induced flies did not prematurely habituate after

training with 4 shocks (p-value=0.7080), either. Therefore, the phenotype of the mutants was not uncovered once again (Acevedo, 2004).

In order to investigate if different days of induction of the RNAi expression would be more efficient in eliminating the 14-3-3ε protein enough for the phenotype to emerge, a Western blot analysis of the 14-3-3ε protein expression was performed in *elav;Gal80^{ts}>#34884* flies, uninduced and induced for 1 or 2 days.



A. B.

Figure 5. Western blot analysis of different days of induction of RNAi for 14-3-3ε expression. There is no significant difference between the different induction states. (A) Western blot. (B) Quantification for relative protein expression.

Data are presented as mean±SEM.

No significant difference in the protein's expression was spotted between the uninduced and induced state for any of the different induction periods, which explains why a significant phenotype did not appear since the beginning. The p-value for the 1-day-induction compared to the uninduced control was 0.6031 and for the 2-days-induction compared to the control 0.5288.

1.2.3 Expression of the RNAi outside of the MBs does not lead to premature habituation

As mentioned in the introduction, the mushroom bodies (MBs) are essential for protection from premature habituation either to olfaction or footshock (Acevedo et al., 2007). More particularly for the footshock, there is need for intact and functional a/β lobes. In order to investigate if the premature habituation phenotype that supposing should have been observed with RNAi expressed in the entire CNS is led by the neurons outside of the mushroom bodies, an excluding of these structures and an expression everywhere else but in the MBs were performed.

elav;247Gal80,Gal80^{ts} female flies were crossed with males form the *#34884 RNAi* strain and raised at 18°C in food with ampicillin. A group of 1-3days old flies of the F1 generation were transferred to 30°C 48 hours before the behavioral experiment in order to induce the RNAi expression. Flies of the same young age that were constantly kept at the standard 18°C conditions were used as controls. All these flies were subjected to the electroshock habituation assay as described at the Materials and Methods' section.

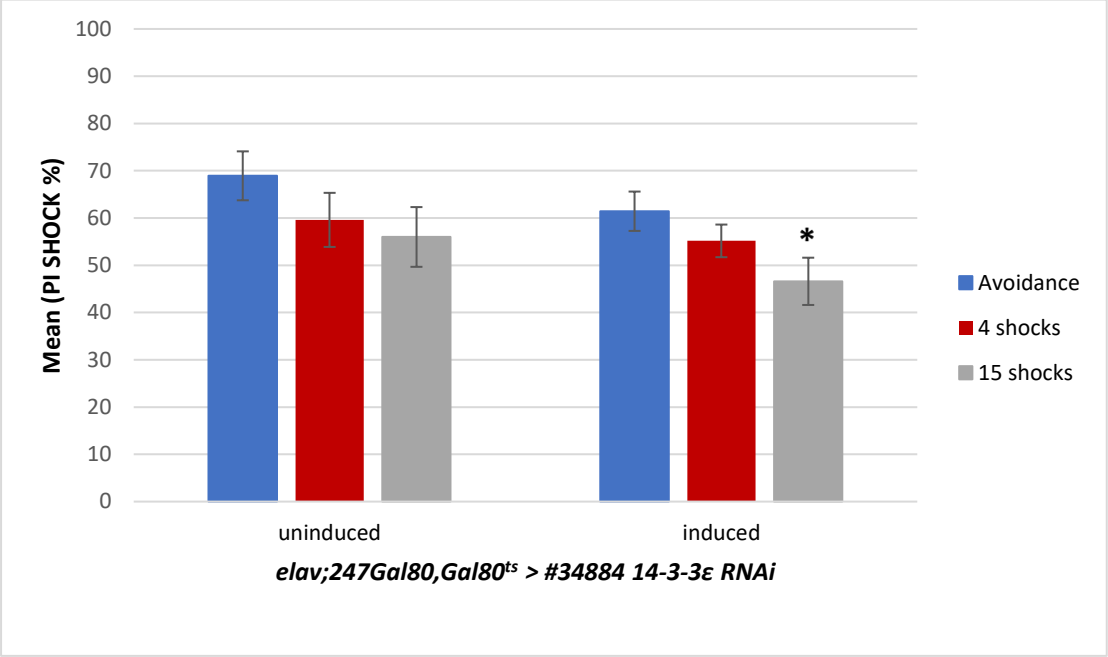


Figure 6. Electroshock habituation for *elav;247Gal80,Gal80^{ts} >#34884 14-3-3ε RNAi*. Both uninduced and induced flies did not habituate prematurely after training with 4 shocks (p-values 0.2004 and 0.3702 respectively). Although uninduced flies did not habituate after training with 15 shocks either (p-value=0.0771), induced flies did (p-value 0.0362).

Data are presented as mean±SEM.

Uninduced flies did not habituate as normally expected after 15-shock training, with a p-value=0.0771 compared to the avoidance performance as they also did not prematurely habituate after training with 4 shocks either, with a p-value=0.2004 compared to the avoidance performance once again. 48-hour-induced flies habituated normally after training with 15 shocks (p-value=0.0362), but did not habituate prematurely after training with 4 shocks (p-value=0.3702). Unfortunately, these results cannot be entrusted since the control group did not behave as expected for the assay and no defective phenotype could be determined.

Finally, in an effort to investigate if these flies with reduced expression of 14-3-3ε through RNA interference show any defect in behavior, a group of them was subjected to a learning assay.

1.3 Learning assay

Since habituation is considered a form of non-associative learning (Lieberman, 2000), learning performance in a specific and well-designed behavioral assay would be an indirect way to study habituation.

elav;Gal80^{ts} female flies were crossed with males of the *#34884 RNAi* strain and raised at 25°C in food with ampicillin. A group of 1-3days old flies of the F1 generation were transferred to 30°C 48 hours before testing in the behavioral experiment in order to induce the RNAi's expression. These flies composed the experimental induced group. Flies of the same young age that were constantly kept at 25°C were used as controls composing the uninduced group. The temperature of 25°C was chosen in order for the 48 hours of induction at 30°C to be able to yield sufficient reduction in the 14-3-3ε levels that would probably help a behavioral phenotype to emerge.

1.3.1 Flies with reduced 14-3-3ε avoid both 3-octanol and benzaldehyde

An odor discrimination test was firstly performed in order to check if the *elav;Gal80^{ts}>#34884 RNAi* flies would show preference to any of the two aversive odorants used in the learning assay that would affect the result of the training process.

The flies were left to freely choose for 90 sec between 50% octanol (3-octanol) and 0.05% benzaldehyde, both diluted in iso-propyl myristate. The results are shown in Figure 7.

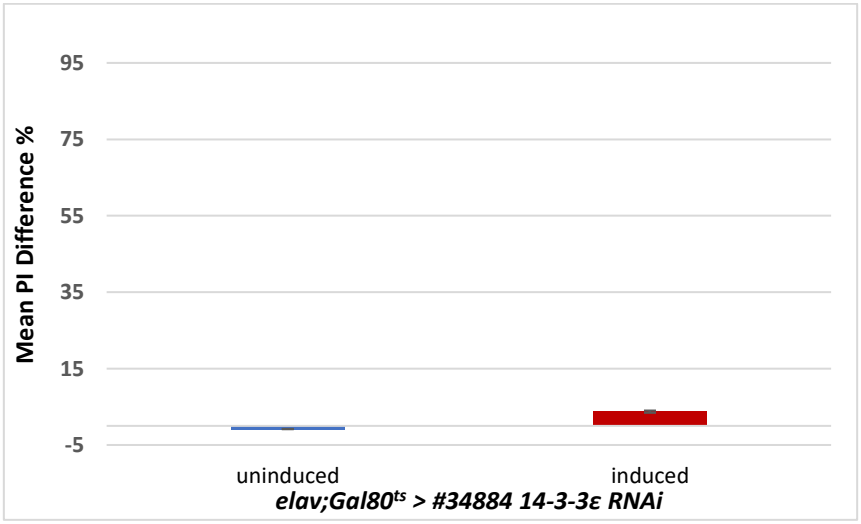


Figure 7. Odor discrimination test for *elav;Gal80^{ts} > #34884 14-3-3ε RNAi*. Both uninduced and induced groups equally avoided 50% octanol (3-octanol) and 0.5% benzaldehyde.

Data are presented as mean±SEM.

Both the induced and uninduced crossed flies showed no significant preference to any of the two odorants, which allowed the proceeds to the performance of the learning assay.

1.3.2 Flies with reduced 14-3-3ε are weak learners

The learning assay was performed as described in the Materials and Methods' section, by training the flies to associate one of the two aversive odorants with a negative stimulus (footshock) and then leaving them to freely choose for 90 sec between the two odorants without the appearance of the extra unconditioned stimulus.

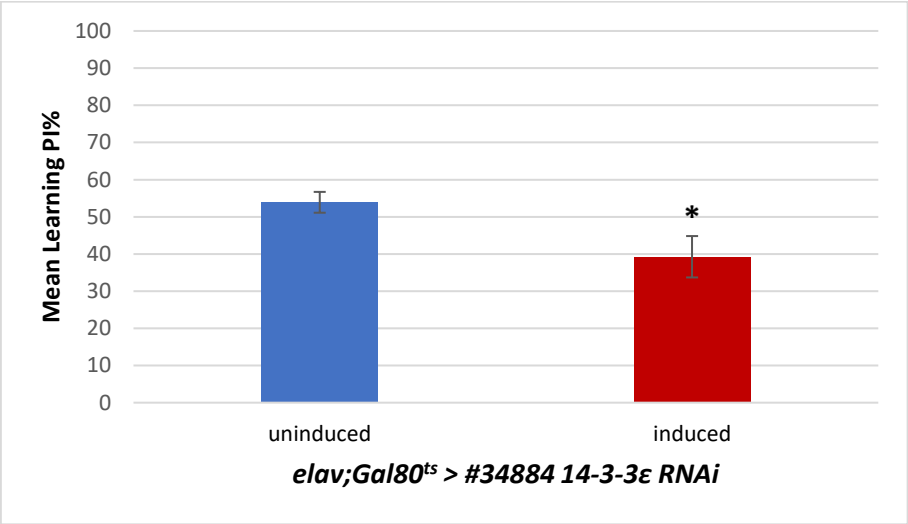


Figure 8. Learning assay for *elav;Gal80ts > #34884 14-3-3ε RNAi*. Induced flies have significantly lower learning performance than the control uninduced ones with a p-value 0.0367.

Data are presented as mean±SEM.

As shown in Figure 8, induced flies are significantly worse learners than the uninduced with a p-value=0.0367. Therefore, it seems safe to think that there may be some deficiency in the habituation process too, which for some reason is not obvious.

2. Protein-tyrosine phosphatase 61F (Ptp61F)

2.1 Electroshock habituation paradigm

In order to confirm previous results mentioned in the introduction that the mushroom bodies are not implicated in the protection from premature habituation respectively to ptp61F protein (Christodoulou, 2018) and taking into account what was previously published by the lab (Acevedo et al., 2007) that the mushroom bodies are essential for the protection from premature habituation to footshock where intact a/β lobes are needed, *739,Gal80^{ts}* female flies were crossed with male flies from the #56510 RNAi strain.

2.1.1 α/β MB lobes are not implicated in protection from premature habituation in respect to ptp61F

The crossed flies were raised at 18°C. A group of 1-3days old flies of the F1 generation were transferred to 30°C 48 hours before the behavioral experiment in order to induce the RNAi expression and eliminate the protein's concentration only in the α,β lobes of the MBs. These flies composed the experimental induced group. Uninduced flies of the same young age that were constantly kept at 18°C were used as the control group.

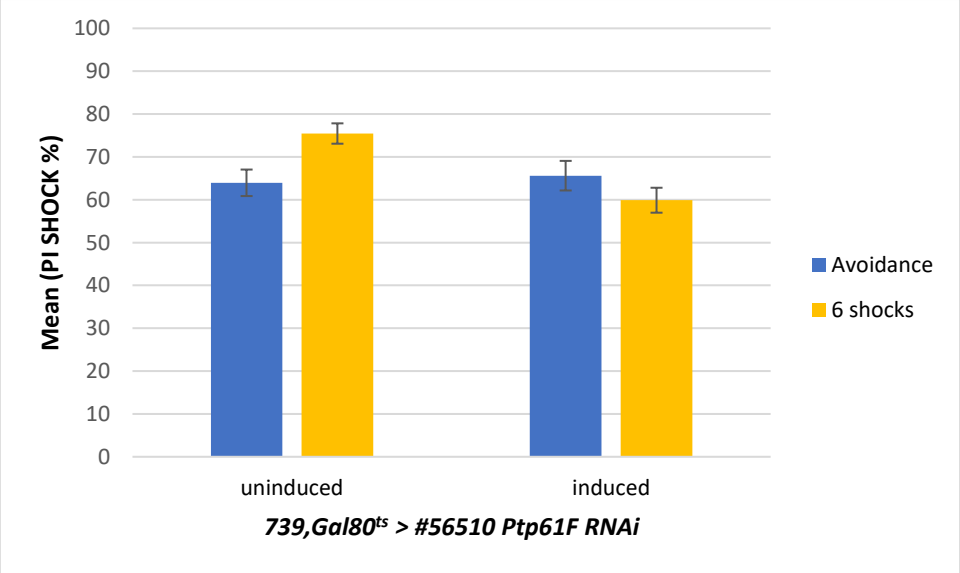


Figure 9. Electroshock habituation for *739,Gal80^{ts} > #56510 Ptp61F RNAi*. None of the experimental induced group nor the control uninduced group of flies prematurely habituated after training with 6 shocks (p-values 0.1781 and 0.0077 respectively).

Data are presented as mean±SEM.

The induced group of flies did not prematurely habituate to 45V-footshock after training with 6 shocks (p-value=0.1781), as the control group (p-value=0.0077). This result partially explains what Christodoulou (2018) showed by reducing the protein's concentration in the whole MBs with *leo,Gal80^{ts}*, that the MBs are not implicated in protection from premature habituation as for the *ptp61F* gene.

2.1.2 Reduction of ptp61F everywhere outside of the MBs does not lead to premature habituation (not expected)

In order to investigate if the premature phenotype depends in neuronal circuits outside the mushroom bodies, *elav;247Gal80,Gal80^{ts}* female flies were crossed with males from the #56510 RNAi strain and raised at 18°C. A group of the F1 flies was induced by being transferred to 30°C 48 hours before the experiment, so that the protein's expression gets reduced everywhere but in the mushroom bodies. The uninduced group of flies that were continuously kept at 18°C were used as negative control. All F1 flies were subjected to the electroshock habituation assay.

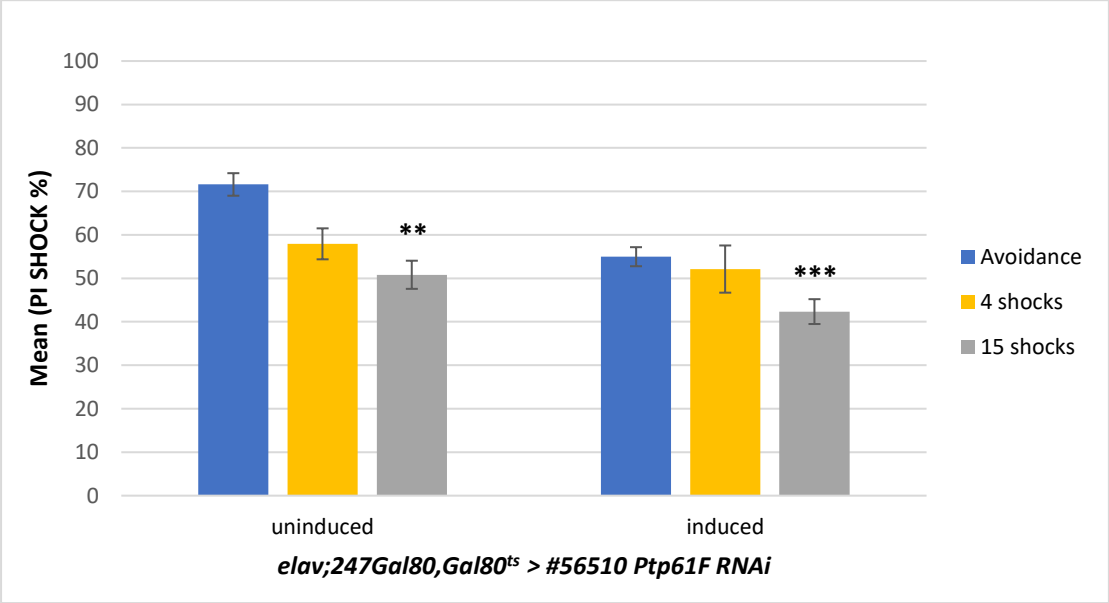


Figure 10. Electroshock habituation for *elav;247Gal80,Gal80^{ts} > #56510 Ptp61F RNAi*. Both uninduced and induced groups habituated normally after training with 15 shocks (p-values 0.0063 and 0.0001 respectively). The induced group did not prematurely habituate (p-value=0.5190), while the control uninduced group prematurely habituated after training with 4 shocks (p-value=0.0062), which makes all the experiment unreliable.

Data are presented as mean±SEM.

The induced group of flies did not prematurely habituate to 45V-footshock (p-value=0.519), while the uninduced group did (p-value=0.0062), a fact which is odd and

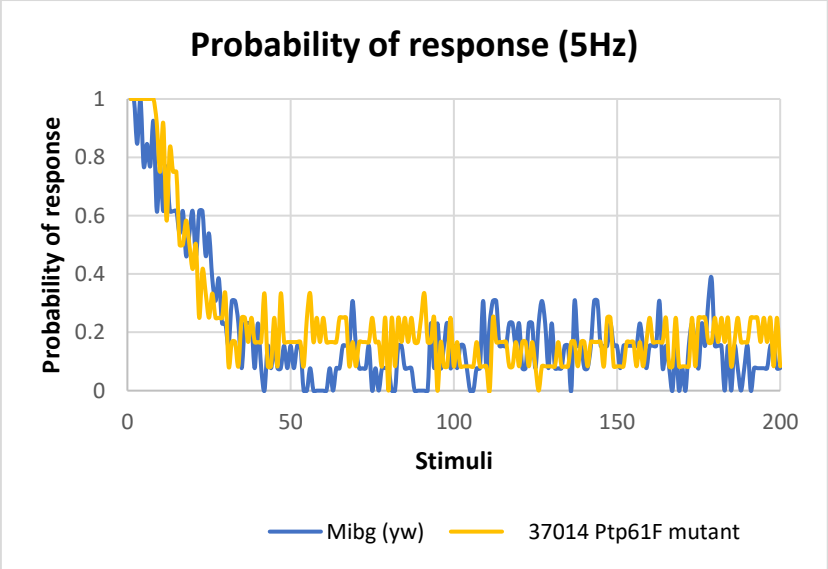
decreases the credibility of the results. On the other hand, both groups of flies normally habituated after training with 15 shocks, with p-values for the induced group equal to 0.0063 and for the uninduced equal to 0.0001. Since the control prematurely habituated, this data are unreliable.

2.2 Electrophysiology

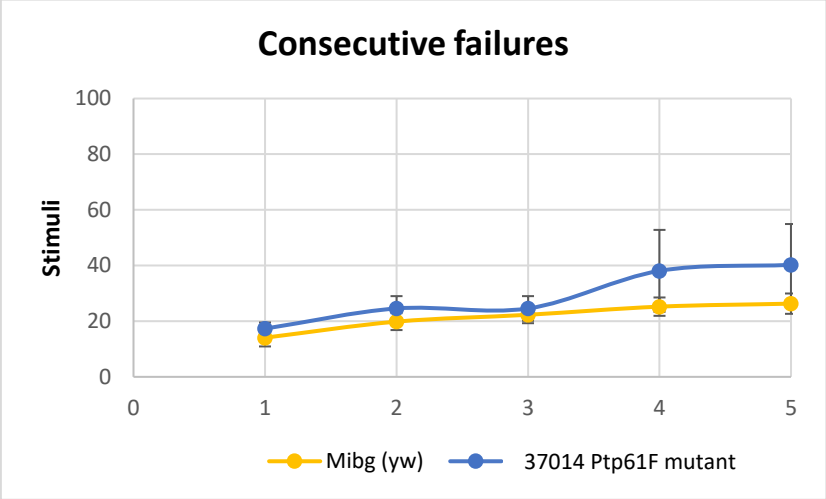
In order to investigate if the premature habituation phenotype presented in the behavioral assay, can also be revealed in different neuronal sets and systems at the same time, a series of electrophysiological recordings of the giant fiber system was performed in 2-6 days old flies of the mutant #37014 (*y1 w*; Mi{MIC}Ptp61FMI03132*), of *yellow,white* (Mibg) background. Mibg flies were used as genetic controls. All flies were raised at 25°C.

Firstly, in an attempt to assess the rhythm of habituation at 5Hz after 1000 stimuli for both the mutant and its control, both genotypes habituated very quickly and any difference between the two if existed was impossible to get spotted.

A.



B.



p-values	Consecutive failures				
Genotype	**1**	**2**	**3**	**4**	**5**
#37014 vs. Mibg	0.9985	0.9782	0.4087	0.4089	0.4154

Figure 11. Rhythm of habituation of GFS at 5Hz repetitive electric stimulation.

(A) Assessment of probability of response.
(B) Consecutive failures. The p-values are shown in the table underneath.

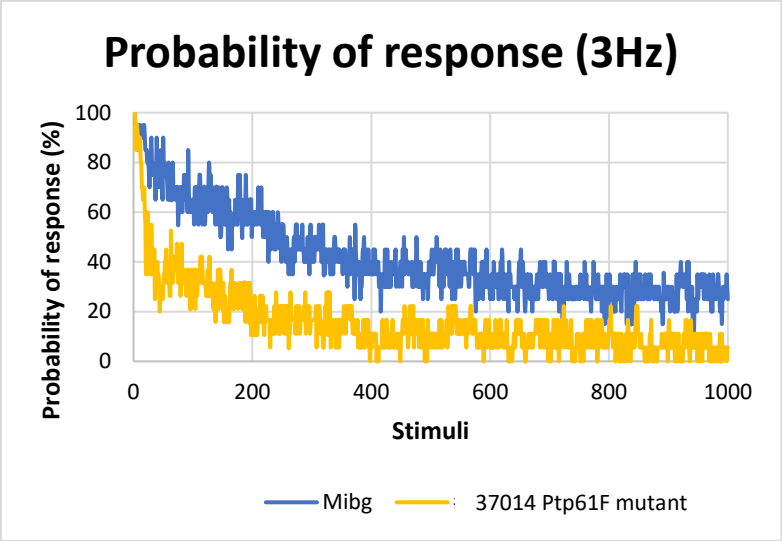
Data are presented as mean±SEM.

Then, the rhythm of habituation at 2Hz was assessed, which is the other frequency commonly studied in literature, but neither genotype habituated at all after the application of 1000 stimuli (graph not shown).

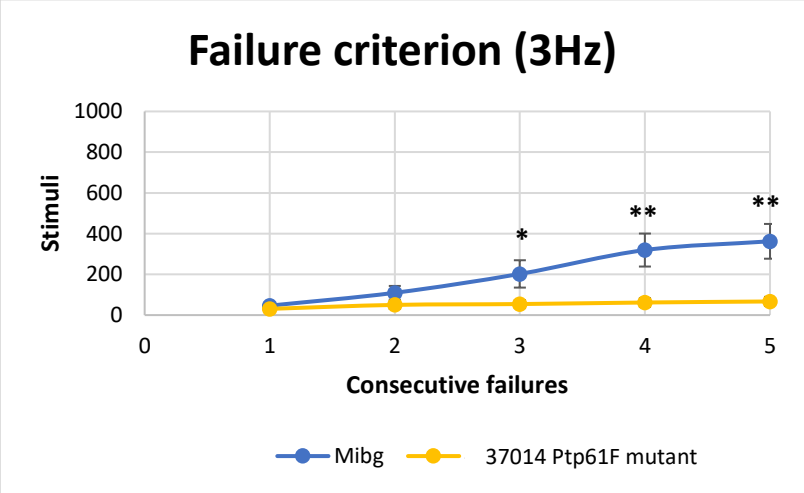
As increasing the frequency of stimulus application is accelerating habituation (Engel & Wu, 1994b), habituation rhythm was studied at 3Hz- an intermediate frequency that could probably reveal differences between the two genotypes if existed.

2.2.1. ptp61F knockdown leads to premature LLR habituation in the GFS

A.



B.



p-values	Consecutive failures				
Genotype	**1**	**2**	**3**	**4**	**5**
#37014 vs. Mibg	0.1711	0.0909	0.0442*	0.0069**	0.0042**

Figure 12. Rhythm of habituation of GFS at 3Hz repetitive electric stimulation in #37014 Ptp61F mutant and its genetic background.

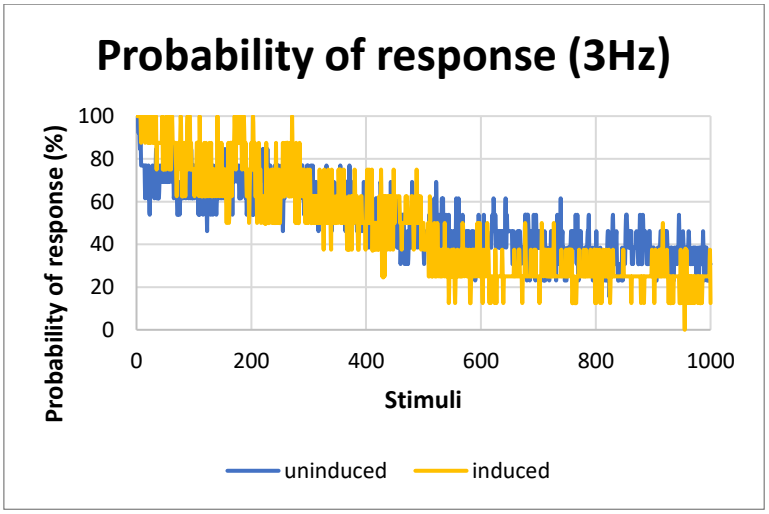
(A) Assessment of probability of response.
(B) Consecutive failures. The p-values are shown in the table underneath.

Data are presented as mean±SEM.

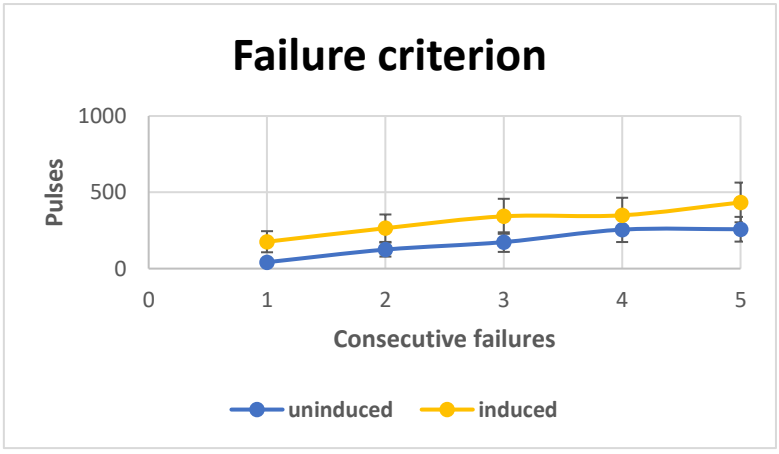
The Ptp61F mutant seems to prematurely habituate in comparison to the control line, which agrees to what was presented in behavior also (Christodoulou, 2018).

In an effort to investigate if the results can be replicated with RNA interference, *elav;Gal80^{ts}* female flies were crossed with males from the #56510 RNAi strain and raised at 18°C, in order for the RNAi to not be expressed during development. A group of 1-5 days old flies of the F1 generation were transferred to 30°C 48 hours before the performance of the experiment in order to induce the RNAi's expression. Flies of the same young age that were constantly kept at the standard 18°C conditions were used as negative controls. All flies were subjected to the electrophysiological habituation paradigm of the giant fiber system as described at the Materials and Methods' section and obtained the following results:

A.



B.



p-values	Consecutive faillures				
Genotype	**1**	**2**	**3**	**4**	**5**
#56510 ind. vs. unind.	0.1162	0.2187	0.2520	0.5409	0.3020

Figure 13. Rhythm of habituation of GFS at 3Hz repetitive electric stimulation in *elaV;Ga80^{ts}>#56510ptp61F RNAi induced and uninduced flies.*

(A) Assessment of probability of response.
(B) Consecutive failures. The p-values are shown in the table underneath.

Data are presented as mean±SEM.

The phenotype of the mutant seems to not be replicated with RNA interference.

3. Protein-tyrosine phosphatase corkscrew (CSW)

MBs were shown to not be implicated in protection from premature habituation for the csw protein (Christodoulou, 2018). In order to confirm this result with RNA interference, *739,Gal80^{ts}* female flies were crossed with #33619 (*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00012}attP2)* and #60448 (*y[1]v[1];P{y[+t7.7]v[+t1.8]=TRiP.HMJ22804}attP40*) RNAi strains for *csw*. The flies were raised at 18°C so that the RNAi would not be expressed. 48 hours before the experiment, a group of the F1 generation's 1-3 days old flies were transferred to 30°C in order to induce the RNAi's expression and reduce the protein concentration. These composed the induced experimental group, while flies that were constantly kept at 18°C were used as the uninduced control group. All flies were subjected to the electroshock habituation assay.

3.1 Electroshock habituation assay

3.1.1 α/β MB lobes are not implicated in protection from premature habituation in respect to csw

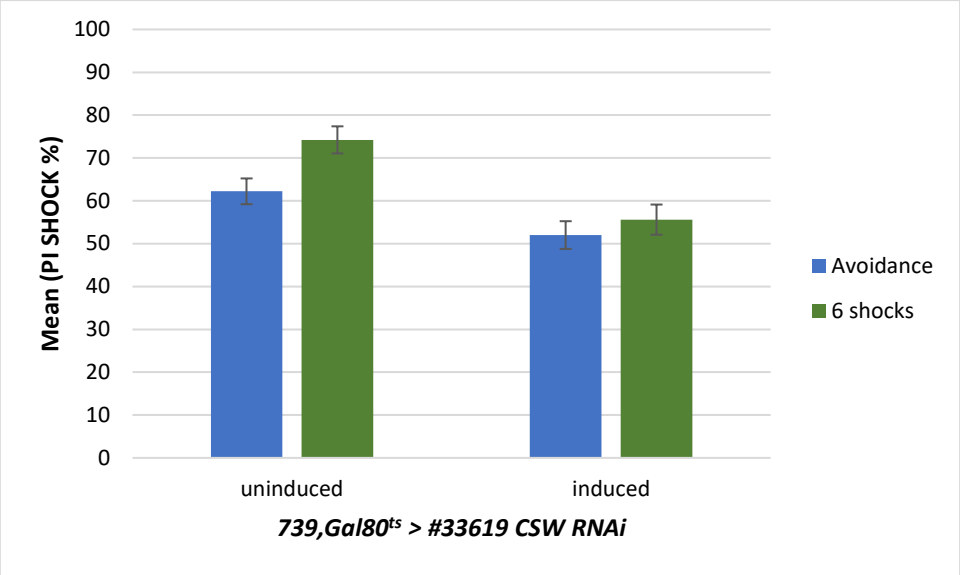


Figure 14. Electroshock habituation for *739,Gal80^{ts} > #33619 CSW RNAi.* None of the experimental induced group nor the control uninduced group of flies prematurely habituated after training with 6 shocks (p-values 0.4345 and 0.0106 respectively).

Data are presented as mean±SEM.

The induced group did not prematurely habituate (p-value=0.4345) after training with 6 shocks, as did not the control group (p-value=0.0106).

In order to replicate this result, a second RNAi was also used:

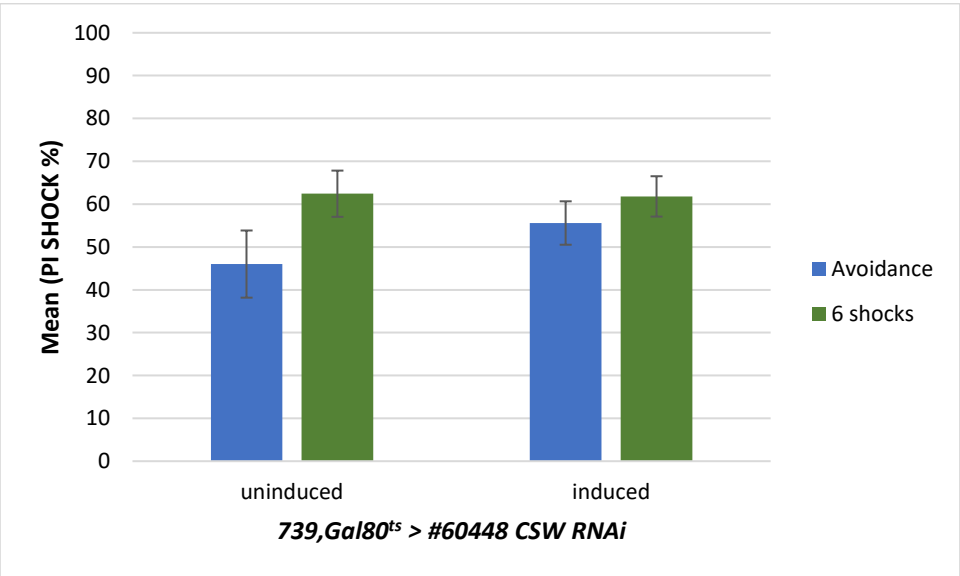


Figure 15. Electroshock habituation for *739,Gal80^{ts} > #60448 CSW RNAi.* None of the experimental induced group nor the control uninduced group of flies prematurely habituated after training with 6 shocks (p-values 0.4357 and 0.0634 respectively).

Data are presented as mean±SEM.

The induced group of flies did not prematurely habituate (p-value=0.4357) after training with 6 shocks, like the control (p-value=0.0634).

Both these results partially confirm previous results from Christodoulou (2018) with *leo,Gal80^{ts}* that the MBs are not implicated in protection from premature habituation referring to the *csw* gene.

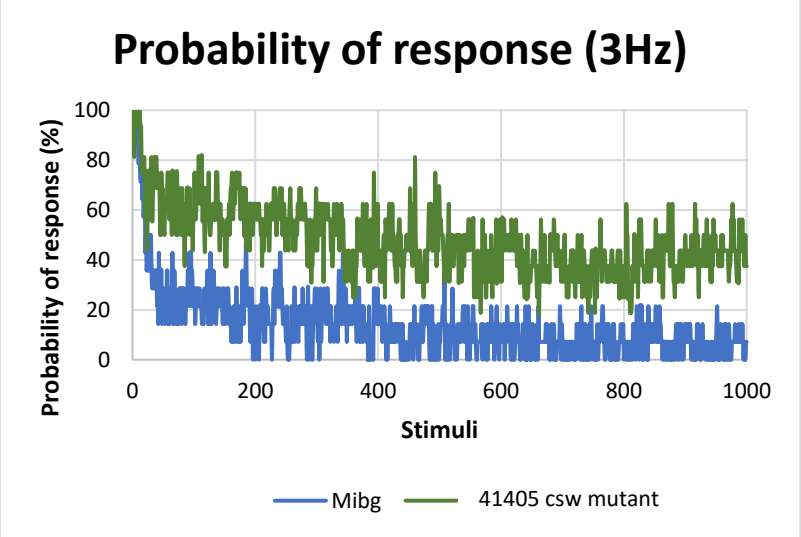
3.2 Electrophysiology

3.2.1 csw knockdown leads to delayed LLR habituation of the GFS

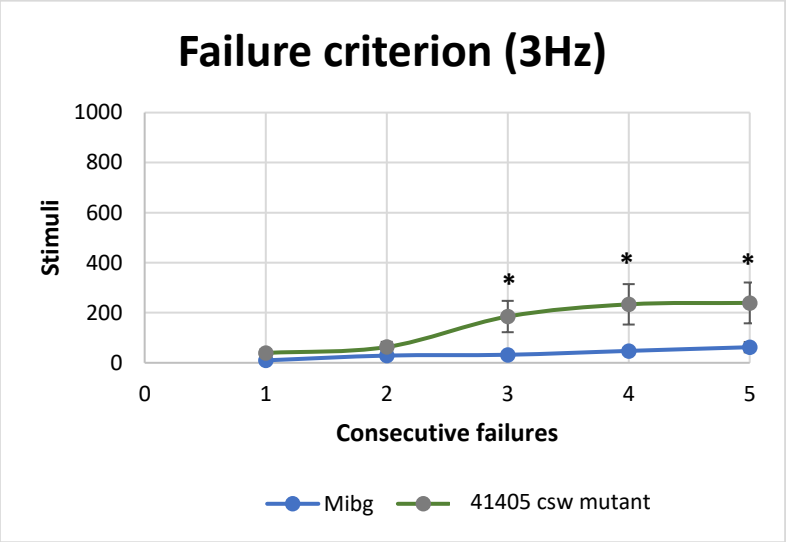
In order to investigate if the premature habituation phenotype in the behavioral paradigm can also be spotted in other neuronal systems and circuits as well, a series of electrophysiological recordings of the giant fiber system was performed in 2-6 days old flies of the mutant #41405 (*y1 w*; Mi{MIC}cswMI05169*), of *yellow,white* background. *Yellow,white* flies (Mibg) were used as genetic controls. All flies were raised at 25°C.

In order to maintain consistence of data in this study, we performed the experiments with application of 1000 stimuli at a frequency of 3Hz.

A.



B.



p-values	Consecutive failures				
Genotype	**1**	**2**	**3**	**4**	**5**
#41405 vs. Mibg	0.0662	0.0689	0.0258*	0.0364*	0.0353*

Figure 16. Rhythm of habituation of GFS at 3Hz repetitive electric stimulation in #41405 csw mutants and their genetic background.

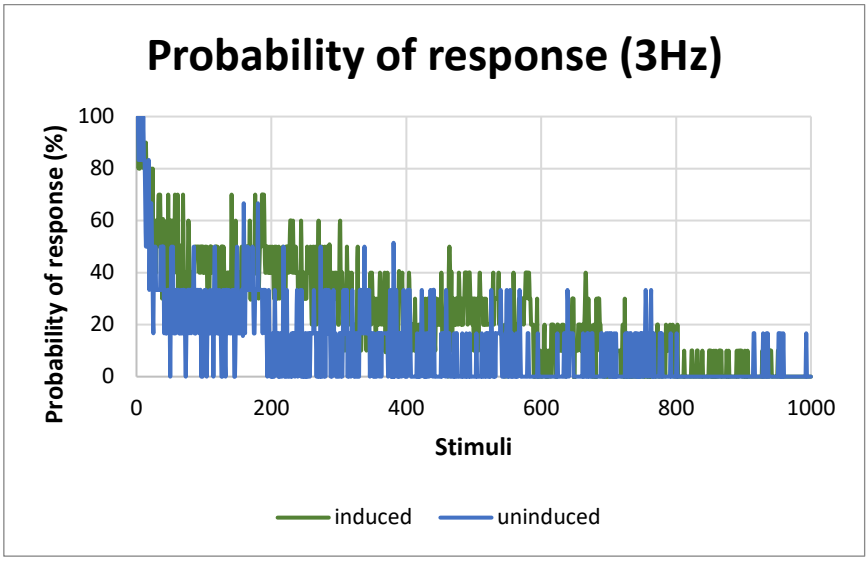
(A) Assessment of probability of response.
(B) Consecutive failures. The p-values are shown in the table underneath.

Data are presented as mean±SEM.

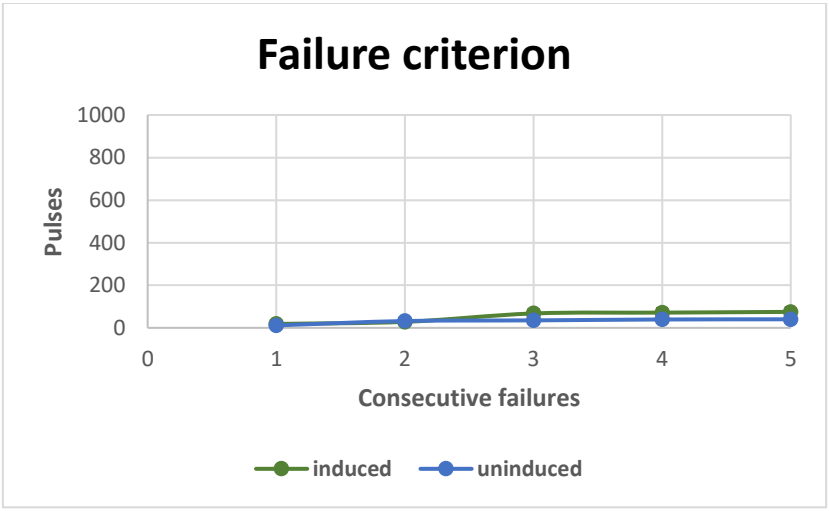
The csw mutant does not prematurely habituate, rather habituates later in comparison to the *yellow,white* control line. This result is opposite to the behavioral phenotype (Christodoulou, 2018).

In order to confirm the results with RNA interference, *elav;Gal80^{ts}* female flies were crossed with males from the #33619 RNAi strain and raised at 18°C, in order for the RNAi to not be expressed during development. A group of 1-5 days old flies of the F1 generation were transferred to 30°C 48 hours before the performance of the experiment in order to induce the RNAi's expression. Flies of the same young age that were constantly kept at the standard 18°C conditions were used as negative controls. All flies were subjected to the electrophysiological habituation paradigm of the giant fiber system as described at the Materials and Methods' section and obtained the following results:

A.



B.



p-values	Consecutive failures				
Genotype	**1**	**2**	**3**	**4**	**5**
#33619 ind. vs. unind.	0.1893	0.8394	0.2506	0.2687	0.2334

Figure 17. Rhythm of habituation of GFS at 3Hz repetitive electric stimulation in *elaV;Ga80^{ts}>#33619csw RNAi induced and uninduced flies*.

(A) Assessment of probability of response.
(B) Consecutive failures. The p-values are shown in the table underneath

Data are presented as mean±SEM.

The induced group shows a smaller, yet similar tendency to habituate later than its uninduced control, which resembles the result in the mutant.

Discussion

Habituation to a repetitive stimulus is a fundamental process that helps the organism focus their attention and save energy to respond to those stimuli that have high importance for their survival and reproductive success (Giles and Rankin, 2009). Premature habituation is not considered evolutionarily advantageous as it does not allow the organism to efficiently discriminate between novel and pre-experienced stimuli they receive from their environment and attach the appropriate importance to them, which may lead to exhaustion, danger and potentially death.

In *D. melanogaster*, 14-3-3ε, ptp61F and csw proteins have been associated with protection from premature habituation to electric footshock (Acevedo *et al.*, 2007; Acevedo, 2004; Christodoulou 2018), through experiments performed with mutants and RNA interference.

1. 14-3-3ε reduction through strong RNAi does not cause premature habituation

14-3-3ε elimination leads to premature habituation in the electroshock behavioral paradigm in mutants (Acevedo, 2004). Pan-neuronal diminution of 14-3-3ε throughout development with RNA interference does not lead to either normal or premature habituation. Taking into account the low avoidance PIs in both experimental groups, someone could assume that total absence of the protein due to strong expression of the RNAi may lead to a strong effect so that the flies would prematurely habituate to electroshock within the 90-sec-period of the avoidance phase of the protocol or would increase the flies' indolence and susceptibility to infections (Acevedo, 2004) and therefore, make them struggle moving between the arms of the maze. Either way, it is necessary to optimize the avoidance phase of the protocol for premature-habituation-related genes, starting by reducing its duration. Pan-neuronal reduction of 14-3-3ε only during adulthood, leads to normal habituation, but not premature habituation to electroshock. Considering 14-3-3ε's high abundance in the CNS, 2 days of induction of the RNAi's expression may not be sufficient to eliminate enough protein for the phenotype to emerge, which was also supported by Western blot. Regardless that, after the knockdown of a protein, the system tries to balance itself and needs time for a strong effect to appear. More days of induction though, would interfere with the reliability of the results, as the particular assay was designed for testing 2-5 days old flies (Acevedo *et al.*, 2007).

Although the neuronal circuit that underlies habituation to footshock is still unknown, Acevedo *et al.* (2007) showed that functional MBs are essential for the protection from premature habituation to either olfaction or footshock, where intact and functional α/β lobes are necessary in particular. Reduction of 14-3-3ε's concentration everywhere but in these structures through RNA interference did not lead to a premature habituation phenotype, probably because of insufficiency of 48-hours-induction to eliminate enough protein, although the whole experiment cannot be trusted due to incapacity of the control group to normally habituate after training with 15 shocks. A Western blot would enlighten this hypothesis.

In order to investigate if any kind of defective behavior due to reduction of 14-3-3ε would be spotted, a learning assay was performed and pointed out that flies with reduced 14-3-3ε are weak learners. Learning performance though, seems to be concentration-related either with loss of 14-3-3ε, or why not with increase of leo, since the control group is also weak in learning itself compared to normal controls.

Overall, most parts of the study have and continue undergoing troubleshooting. All experiments should be repeated in three different biological replicates and Western blot or more sensitive analysis should be performed to explore the possible role of leo.

2. Leo may compensate for the loss of 14-3-3ε

Increase in the concentration of 14-3-3ε has been proposed to occasionally compensate for the loss of the other 14-3-3 Drosophila protein, the leonardo isoform (Skoulakis and Davis, 1998; Acevedo, 2004), but could this also happen vice versa? Conventional Western blotting did not reveal differential concentration of leo in flies with low and normal 14-3-3ε expression, as this protein is also quite abundant in the CNS and there is need for more sensitive methods like RNA sequencing or analysis of the temporal, quantitative and spatial distribution of the transcripts in response to the loss of ε isoform either in developmental cases like mutants and crosses with *elavGal4* driver or after the temporal expression of the RNAi against the *14-3-3ε* gene.

3. 14-3-3ε may be a stress-inducible protein

Research has shown that the 14-3-3 protein family is implicated in stress (Roberts, Salinas and Collinge, 2002; Sluchanko and Gusev, 2017; Pennington et al., 2018) with overexpression of 14-3-3s being stress-relieving and that 14-3-3 proteins are heat-shock-related proteins (Hsp) that protect cells against physiological stress (Yano et al., 2006). In our study, the 48-hour induction process at 30°C can be a major stressor for the flies that corresponds to chronic stress conditions. Elimination of 14-3-3ε may lead to induction of stress response to heat-shock or may be prevented as a compensating mechanism of the fly to deal with this stressful condition. There is evidence that 14-3-3ζ (leo) isoform is a heat-inducible molecular chaperone. Experiments performed in Drosophila cells showed that leo, but not 14-3-3ε, gets up-regulated under heat-shock conditions (Yano et al., 2006). Other research showed that 14-3-3ε has a chaperone-like activity too, regulating different cellular pathways in response to an acute stressor or chronic-stress (Wang et al., 2003; Zhao et al., 2021). Complete ablation of the human YWHAE gene that codes for 14-3-3ε was shown to result in multiple defects in neuropsychiatric behaviors in mice (Wachi, Cornell and Toyo-oka, 2017).

Could these effects be responsible for the absence of a defective habituation phenotype when inducing RNA interference with heat-shock? It would be interesting if the impaired and premature habituation phenotypes of 14-3-3ε mutants or low-14-3-3ε-expressing flies of previous works were attributed to the increase of leo -if true- and not the loss of 14-3-3ε itself.

4. α/β MB lobes are not implicated in premature habituation after reduction of ptp61F or csw concentrations

Despite what was previously published (Acevedo *et al.*, 2007), R.Christodoulou (2018) showed that the MBs are not implicated in protection from premature habituation regarding ptp61F and csw proteins. In order to test that ourselves, we diminished their concentration temporally only in the α/β lobes, whose role was emphasized in the paper, and partially confirmed the previous results. Experimenting with flies expressing the RNAi everywhere outside of the MBs did not give us trustworthy results, as the control flies prematurely habituated. This could happen due to possible leakiness of the driver used, whose pattern should be confirmed crossing it with Gal4-driven UAS-GFP flies. Unfortunately, there is no clear idea of where protection from premature habituation lies in respect to either ptp61F or csw.

5. Abrogation of ptp61F and csw alters LLR habituation in the GFS

Experimenting with electrophysiology, D.Kadas (2011) showed that loss of 14-3-3ε in mutant strains leads to premature habituation upon repetitive stimulation of the giant fiber system. In our study, we electrophysiologically characterized habituation in the GFS of ptp61F and csw mutant flies. The rhythm of habituation of ptp61F mutants and their respective genetic control, yellowhite (genetic background), was initially tried to be assessed after exposure to 1000 stimuli with a frequency of 5Hz, but both genotypes habituated so quickly that it was impossible to detect any differences if existed. The rhythm of habituation at 2Hz was then assessed, but neither genotype habituated at all after the appliance of 1000 stimuli (graph not shown). Taking into account the fact that increasing the frequency of stimulus application accelerates habituation (Engel and Wu, 1994), habituation was chosen to be studied at the intermediate frequency of 3Hz.

The ptp61F mutant habituated prematurely in comparison to its genetic control, as in behavior as well. RNA interference did not confirm this result. Such a particularity could lie in the fact that mutations and RNAis do not always work in parallel. The protein of interest possibly shows multidomain functionalities that are presented in the mutant but not in the RNAi crossed strain, since the mutation may have caused loss or gain of function or different functionality, but not loss of protein. Moreover,

the ptp61F RNAi used may not be strong enough to eliminate the required amount of protein for a strong impaired phenotype to appear.

On the other hand, the csw mutant habituated later than its genetic control, which is an opposite-looking phenotype than the one in behavior. This result was confirmed with RNA interference. More particularly, the effect is not confirmed when reducing the protein with RNAi, but the tendency to a slower habituation rhythm remains.

Differences in the habituation phenotypes of the flies originated from the same gene manipulations, indicate that the neuronal subsets implicated in the response to the two assays of this study are independent of each other. An immunohistochemistry in order to detect ptp61F and csw concentrations in the whole neuronal system of the fly and the textures of all structures in their absence would clarify the image of the results we received for each case.

The fact that the *elav;Gal80^{ts}* crosses did not reflect the mutant results both in electrophysiology and behavior could also indicate that this regulated expression system is not the most efficient one. Different ways of RNAi's induction could prove to be more potent, like the Gal4:GeneSwitch system, carrying the *H.sapiens* progesterone receptor. The GAL4 domain can bind to a UAS regulatory sequence, which can only occur when anti-progestin mifepristone (RU486) is present (Flybase; Osterwalder et al., 2001; Rana, Rera and Walker, 2013). This study was exploratory and suggested what not to do in this type of studies. All experiments should be repeated in three different biological replicates.

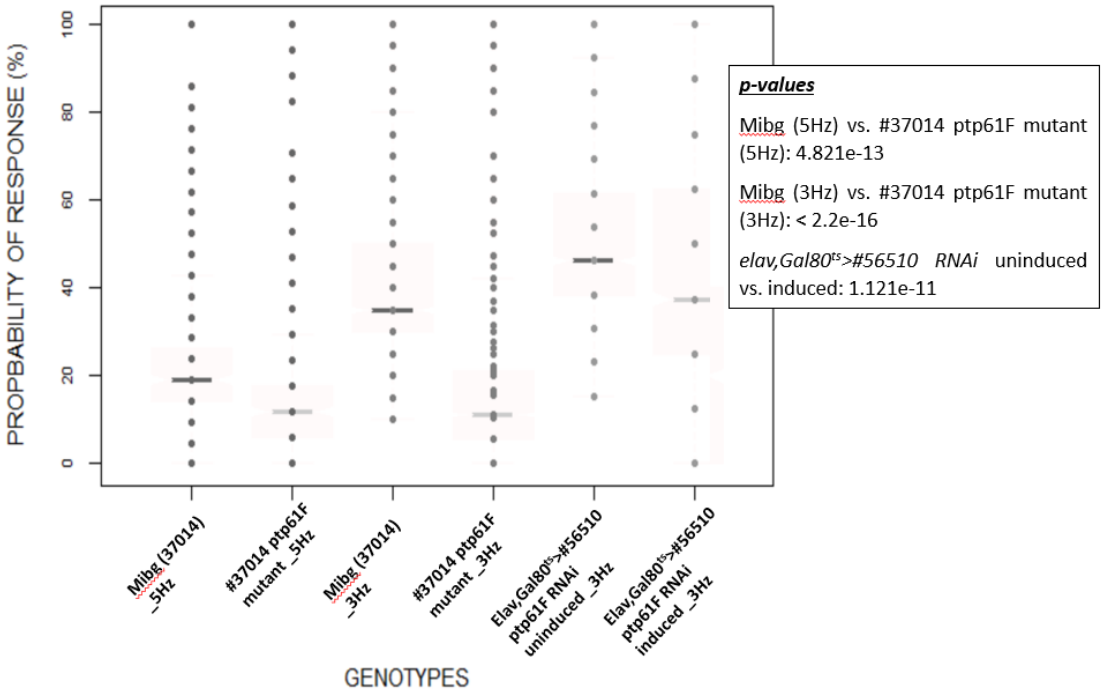
Acknowledgements

I am grateful to Dr. Efthimios Skoulakis for supervising, mentoring, guiding me and scientifically and personally supporting me throughout the project. I am also grateful to the other two members of my supervising committee, Prof. Christos Consoulas and Prof. Antonios Stamatakis, for their mentoring and support during my thesis project. I am thankful to Alexandros Gaitanidis for teaching me electrophysiology and also supporting and guiding me the whole time, as well as Kyriaki Foka for the exact same reasons in the rest of the study. Special thanks to Ekin Daplan for her moral support and help with R statistics and hypothesis testing, as well as to Anastasios Galanis, Ioannis Maragkos and to all Skoulakis' lab members. Last but not least, I am grateful to Prof. Spiros Efthimiopoulos for his high urge to motivate and support us throughout our master studies.

Supplementary

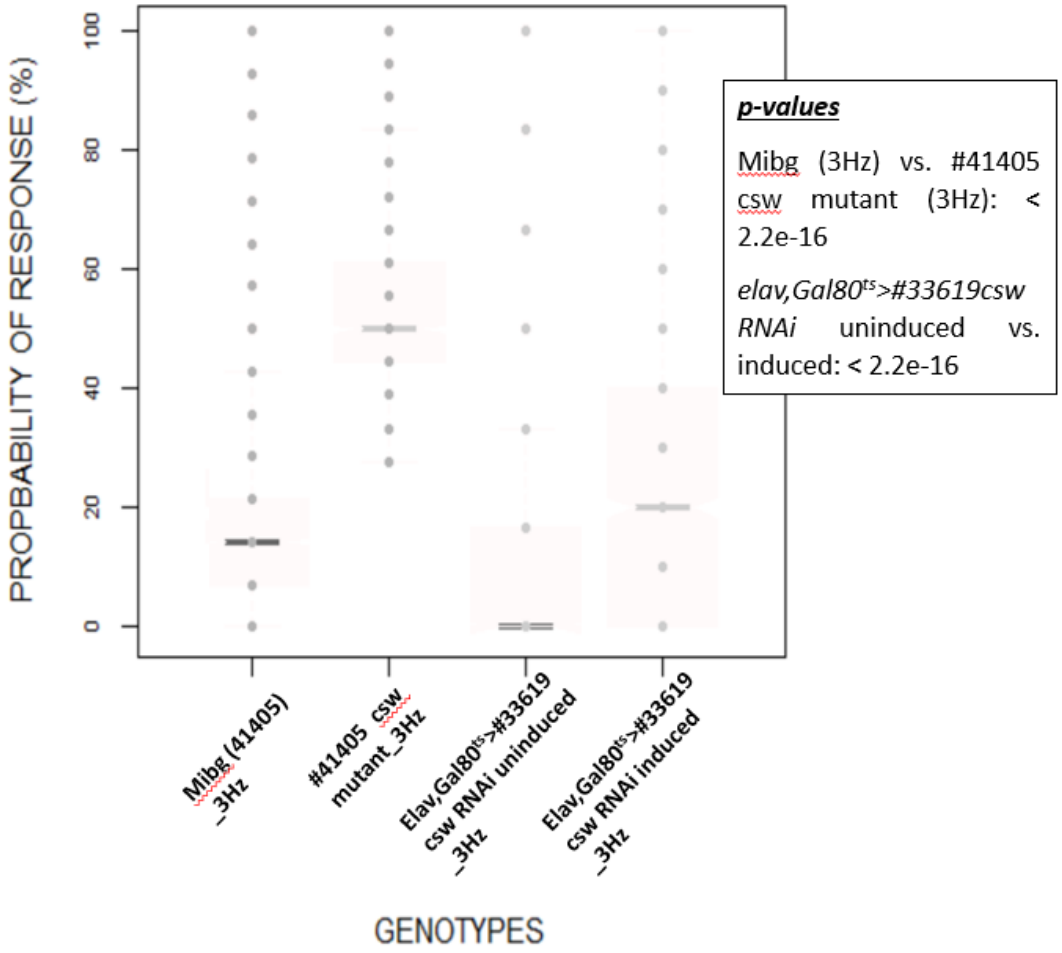
Hypothesis testing for electrophysiological results after ptp61F and csw abrogation

In an effort to evaluate the difference of the probability of response curves of each genotype compared to its genetic control, hypothesis testing was performed using R statistics. The groups of interest were compared through a Wilcoxon non-parametric test.



Box plot 1. Wilcoxon non-parametric hypothesis testing box plot after ptp61F abrogation.

Although each point in the graph corresponds to the probability of response of the genotype to a different stimulus, the mean values from the Wilcoxon test represent the tendency of the data and gives an indication of how early or late habituation occurs. The lower the mean, the lower the probabilities of response in total as well as their sum, the faster the habituation occurs. As shown in Figure 15, hypothesis testing confirmed that ptp61F abrogation leads to premature LLR habituation of the GFS both in mutants and crossed flies and both at 5Hz and 3Hz of repetitive stimulation.



Box plot 2. Wilcoxon non-parametric hypothesis testing box plot after csw abrogation.

Hypothesis testing performed in R statistics using the non-parametric Wilcoxon test regarding corkscrew, revealed that its abrogation through mutations or RNA interference leads to delayed LLR habituation of the GFS compared to its genetic control after 3Hz repeated stimulation.

References

Acevedo, S. F. (2004) 'The dynamics, interactions and phenotypes associated with the three members of the 14-3-3 family in *Drosophila* melanogaster' (Doctoral dissertation), Texas A&M University, USA.

Acevedo, S. F. *et al.* (2007) 'Protection from premature habituation requires functional mushroom bodies in Drosophila.', *Learning & memory (Cold Spring Harbor, N.Y.)*. doi: 10.1101/lm.566007.

Benton, R., Palacios, I. M. and Johnston, D. S. (2002) 'Drosophila 14-3-3/PAR-5 is an essential mediator of PAR-1 function in axis formation', *Developmental Cell*. doi: 10.1016/S1534-5807(02)00320-9.

Chang, H. C. and Rubin, G. M. (1997) '14-3-3ε positively regulates Ras-mediated signaling in Drosophila', *Genes and Development*. doi: 10.1101/gad.11.9.1132.

Chen, F. *et al.* (2007) 'Multiple Protein Phosphatases Are Required for Mitosis in Drosophila', *Current Biology*. doi: 10.1016/j.cub.2007.01.068.

Clemens, J. C. *et al.* (1996) 'A Drosophila protein-tyrosine phosphatase associates with an adapter protein required for axonal guidance', *Journal of Biological Chemistry*. doi: 10.1074/jbc.271.29.17002.

Coppola, G., Pierelli, F. and Schoenen, J. (2009) 'Habituation and migraine', *Neurobiology of Learning and Memory*. doi: 10.1016/j.nlm.2008.07.006.

Engel, J. E. *et al.* (2000) 'A cGMP-dependent protein kinase gene, foraging, modifies habituation-like response decrement of the giant fiber escape circuit in Drosophila', *Learning and Memory*. doi: 10.1101/lm.31600.

Engel, J. E. and Wu, C. F. (1992) 'Interactions of membrane excitability mutations affecting potassium and sodium currents in the flight and giant fiber escape systems of Drosophila', *Journal of Comparative Physiology A*, 171(1), pp. 93–104. doi: 10.1007/BF00195964.

Engel, J. E. and Wu, C. F. (1994) 'Altered mechanoreceptor response in Drosophila bang-sensitive mutants', *Journal of Comparative Physiology A*. doi: 10.1007/BF00192986.

Engel, J. E. and Wu, C. F. (1996) 'Altered habituation of an identified escape circuit in Drosophila memory mutants', *Journal of Neuroscience*. doi: 10.1523/jneurosci.16-10-03486.1996.

Engel, J. E. and Wu, C. F. (1998) 'Genetic dissection of functional contributions of specific potassium channel subunits in habituation of an escape circuit in Drosophila', *Journal of Neuroscience*. doi: 10.1523/jneurosci.18-06-02254.1998.

Engel, J. E. and Wu, C. F. (2009) 'Neurogenetic approaches to habituation and dishabituation in Drosophila', *Neurobiology of Learning and Memory*. doi: 10.1016/j.nlm.2008.08.003.

Gilbert, C. and Strausfeld, N. J. (1991) 'The functional organization of male-specific visual neurons in flies', *Journal of Comparative Physiology A*. doi: 10.1007/BF00197653.

Giles, A. C. and Rankin, C. H. (2009) 'Neurobiology of Learning and Memory Behavioral and genetic characterization of habituation using Caenorhabditis elegans', *Neurobiology of Learning and Memory*. Elsevier Inc., 92(2), pp. 139–146. doi: 10.1016/j.nlm.2008.08.004.

Gillberg, C. (2003) 'Deficits in attention, motor control, and perception: A brief review', *Archives of Disease in Childhood*. doi: 10.1136/adc.88.10.904.

Gouzi, J. Y. *et al.* (2011) 'The receptor tyrosine kinase alk controls neurofibromin functions in drosophila growth and learning', *PLoS Genetics*. doi: 10.1371/journal.pgen.1002281.

Groves, P. M. and Thompson, R. F. (1970) 'Habituation: A dual-process theory', *Psychological Review*. doi: 10.1037/h0029810.

Hammer, T. B. *et al.* (2011) 'Stability of prepulse inhibition and habituation of the startle reflex in schizophrenia: A 6-year follow-up study of initially antipsychotic-naive, first-episode schizophrenia patients', *International Journal of Neuropsychopharmacology*. doi: 10.1017/S1461145711000034.

Huang, Z. *et al.* (2007) 'Crk-associated substrate (Cas) signaling protein functions with integrins to specify axon guidance during development', *Development*. doi: 10.1242/dev.004242.

Kadas, D. *et al.* (2012) 'Constitutive activation of Ca 2+/calmodulin-dependent protein kinase II during development impairs central cholinergic transmission in a circuit underlying escape behavior in Drosophila', *Journal of Neuroscience*, 32(1), pp. 170–182. doi: 10.1523/JNEUROSCI.6583-10.2012.

Kalita, J., Bhoi, S. K. and Misra, U. K. (2014) 'Is lack of habituation of evoked potential a biological marker of migraine?', *Clinical Journal of Pain*. doi: 10.1097/AJP.0000000000000034.

Keramidis, I. *et al.* (2020) 'Functional Interactions of Tau Phosphorylation Sites That Mediate Toxicity and Deficient Learning in Drosophila melanogaster', *Frontiers in Molecular Neuroscience*. doi: 10.3389/fnmol.2020.569520.

Koenig, J. H. and Ikeda, K. (2005) 'Relationship of the reserve vesicle population to synaptic depression in the tergotrochanteral and dorsal longitudinal muscles of Drosophila', *Journal of Neurophysiology*. doi: 10.1152/jn.00323.2005.

Lee, S. J., Feldman, R. and O'Farrell, P. H. (2008) 'An RNA interference screen identifies a novel regulator of target of rapamycin that mediates hypoxia suppression of translation in Drosophila S2 cells', *Molecular Biology of the Cell*. doi: 10.1091/mbc.E08-03-0265.

Lieberman, M. D. (2000) 'Intuition: A social cognitive neuroscience approach.', *Psychological Bulletin*. US: American Psychological Association, pp. 109–137. doi: 10.1037/0033-2909.126.1.109.

Massa, J. and O'Desky, I. H. (2012) 'Impaired Visual Habituation in Adults With ADHD', *Journal of Attention Disorders*. doi: 10.1177/1087054711423621.

McDiarmid, T. A., Bernardos, A. C. and Rankin, C. H. (2017) *Habituation is altered in neuropsychiatric disorders—A comprehensive review with recommendations for experimental design and analysis*, *Neuroscience and Biobehavioral Reviews*. Elsevier Ltd. doi: 10.1016/j.neubiorev.2017.05.028.

McDiarmid, T. A., Yu, A. J. and Rankin, C. H. (2019) 'Habituation Is More Than Learning to Ignore: Multiple Mechanisms Serve to Facilitate Shifts in Behavioral Strategy', *BioEssays*, 41(9), pp. 1–10. doi: 10.1002/bies.201900077.

Meincke, U. *et al.* (2004) 'Prepulse inhibition of the acoustically evoked startle reflex in patients with an acute schizophrenic psychosis - A longitudinal study', *European Archives of Psychiatry and Clinical Neuroscience*. doi: 10.1007/s00406-004-0523-0.

Morello, F. *et al.* (2020) 'ADHD-like behaviors caused by inactivation of a transcription factor controlling the balance of inhibitory and excitatory neuron development in the mouse anterior brainstem', *Translational Psychiatry*. doi: 10.1038/s41398-020-01033-8.

Oishi, K. *et al.* (2009) 'Phosphatase-defective LEOPARD syndrome mutations in PTPN11 gene have gain-of-function effects during Drosophila development', *Human Molecular Genetics*. doi: 10.1093/hmg/ddn336.

Osterwalder, T. *et al.* (2001) 'A conditional tissue-specific transgene expression system using inducible GAL4', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.221303298.

Pennington, K. *et al.* (2018) 'The dynamic and stress-adaptive signaling hub of 14-3-3: emerging mechanisms of regulation and context-dependent protein–protein interactions', *Oncogene*. doi: 10.1038/s41388-018-0348-3.

Perkins, L. A. *et al.* (1996) 'The nonreceptor protein tyrosine phosphatase Corkscrew functions in multiple receptor tyrosine kinase pathways in Drosophila', *Developmental Biology*. doi: 10.1006/dbio.1996.0285.

Pézier, A. P. *et al.* (2016) 'Shaking B mediates synaptic coupling between auditory sensory neurons and the giant fiber of Drosophila melanogaster', *PLoS ONE*. doi: 10.1371/journal.pone.0152211.

Philip, N., Acevedo, S. F. and Skoulakis, E. M. C. (2001) 'Conditional rescue of olfactory learning and memory defects in mutants of the 14-3-3ζ gene leonardo', *Journal of Neuroscience*, 21(21), pp. 8417–8425. doi: 10.1523/jneurosci.21-21-08417.2001.

Qiao, H. *et al.* (2014) '14-3-3 Proteins Are Required for Hippocampal Long-Term Potentiation and Associative Learning and Memory', *Journal of Neuroscience*, 34(14), pp. 4801–4808. doi: 10.1523/JNEUROSCI.4393-13.2014.

Rana, A., Rera, M. and Walker, D. W. (2013) 'Parkin overexpression during aging reduces proteotoxicity, alters mitochondrial dynamics, and extends lifespan', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.1216197110.

Rankin, C. H., Gannon, T. and Wicks, S. R. (2000) 'Developmental analysis of habituation in the nematode C. elegans', *Developmental Psychobiology*. doi: 10.1002/(SICI)1098-2302(200005)36:4<261::AID-DEV1>3.0.CO;2-7.

Reflex, T. H. E. O. *et al.* (1962) 'The i nvestigation of higher nervous functions demands detailed analysis of the orienting reflex as a part of the complex exploratory behavior', (September).

Roberts, M. R., Salinas, J. and Collinge, D. B. (2002) '14-3-3 Proteins and the response to abiotic and biotic stress', *Plant Molecular Biology*. doi: 10.1023/A:1021261614491.

Rose, J. K. and Rankin, C. H. (2001) 'Analyses of habituation in Caenorhabditis elegans', *Learning and Memory*. doi: 10.1101/lm.37801.

Roseboom, P. H. *et al.* (1994) 'Cloning and Characterization of the ∈ and ζ Isoforms of the 14-3-3 Proteins', *DNA and Cell Biology*, 13(6), pp. 629–640. doi: 10.1089/dna.1994.13.629.

Semelidou, O., Acevedo, S. F. and Skoulakis, E. M. C. (2018) 'Temporally specific engagement of distinct neuronal circuits regulating olfactory habituation in Drosophila', *eLife*. doi: 10.7554/eLife.39569.

Skoulakis, E. M. C. and Davis, R. L. (1998) '14-3-3 Proteins in neuronal development and function', *Molecular Neurobiology*. doi: 10.1007/BF02741386.

Slaats-Willemse, D. *et al.* (2003) 'Deficient response inhibition as a cognitive endophenotype of ADHD', *Journal of the American Academy of Child and Adolescent Psychiatry*. doi: 10.1097/00004583-200310000-00016.

Sluchanko, N. N. and Gusev, N. B. (2017) 'Moonlighting chaperone-like activity of the universal regulatory 14-3-3 proteins', *FEBS Journal*. doi: 10.1111/febs.13986.

Smith, R. K. *et al.* (2002) 'MASK, a large ankyrin repeat and KH domain-containing protein involved in Drosophila receptor tyrosine kinase signaling', *Development*.

Su, T. T. *et al.* (2001) 'Cell cycle roles for two 14-3-3 proteins during Drosophila development', *Journal of Cell Science*.

Tchankouo-Nguetcheu, S. *et al.* (2014) 'Negative regulation of MAP kinase signaling in Drosophila by Ptp61F/PTP1B.', *Molecular genetics and genomics : MGG*. doi: 10.1007/s00438-014-0852-2.

Thompson, R. F. and Spencer, W. A. (1966) 'HABITUAT1ON ':, 73(1), pp. 16–43.

Tsigkari, K. K., Acevedo, S. F. and Skoulakis, E. M. C. (2012) '14-3-3ε is required for germ cell migration in drosophila', *PLoS ONE*. doi: 10.1371/journal.pone.0036702.

Wachi, T., Cornell, B. and Toyo-oka, K. (2017) 'Complete ablation of the 14-3-3epsilon protein results in multiple defects in neuropsychiatric behaviors', *Behavioural Brain Research*. doi: 10.1016/j.bbr.2016.11.016.

Wang, X. *et al.* (2003) 'Regulation of Molecular Chaperone Gene Transcription Involves the Serine Phosphorylation, 14-3-3ε Binding, and Cytoplasmic Sequestration of Heat Shock Factor 1', *Molecular and Cellular Biology*. doi: 10.1128/mcb.23.17.6013-

6026.2003.

Williams, L. E. *et al.* (2013) 'Reduced habituation in patients with schizophrenia', *Schizophrenia Research*. doi: 10.1016/j.schres.2013.10.017.

Willoughby, L. F. *et al.* (2017) 'Differential regulation of protein tyrosine kinase signalling by Dock and the PTP61F variants', *FEBS Journal*. doi: 10.1111/febs.14118.

Wu, C. L. *et al.* (2011) 'Dock/Nck facilitates PTP61F/PTP1B regulation of insulin signalling', *Biochemical Journal*. doi: 10.1042/BJ20110799.

Yano, M. *et al.* (2006) 'A novel function of 14-3-3 protein: 14-3-3ζ is a heat-shock-related molecular chaperone that dissolves thermal-aggregated proteins', *Molecular Biology of the Cell*. doi: 10.1091/mbc.E06-03-0229.

Zhao, Y. *et al.* (2021) 'Identification of 14-3-3 epsilon as a regulator of the neural apoptotic pathway for chronic-stress-induced depression', *iScience*. doi: 10.1016/j.isci.2021.102043.

RESEARCH PROPOSAL

Premature habituation and ADHD

1.Summary

Habituation is a form of non-associative learning and a core automatic mechanism of decreasing responsiveness to a repetitive stimulus. Premature habituation does not permit effective discrimination between novel and pre-experienced stimuli as the information content does not stay long enough for the organism to process it correctly. Acevedo *et al.* (2007) showed that the mushroom bodies, and particularly the α/β lobes are essential structures for the protection from premature habituation to footshock. Acevedo (2004) and Christodoulou (2018) pointed out the role of 14-3-3ε, ptp61F and corkscrew genes in protection from premature habituation. Kadas (2011) showed that loss of 14-3-3ε leads the long latency response of the giant fiber system to prematurely habituate to repetitive stimulation as well. We showed that ptp61F and corkscrew mutations lead to differential habituation in the GFS, while we could not conclude for the role of all 3 genes in behavior. Our future studies will focus on identifying and characterizing the pathway underlying premature habituation to footshock. We will employ several genetic approaches such as the GAL4/UAS system and overexpression of the temperature-sensitive allele of shibire (shi^{ts1}), which blocks neurotransmission in a specific set of neurons, along with imaging techniques such as calcium imaging. We will also try to search for a pharmacological rescue (Ritalin etc.) of the impaired phenotypes and possible implications in specific disorders like ADHD. Finally, in an attempt to uncover the physiology readout of neuropsychiatric disorders on habituation, we will perform a genetic screen for habituation in the GFS of genes known to be involved in these disorders.

2. Project description

2.1 Specific Aims

Previous work in Skoulakis' lab (Biomedical Sciences Reasearch Center "Alexander Fleming") showed that 14-3-3ε, ptp61F and corkscrew genes are implicated in protection from premature habituation to footshock and that mushroom bodies (MBs) are essential structures in the process. Consoulas' lab (Medical School, University of Athens) has shown that ε leads the long latency response (LLR) of the giant fiber system (GFS) to prematurely habituate. In an attempt to identify specific

genes and molecules that protect from premature habituation and characterize the pathways that are implicated we will perform:

1. Behavioral screening of genes governing shock habituation latency in the electroshock habituation paradigm (Acevedo *et al.*, 2007). Special focus on human ADHD-related genes

- Mutated flies on specific genes
- Heatshock in RNAi genes for temporal- and temperature-controlled expression without need for $Gal80^{ts}$ and use of an artificial ligand-inducible GAL4:GeneSwitch system
- RNA interference in the MBs as a whole, in individual parts (α/β, α'/β', γ lobes) and in the whole CNS excluding the MBs in order to examine their role
- Study potential compensation of 14-3-3ε's results by leonardo isoform with RNAseq and proteomics
- shibiri (UAS-shi^{ts}) in order to block neurotransmission in the MBs and check if the effect is afferent or efferent to them
- Calcium-imaging on the dendrites of the MBs-cells to see if the effect is presynaptic or postsynaptic to them

2. Identify and characterize the whole neuronal pathway implicated in the process

- shibiri (UAS-shi^{ts}) in order to block neurotransmission in different neuronal populations like dopaminergic neurons, glutamatergic neurons etc. to check if they are implicated in the effect
- Calcium-imaging on the dendrites of the postsynaptic party of the identified neuronal set
- Recovery of habituation with specific dishabituators like air/yeast puff

3. Pharmacological rescue of premature habituation phenotype

- Employ Ritalin and other attention-related drugs through food in an attempt to rescue the premature habituation phenotype when existed

4. Screening molecular elements responsible for defected habituation of LLR of the GFS (Engel and Wu, 1996)

- Electrophysiological screening of different genes and molecules associated with premature or impaired habituation in different neuronal systems
- Express human ADHD-related genes and study electrophysiology in the GFS
- Pharmacological rescue with Ritalin or other drugs

2.2 Introduction and Significance

Habituation is considered a form of non-associative learning apparent across all animal species and one of the simplest forms of neuronal plasticity (Lieberman, 2000; Asztalos, Arora and Tully, 2007). It is actually a major mechanism to decrease responsiveness to repetitive or prolonged non-reinforced stimuli with little predictive value and importance for survival (Thompson and Spencer, 1966; Giles and Rankin, 2009). Premature habituation does not permit efficient discrimination between novel and pre-experienced stimuli and therefore may lead to a non-appropriate response (Acevedo *et al.*, 2007). In humans, it has been linked to migraines (Siniatchkin, Kropp and Gerber, 2003) and ADHD (Gillberg, 2001; Zelaznik *et al.*, 2012). To gain even more insight about premature habituation we will take advantage of two paradigms developed in *Drosophila melanogaster*, the electroshock habituation paradigm (Acevedo *et al.*, 2007) and the habituation of the giant fiber system escape response (Engel and Wu, 1992;1994).

Studying the gill-withdrawal reflex in Aplysia, Kandel et al. showed that habituation is a presynaptic process decreased neurotransmitter release (Castellucci and Kandel, 1974). Previous work in the lab showed that the mushroom bodies are essential for the protection from premature habituation to electric footshock (Acevedo *et al.*, 2007), yet the pathway underlying it/implicated is still unknown. In an attempt to identify this pathway, we will block neurotransmission with shibiri (UAS-shi^{ts}) in different neuronal populations afferent to the MBs and the MBs themselves along with their individual counterparts (α/β, α'/β', γ lobes) in order to determine the responsible neurons (Semelidou et al., 2018). To verify the result, we will additionally perform calcium-imaging on the dendrites of the postsynaptic party of the identified neuronal set/population.

A variety of genes have already been identified that play a role in protection from premature habituation. Taking into consideration the works of Acevedo *et al.* (2007), Kadas (2011), Christodoulou (2018), Roussou *et al.* (2019) and Karamolegkou (2021), we will perform a screening of different genes, with special focus on some attention- and human ADHD-related ones, in order to determine their potential role in protection from premature habituation through specific mutations and RNA interference using the GAL4-UAS system which is used for cell- or neuronal population-specific genetic manipulation to both electric footshock and long-latency response habituation of the GFS. In all cases, we will confirm that if an effect, it spontaneously goes away/disappears when a dishabituator (yeast- or air-puff) is employed in the system. Finally, based again on previous results of the lab, we will try to pharmacologically rescue premature habituation when exhibited, employing Ritalin and other ADHD- and attention-related drugs and compounds.

2.3 Research Strategy

Neuron-specific expression and ablation

The Drosophila strains needed for our screening experiments will be obtained by expressing the RNAis in specific neuronal populations either by heatshocking them if constructed so or by using the artificial ligand-inducible transcriptional activator GAL4-GeneSwitch system. This system contains a DNA-binding domain from the *Saccharomyces cerevisiae* GAL4 gene, a truncated ligand-binding domain from the *Homo sapiens* progesterone receptor (PGR) and the transcriptional activation domain from the *Homo sapiens* RELA gene. The GAL4 domain can bind to a UAS regulatory sequence, but the PGR domain ensures that its binding and transcriptional activation occurs only when anti-progestin mifepristone is present (Osterwalder *et al.*, 2001; Rana, Rera and Walker, 2013).

The Drosophila strains needed for our pathway-identifying experiments will be obtained by crossing neurons- or neuronal populations-GAL4 drivers to shibiri (UAS-shi^{ts}) in order to block neurotransmission from these neurons to efferent pathways. This transgene carries a temperature-sensitive mutation of the dynamin encoded by the gene *shibire* that seems to prevent neurotransmitter recycling to the presynaptic neurons, causing their functional depletion (Bengtson and Kitamoto, 2001). The control lines will contain each GAL4 driver and UAS-shi^{ts} crossed to w^{1118}.

The Drosophila mutants and UAS-RNAi lines will be obtained from Bloomington Indiana Stock Center and collaborating labs.

All Drosophila strains will be cultured in standard wheat-flour-sugar food supplemented with soy flour and $CaCl_2$ at 22–25°C. Crosses expressing UAS-shi^{ts} will be transferred to 32°C 30 minutes prior to testing. Ritalin will be delivered to the animals through food, 48 hours prior to testing.

Behavioral assay of habituation to electric shock

All behavioral experiments will be carried out in a balanced design, where all genotypes involved in an experiment will be tested per day. The experimenter will be blind to the genotype and the order of genotypes submitted to training and testing will be randomized. Behavioral experiments will be replicated at least 3 independent times and at least once with flies from different crosses and a different time period (biological replicates).

All flies will be tested 2-5 days after emergence. They will be collected after brief anesthesia with CO_2 at least 2 days before testing and will be kept in 3mL-food vials in groups of 50-70 flies. All flies will be transferred to fresh 3mL-food vials 1-1,5 hour before the experiment and will be kept in a dark box for the whole time so that they get used to no-light conditions and the only stimulus driving the tested behavior will be the experimental one. Crosses expressing UAS-shi^{ts} will be transferred to 32°C 30 minutes prior to testing.

All experiments will be performed under dim red light at 24°-25°C and 65%-70% humidity.

Electroshock avoidance test

Experiments were performed at 24°C and 67% humidity. Approximately 50 naive flies were placed at the choice point of a T-maze and left to choose between an electrified and an otherwise identical inert standard copper grid for 90 seconds. In the electrified grid, there were delivered 1.2sec-duration stimuli of 45V intensity every 4 sec. The airflow in all arms of the maze was kept constant at 500mL/min at all times. The performance index (PI) was calculated as the fraction of flies avoiding the electrified grid minus the fraction of flies that did not.

Electroshock habituation assay

Experiments was performed as previously described by Acevedo et al. (2007), at 24°C and 67% humidity. During the training stage, a set of around 50 flies was trained in the upper arm of a standard T-maze lined with an electrifiable grid, by receiving 6 (in premature habituation training) or 15 (in normal habituation training) x1.2-sec electroshocks of 45V each. Right afterwards, the flies were left to rest for 30 seconds before placed in the lower choice point of the maze and left to choose between an electrified and an otherwise identical inert standard copper grid for 90 seconds as above. At the end of this period, the flies in each arm were trapped and counted, and the performance index was calculated as previously described.

Electrophysiology

All electrophysiological experiments were carried out in a balanced design, where all genotypes involved in an experiment were tested per day. The order of genotypes submitted to testing was in the consecutive order of 1 control to 1 experimental. The experiments were replicated at least 3 independent times and were performed on at least 8-10 animals of each genotype so that the data size and distribution permit

statistical analysis. They still need to be replicated on flies from different crosses and a different time period (biological replicates). Data are shown as mean ±SEM.

Preparation for recording

We briefly anesthetize the flies with CO_2. We glue each fly to a thin metal wire on the back of their neck using super glue, let it dry for a few seconds and then place the wire with the fly in a humid chamber (petri dish with a wet piece of paper inside) (Engel and Wu, 1996) and leave it to recover for at least 1h at either 21-24^{o}C or 30^{o}C if the flies are induced carrying the Gal80^{ts} temporal expression gene. We place the fly on the electrophysiology stage, with the legs touching the ground so that it can step on a solid surface during the whole procedure and noise from excessive movement gets diminished. We use uninsulated tungsten electrodes to penetrate the tissues (Kadas et al., 2012). We place the electrodes as follow: 1 stimulation electrode in each one of the eyes, 1 recording electrode in target the upper part of the back and close to the middle trying to target the 5th-6th DLM fibers and finally 1 reference electrode in the abdomen (right below the end of the back) (Engel and Wu, 1992). We confirm that all electrodes are placed correctly by giving a low-Voltage stimulus, we correct when needed and try to remove noise from the spontaneous activity recorded.

Electrophysiological recordings

Since the electrophysiology setup had not been used for a long time, we firstly needed to set it up from the beginning and optimize the habituation protocol so that our data are in line with literature. During this procedure, we used wild-type Canton-S flies. The work and data we obtained are presented in the Supplementary appendix. The habituation protocol that we therefore decided to perform is presented right below.

We leave the fly to relax for 10min with the electrodes penetrating the tissues. We apply single (0.1ms) pulses with 30sec interstimulus interval to avoid habituation of the response with a Grass S88 stimulator (Grass Technologies), starting with low voltages (4V) and subsequently increasing by 1-2V the intensity of the next stimulus delivered after 30sec until we get a clear short latency response. Data are digitized with an analog-to-digital converter (Digidata 1200; Molecular Devices) without filtering and are analyzed and displayed with SignalExpress LabView 2009 software (National Instruments). We check the recording and determine the response thresholds for short-latency and long-latency. We leave the fly to rest for 10min before applying the habituation protocol. Humidity in the room during habituation needs to be around 40-60%.

We apply 1000 stimuli with an intensity near the top of the long-latency stimulus range (0.5-1V below the upper threshold) with a frequency of 2Hz, 3Hz or 5Hz. When finished, we remove the fly from the stage. We count the “yes” or “no” response of the fly after each given stimulus/ how many times and at which given stimuli the fly responded and address the probability of response per stimulus and the failure criterion for each genotype tested. The probability of response is defined as the number of animals that respond at a certain stimulus. The failure criterion involves the average number of stimuli at which the animals fail to respond for 1, 2, 3, 4 and 5 consecutive times. Flies that failed to respond to either or both the first two stimuli, were excluded from the analysis.

Calcium imaging and microscopy

The calcium imaging setup consists of five parts: an upright microscope, a light source, a charge-coupled device (CCD) camera, a beam-splitter device, and computer hardware and software to control data acquisition.

The genetically-encoded calcium indicators GCaMP6f or GCaMP6m will be expressed using the Gal4/UAS- system in various driver lines. Regions of interest will be selected by hand in the dendritic armors of these neurons and in the axon terminals outlining the cellular locus of the effect. We will carefully perform a dissection in order to expose the brain and the GF track, leaving the eyes intact. We will perform in vivo calcium imaging while delivering specific pulses with a 5Hz or 3Hz (as in the habituation protocol) frequency to the eyes.

Analysis of imaging data

Analysis of two-photon images will be performed using MATLAB scripts (The MathWorks, Inc.).

References

Acevedo, S. F. (2004) ‘The dynamics, interactions and phenotypes associated with the three members of the 14-3-3 family in Drosophila melanogaster’ (Doctoral dissertation), Texas A&M University, USA.

Acevedo, S. F. *et al.* (2007) ‘Protection from premature habituation requires functional mushroom bodies in Drosophila.’, *Learning & memory (Cold Spring Harbor, N.Y.)*. doi: 10.1101/lm.566007.

Asztalos, Z., Arora, N. and Tully, T. (2007) 'Olfactory jump reflex habituation in Drosophila and effects of classical conditioning mutations', *Journal of Neurogenetics*. doi: 10.1080/01677060701247508.
Bengtson, J. and Kitamoto, T. (2001) 'Conditional modification of behavior by targeted expression of a temperature-sensitive shibire allele in subsets of neurons', *CSH Neurobiology of Drosophila*.
Castellucci, V. F. and Kandel, E. R. (1974) 'A quantal analysis of the synaptic depression underlying habituation of the gill-withdrawal reflex in Aplysia.', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.71.12.5004.
Engel, J. E. and Wu, C. F. (1992) 'Interactions of membrane excitability mutations affecting potassium and sodium currents in the flight and giant fiber escape systems of Drosophila', *Journal of Comparative Physiology A*, 171(1), pp. 93–104. doi: 10.1007/BF00195964.
Engel, J. E. and Wu, C. F. (1996) 'Altered habituation of an identified escape circuit in Drosophila memory mutants', *Journal of Neuroscience*. doi: 10.1523/jneurosci.16-10-03486.1996.
Giles, A. C. and Rankin, C. H. (2009) 'Neurobiology of Learning and Memory Behavioral and genetic characterization of habituation using Caenorhabditis elegans', *Neurobiology of Learning and Memory*. Elsevier Inc., 92(2), pp. 139–146. doi: 10.1016/j.nlm.2008.08.004.
Gillberg, C. (2001) 'Child neuropsychiatrie perspectives contribute to understanding psychosocial disadvantage', *Lakartidningen*.
Kadas, D. *et al.* (2012) 'Constitutive activation of Ca 2+/calmodulin-dependent protein kinase II during development impairs central cholinergic transmission in a circuit underlying escape behavior in Drosophila', *Journal of Neuroscience*, 32(1), pp. 170–182. doi: 10.1523/JNEUROSCI.6583-10.2012.
Lieberman, M. D. (2000) 'Intuition: A social cognitive neuroscience approach.', *Psychological Bulletin*. US: American Psychological Association, pp. 109–137. doi: 10.1037/0033-2909.126.1.109.
Osterwalder, T. *et al.* (2001) 'A conditional tissue-specific transgene expression system using inducible GAL4', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.221303298.
Rana, A., Rera, M. and Walker, D. W. (2013) 'Parkin overexpression during aging reduces proteotoxicity, alters mitochondrial dynamics, and extends lifespan', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.1216197110.
Roussou, I. G. *et al.* (2019) 'Drosophila Bruton's Tyrosine Kinase Regulates Habituation Latency and Facilitation in Distinct Mushroom Body Neurons', *The Journal of neuroscience : the official journal of the Society for Neuroscience*. doi: 10.1523/JNEUROSCI.0633-19.2019.
Siniatchkin, M., Kropp, P. and Gerber, W. D. (2003) 'What kind of habituation is impaired in migraine patients?', *Cephalalgia*. doi: 10.1046/j.1468-2982.2003.00434.x.
Thompson, R. F. and Spencer, W. A. (1966) 'HABITUAT1ON ':, 73(1), pp. 16–43.
Zelaznik, H. N. *et al.* (2012) 'Motor timing deficits in children with Attention-Deficit/Hyperactivity disorder', *Human Movement Science*. doi: 10.1016/j.humov.2011.05.003.

BUDGET

BUDGET		
Category		**Total in €**
Direct Costs Personnel		
Post-Doc Researcher(s) (1x48months)		**24600** (1x48months)
PhD Candidate(s)		**-**
Total Direct costs for Personnel		**24600**
Other Direct Costs	**Justification**	
6.1.2 Consumables	Fly food and housing (cotton and vials)	**10000**
	CO_2	
	Electric grids	**500**
	Electrophysiology consumables (tungsten wires)	**200**
	Chemicals and Solutions	**2500**
	Drugs	**1000**
	Molecular Biology plastics	**1000**
6.1.3 Travel	Present work progress and findings in conferences	**2000**
6.1.4 Dissemination	2 publications	**6000**
6.1.5 Use and/or Access to equipment etc.	Calcium imaging equipment	**5000**
6.1.6 Equipment	-	**-**
6.1.7 Other Costs	Office supplies	**1000**
6.1.8 Purchase of animals	Drosophila form BDSC	**500**
Total "other direct costs"		**29700**
Total Direct Costs		**54300**
Indirect Costs (Institution overhead, 10%)		**5430**
Total Budget		**59730**

CV

Eleni Giannopoulou
17 Efpatorias Str, Athens, 11522, Greece
Email: elen94gr@gmail.com, elenigian@biol.uoa.gr
Date of Birth: 22/04/1994

Education

2018-2021: International masters' program in Neurosciences, National and Kapodistrian University of Athens
2013-2018: Bachelor Degree in Biology, Department of Biology, School of Sciences, National and Kapodistrian University of Athens
2012: Graduation from 4th Lyceum of Tripolis, Greece

Experience

02/2021-ongoing: Technician assistant, Molecular Cognitive Neuroscience lab, Biomedical Sciences Research Center "Alexander Fleming"
"Flagship action Bee routes: Bee toxicity - methods of evaluation, detoxification time"

09/2019-11/2020: Masters' Diploma Thesis, Molecular Cognitive Neuroscience lab, Biomedical Sciences Research Center "Alexander Fleming"
"Behavioral and Electrophysiological characterization of genes governing shock habituation latency in *Drosophila melanogaster*"

06/2019-09/2019: Voluntary traineeship, Molecular Cognitive Neuroscience lab, Biomedical Sciences Research Center "Alexander Fleming"
"Effects of sulfoximine exposure on bee cognition"

05/2019-07/2019: 2nd rotation internship (for the MSc), Molecular Cognitive Neuroscience lab, Biomedical Sciences Research Center "Alexander Fleming"
"Characterization of genes governing shock habituation latency in Drosophila melanogaster"

02/2019-04/2019: 1st rotation internship (for the MSc), Experimental Physiology lab, Faculty of Health Sciences, School of Medicine, National and Kapodistrian University of Athens
"How starvation affects courtship behavior in *Drosophila melanogaster*?"

03/2018-06/2018: Internship with Erasmus+ Placement, Structural and Functional Plasticity of the Nervous System research group, Faculty of Science, Swammerdam Institute for Life Sciences, University of Amsterdam
"Effects of early life stress on the expression of GR, MR, NMDAR2A/2B, NGF1-A in P9 and P21 mice. Special focus on transcription factor Egr1."

2016-2017: Bachelor Diploma Thesis (External), Laboratory of Biology and Biochemistry, School of Health Sciences, Faculty of Nursing, National and Kapodistrian University of Athens
"Effect of early life experiences on the myelination of the prefrontal cortex of the rat brain"

06/2016-08/2016: Internship as Lab Assistant, Laboratory of Biology and Biochemistry, School of Health Sciences, Faculty of Nursing, National and Kapodistrian University of Athens
"Immunolocalization of MBP antigen in slices of rat brains"
11/2015-06/2016: Voluntary traineeship, Laboratory of Biology and Biochemistry, School of Health Sciences, Faculty of Nursing, National and Kapodistrian University of Athens

Languages

English: Excellent knowledge (C2 University of Michigan)
Spanish: Good knowledge (B2 Instituto Cervantes)
Greek: Native language