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Thesis: The locus of habituation in the giant fiber circuit of Drosophila

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The locus of habituation in the giant fiber circuit of Drosophila

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Summary

Habituation, the response decrement after repeated stimulation, is the most primitive non-associative form of learning, conserved across species. Electrophysiological studies in model organisms such as Drosophila melanogaster have revealed various genes and mechanisms implicated in habituation. The giant fiber system (GFS) of Drosophila, a well-defined, electrophysiologically amenable, sensory-motor circuit underlies the fly’s escape response. The response can be habituated, and the GFS has been extensively used as a model to study the contribution of several genes in habituation. However, the locus of habituation has been elusive. In the current study we employed a combination of recently available genetic tools in Drosophila and electrophysiology to unravel the cellular substrate of GFS habituation. We downregulated the expression of Shaker A-type channel (homologous to vertebrate Kv1) with RNAi using the binary GAL4-UAS system. We targeted the presynaptic neurons to the giant fiber LPLC2 (lobula plate/lobula columnar, type 2) and LC4 (lobula columnar, type 4). The electrophysiological analysis revealed that shaker downregulation in LC4, but not in LPLC2, neurons eliminates habituation of the giant fiber circuit. This suggests that the group of the 55 LC4 neurons that make ~2500 synaptic contacts with the giant fiber dendrites is the cellular locus of GFS habituation. Our study lays the ground for in depth analysis of the molecular mechanisms underlying habituation at the synaptic level in identified neurons.

Highlights

- Habituation of the giant fiber response to repeated stimulation can be eliminated by downregulating the shaker potassium channels in LC4, but not in LPLC2 neurons- the two groups of neurons that are presynaptic to the giant fiber.
- Behaviorally, visual input for looming speed that is encoded by the LC4 neurons can be habituated. In contrast, visual input for looming size that is encoded by the LPLC2 neurons is not habituated.

Keywords

habituation, giant fiber system, drosophila

Abbreviations

GFS – Giant fiber system

LPLC2 – Lobula plate/lobula columnar, type 2

LC4 – Lobula columnar, type 4

SLR – Short Latency Response

LLR – Long Latency Response

Introduction

Habituation is a basic form of non-associative, implicit learning conserved across many animal phyla (Giles AC et al., 2009). It describes the decrease in behavioral response to repeated stimulation that is not caused by receptor adaptation or muscle fatigue (Thompson and Spencer, 1966). In a more global approach, habituation is the unsupervised method the brain uses in order to ignore irrelevant stimuli and focus in the salient ones even if these are embedded within high background noise, highlighting its role as a cognition filter (McDiarmid TA et al., 2017). So far habituation has been studied extensively in several organisms including Aplysia, Drosophila, C. Elegans (Glanzman DL, 2009; Rose JK and Rankin, 2001; Engel JE and Wu CF, 1988). The neuronal simplicity of such systems along with the knowledge of their anatomical and physiological properties enabled the investigation of several mechanisms implicated in habituation (Glanzman DL, 2009; Rose JK and Rankin, 2001; Engel JE and Wu CF, 1988).

The molecular mechanisms governing habituation remains an unexplored field. Kandel and his colleagues using the gill withdrawal reflex of Aplysia were the first to set the principles for the molecular and cellular study of habituation (Castellucci and Kandel, 1974). They demonstrated that during habituation of the defensive withdrawal reflexes, the sensory -to-motor neuron synapses undergo rapid and profound homosynaptic depression, exclusively due to presynaptic changes (Castellucci and Kandel, 1974).

Additionally, Drosophila has been proved as an exceptional tool for examining several habituated behaviors differentially evoked (Engel and Wu 2009). So far there have been identified several genes linked with habituation and most of the genes are implicated in cAMP metabolism (Engel JE and Wu CF 2009). The most studied paradigms are rutabaga (rut) and dunce (dnc), mostly mentioned as memory mutants,

since their role have been linked to short term memory events (Qiu Y et al., 1993). The mutants rut and dnc, involved in cAMP metabolism, encoding for adenyl cyclase and cAMP phosphodiesterase respectively, were investigated in several model systems in the fly (Engel JE and Wu CF 2009). The second messenger signal transduction pathways such as cAMP signalling are declared mediators of synaptic plasticity, required for short term memory formation (Z. Guan, et al., 2011). In all the evoked behaviors reviewed by Engel and Wu, dnc and rut mutants impaired normal habituation, though in a different way (Engel and Wu, 2009). Another example is cGMP-dependent protein kinase (PKG), a cGMP-dependent protein kinase encoded by the gene for (foraging) and identified for natural variation in food searching behavior (Kent CF et al., 2009) and caki, which encodes a presynaptic membrane-associated protein with domains analogous to calmodulin (CaM), related with neuroplasticity (Martin JR and Ollo R, 1996). Furthermore genes that play a key role in the circadian rhythm found to be involved in habituation (Engel JE and Wu CF, 1988).

Engel and Wu took advantage of the identified neuronal circuit of the fly that mediates escape response, in order to investigate habituation (Phelan et al., 2017). This neural system called giant fiber system includes the sensory part (photoreceptors, retina, lamina, medulla and lobular ColA interneurons) and the motor part (motorneurons and muscles) (Pezier et al., 2016). The part of the giant fiber escape circuit responsible for habituation is likely located in the neurons upstream to the giant fiber, in the central brain and does not involve sensory adaptation or motor fatigue (Engel and Wu, 1996). The subject of this study indicates that habituation of the escape response system is located in the neurons being the primary direct visual inputs to the giant fiber (Ache et al., 2019).

Potassium channels and their potential role in habituation have always concerned scientific research. These channels are an essential component for habituation although they have differential control mechanisms depending on the form of non-associative conditioning (Joiner MA, et al., 2007). In general K^+ currents are a key element of intrinsic properties regulating excitability, frequency coding and firing patterns of a neuron (B. Hille, 1978). Focusing in Drosophila K^+ currents neurons and muscle cells are characterized by two voltage-gated (I_{Kv}) currents, I_A (fast inactivating transient A-type current) and I_K (persistent delayed rectifier) (Salkoff and Wyman, 1981; Solc CK and Aldrich RW, 1988). The shaker gene encodes the structural α (pore forming) subunit of a voltage gated potassium channel (Salkoff and Wyman, 1981). The channels formed are responsible for I_A inactivating transient A-type, a very rapidly inactivating current, which enables the repolarization of the neuron (Martínez-Padrón, et al., 1997). As a matter of fact it is a basic component for electrical excitability in neurons and muscle cells, as well as for the regulation of neurotransmitter release at the synapse (Engel and Wu, 1988) and is involved in several stereotypic behaviors (Engel JE and Wu CF, 1988).

Engel and Wu have investigated the role of several subunits of potassium channels in habituation and found that mutations of Sh enhanced habituation (Engel JE and Wu CF, 1988). In this study we downregulated the expression of Shaker gene applying the UAS-Gal4 system for targeted expression, in the LPLC2 (lobula plate/ columnar type 2) and LC4 (lobula columnar type 4). LPLC2 and LC4 are actually two groups of ColA neurons and they contribute 99.4 % of the giant fiber's direct-input synapses from the optic lobe. Furthermore recent findings suggests that LPLC2 neurons form synapses in LC4 (Ache, et al., 2019). Our work indicates that downregulation of the gene endcoding Shaker subunits in the LC4 neurons, but not in the LPLC2 neurons, leads to a non habituated phenotype. Thus, these results indicate that habituation of GFS is located in the LC4 neurons.

Materials & Methods

Flies were raised on standard corn flour-yeast-agar medium in an incubator at 24^0C and >60% humidity on 12 hours dark/light cycles.

Drosophila strains

The LPLC2 split GAL4 strain [R19G02_p65ADZp (attP40); R75G12_ZpGdbd (attP2)] (Ache et al., 2019), which drives expression in the LPC2 (lobula plate/ lobula columnar, type 2) neurons and the LC4 split GAL4 strain [R47H03_p65ADZp (attP40); JRC_SS00315 R72E01_ZpGdbd (attP2)] (Ache et al., 2019), which drives expression in the LC4 (lobula columnar, type 4) were crossed to the following strain containing UAS insertions: y1 v1; P{UAS-GFP.VALIUM10} attP2 used as a control (Bloomington Stock Center, 35786; RRID:BDSC_35786), and to the y1 sc* v1; P{TRiP.HMC03576}attP40 which expresses dsRNA for RNAi of shaker (Bloomington Stock Center, 53347; RRID:BDSC_53347).

Electrophysiological preparation and recordings

Flies were anesthetized by cooling them on ice, glued to a thin metal wire attached to the neck with cyanoacrylate adhesive. Flies were allowed to recover from anesthesia at least for 1 hour. In order to stimulate the GF neurons and subsequently the following motorneurons, a pair of uninsulated tungsten electrodes were inserted, via micromanipulators, in the eyes. A similar electrode was used to record from the DLM (fibers 5 and 6) or TTM. A fourth tungsten reference electrode was placed into the scutellum or the abdomen (Tanouye and Wyman, 1980; Engel and Wu, 1992; Allen et al., 2000). Brain or thoracic stimulation was performed by delivering stimuli (0.15 ms in duration) with a Grass S88 stimulator (Grass Technologies), while DLM or TTM muscle action potentials were acquired in the frequency range (300 Hz to 10 KHz) and

amplified 100X by a differential AC amplifier (model 1700; A-M Systems). Data were digitized with an analog-to-digital converter (Digidata 1200; Molecular Devices) without filtering and were analyzed and displayed with Clampex 8.1 version software (Molecular Devices).

Measuring "latency response" (the time between the stimulation and muscle depolarization) provides a way to reproducibly and quantitatively assess the functional status of the GFS components, including both central synapses (GF-TTMn, GF-PSI, PSI-DLMn) and the chemical (glutamatergic) neuromuscular junctions (TTMn-TTM and DLMn-DLM). The parameters used in order to assess habituation of the Drosophila’s escape response system were the SLR (Short Latency Response) and the LLR (Long Latency Response). In general, “latency response” is the time between the stimulation and the depolarization of the muscle. Specifically short latency response (SLR), an estimation of the time required for the signal, is initiated by direct electrical stimulation of the GFs, to activate the target muscle. Five recordings were acquired to estimate the average response latency of each fly. For long-latency responses (LLRs), GF neurons were activated synaptically (indirect GF stimulation). Their activation was achieved by low strength- compared to short latency response activation- electrical pulses (Engel and Wu, 1996) delivered through electrodes positioned in more external eye layers.

Mimicking the visual stimuli, evoking the giant fiber response which is actually the escape response of the fly, we stimulate electrically the eyes. Different intensities of electrical stimulation, passed between electrodes in the eyes, can be used to activate afferent elements in the brain (long-latency response) or to bypass them and trigger the thoracic stage (short latency response), enabling the analysis of distinct circuit components with different response properties (Elkins and Ganetzky, 1990; Trimarchi and Schneiderman, 1993; Engel and Wu, 1996; Lin and Nash, 1996). We have demonstrated that the long-latency response shows characteristic parameters of habituation (Thompson and Spencer, 1966).

Results

The GFS of Drosophila is a well anatomically described and electrophysiologically studied circuit (Fig. 1A).

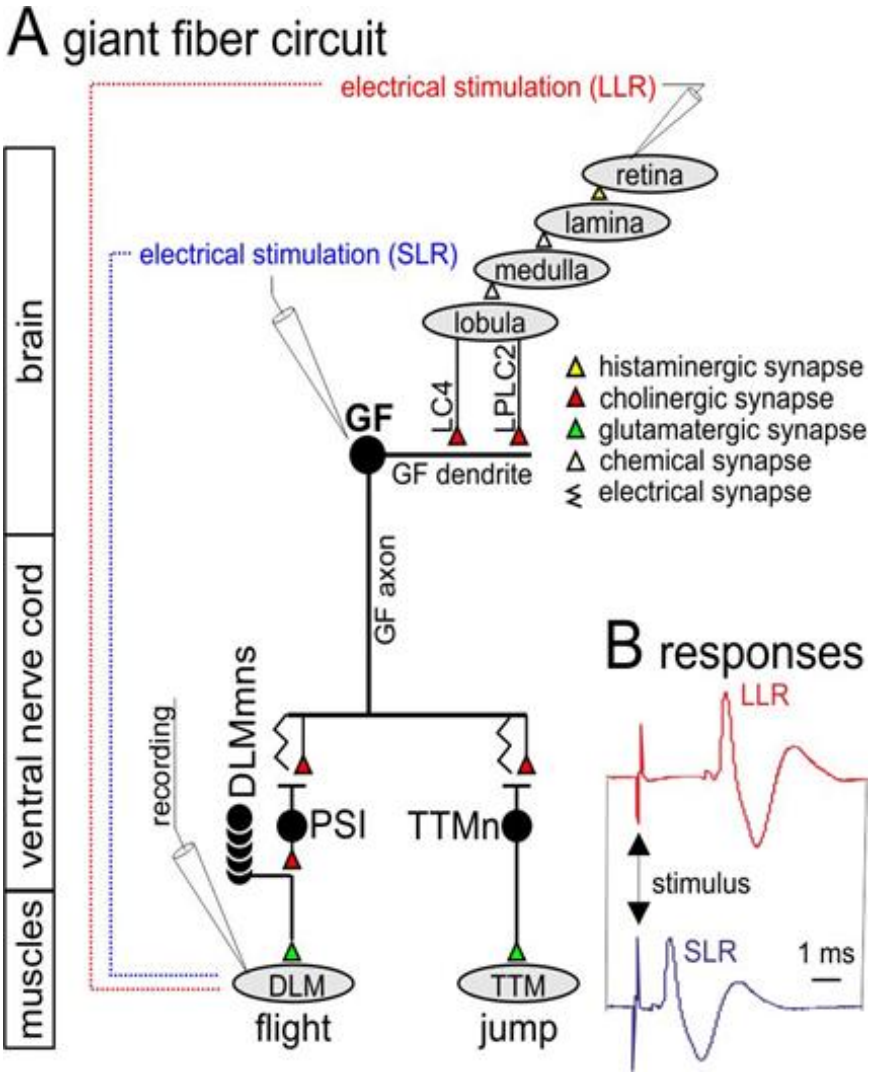

Figure 1. Diagrammatic depiction of the giant fiber circuit. (A) Circuit diagram of the GFS. Visual information is processed in the optic lobe through retina, lamina, medulla and lobula plate/lobula. Escape relevant visual information is relayed mainly by the lobula columnar type 4 (LC4) and the lobular plate/lobular columnar type 2 (LPLC2) projection neurons onto the giant fiber (GF) dendrite. The GF projects into the thorax and makes mixed electrical/chemical synapses with the TTM jump motoneuron (TTMn) and the peripherally synapsing interneuron (PSI). TTMn innervates the tergotrochanteral jump muscle (TTM). PSI forms cholinergic synapses onto the axons of the 5 flight motoneurons (DLMmns) to the dorsal longitudinal flight muscle (DLM), thus initiating flight. Circuit output can be recorded in intact animals from the DLM. A long latency response (red, LLR) of ~5 ms latency can be elicited by electrical stimulation in the eye, whereas a short latency response (1.5 ms, blue, SLR) is induced by direct stimulation of the GF. **(B)** Long (upper trace red) and short latency (lower trace blue) responses recorded from the DLM in the same animal.

Visual input from an approaching predator is processed in the optic lobe through retina, lamina, medulla and lobula plate/lobula to the giant fibers. The so-called giant fibers (GF) are actually a pair of large descending interneurons with axon diameter 6-8μm (Power, 1948; King and Wyman, 1980). They have their cell bodies in the brain and some of their dendrites form electrical synapses with a bundle of giant commissural interneurons (GCIs; Allen et al., 2006). This GF interconnection ensures their simultaneous activation regardless the origin (right or left eye) of visual input (Phelan et al., 2017). The axon of the GF descends to the mesothoracic neuromere, where it forms mixed (cholinergic and electrical) synapses with the peripherally synapsing interneurons (PSI) and the ipsilateral tergotrochanteral muscle motorneurons (TTMns; Phelan et al., 2017). The TTMns exit the nentral nerve cord to innervate the ipsilateral tergotrochanteral muscles (TTMs), the jump muscles responsible for middle leg extension (Phelan et al., 2017). The PSI extend its axon to the contralateral posterior dorsal mesothoracic nerve (PDMN), where it forms chemical synapses with the five dorsal longitudinal muscle motorneurons (DLMn) (Phelan et al., 2017). The DLMns form glutamatergic synapses with the dorsal longitudinal muscles (DLM), the muscles responsible for the downstroke of the wings (wing depressors; Fig. 1). The synapses between the GF and the TTMn and between the GF and the PSI are mixed, consisting of an electrical (shak-B gene) and a chemical component which uses achetylcholine as a neurotransmitter (llen et al., 2007). The PSI – DLMn synapse is a purely chemical synapse and uses achetylcholine and the neuromuscular junctions (TTMn-TTM and DLMn-DLM) are again purely chemical synapses that use the neurotransmitter glutamate (Fig. 1; Phelan et al., 2017).

A strong enough, not habituated visual input initiates action potentials in the GF neurons, and then the pathway efferent to GF guarantees the signal transmission to the muscles. Therefore, the output of the GF circuit can be recorded in intact animals from the flight (DLM) or/and the jump (TTM) muscles and muscle recordings can be used as readouts of GF action potentials. A long latency response (Fig. 1A, B, red, LLR) can be elicited by electrical stimulation in the eyes, whereas a short latency response (Fig. 1A, B, blue, SLR) is induced by direct stimulation of the GF.

It has been shown recently (Ache et al., 2019) that the presynaptic neurons to the giant fibers, the ColA interneurons consist of two group of neurons with dendrites in different visual neuropiles (Fig. 2).

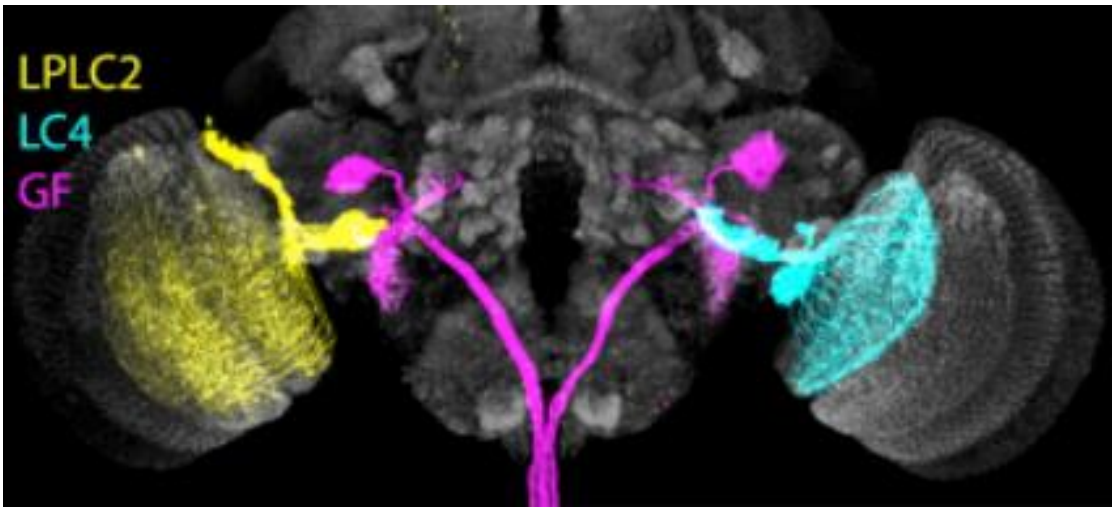

Figure 2. Confocal images from the Drosophila brain showing the LPLC2 (yellow), LC4 (blew) and GF neurons (red; Ache et al., 2019).

Escape relevant visual information (looming size and looming speed of a predator) is relayed mainly by the lobula columnar type 4 (LC4) and the lobular plate/lobular columnar type 2 (LPLC2) projection neurons onto the giant fiber (GF) dendrite. These two groups of interneurons are likely targets for habituation. We downregulate the shaker potassium channels in LC4 and LPLC2 neurons. The visual neuropile was electrically stimulated and record the output (LLR, SLR) of GFS from the flight muscles (Fig. 1A). Long and short latency responses were induced by different strength stimuli (Fig. 3A, B). No statistically significant differences were observed in LLR and SLR duration between LC4-GAL4<UAS-ShRNAi, LPLC2-GAL4<UAS-ShRNAi flies and control flies (Fig. 3C). This suggests that shaker downregulation in the presynaptic-to-GF neurons does not cause substantial changes in action potential conduction velocity or/and synaptic delays.

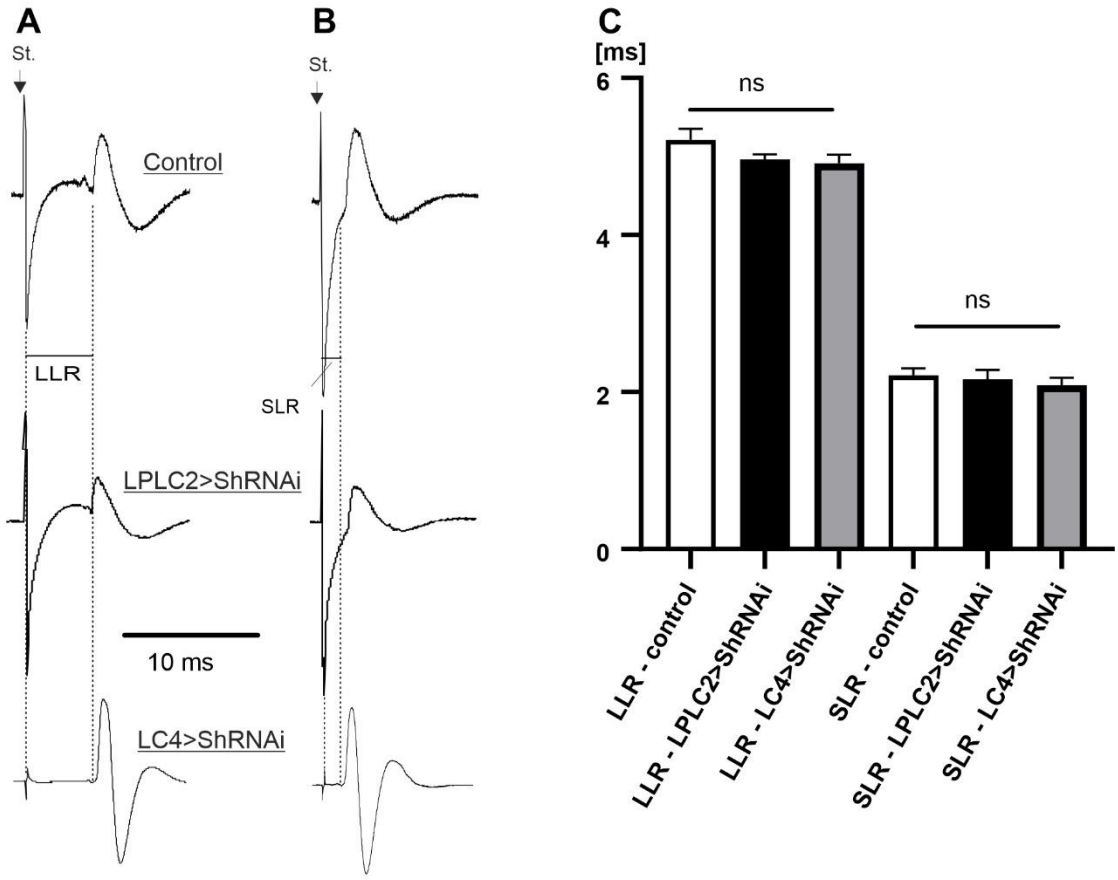

Figure 3. No differences in LLR and SLR duration in flies expressing ShRNAi in LC4 and LPLC2 neurons. A, B. Representative DLM action potentials were recorded after GF stimulation with a 0.15ms voltage pulse across the brain. The short and long latency responses (SLR, LLR) are the intervals between the stimulus artifact and the onset of the initial phase of the muscle potential, as indicated by dotted lines. LLR (A) and SLR (B) muscle responses were evoked with electrical stimuli of different strength delivered in the visual neuropile. C. Quantification of LLR and SLR. No significant difference between measurements (One-way Anova, Tukey’s multiple comparison test).

Downregulation of Shaker subunits expression in LC4 neurons evokes a non-habituated LLR phenotype. Figure 4 shows habitation plots for the first 100 stimuli, for LC4>ShRNAi (Fig. 4A), LPLC2>shRNAi (Fig. 4B) and corresponding controls. The response probability for the first 100 stimuli delivered at 5 Hz in LC4>ShRNAi equals to 1, thus downregulation of shaker channels in LC4 neurons dramatically decreases habituation (Fig. 4A). In contrast LPLC2>ShRNAi flies exhibit a similar to control habituation rate (Fig. 4B, C).

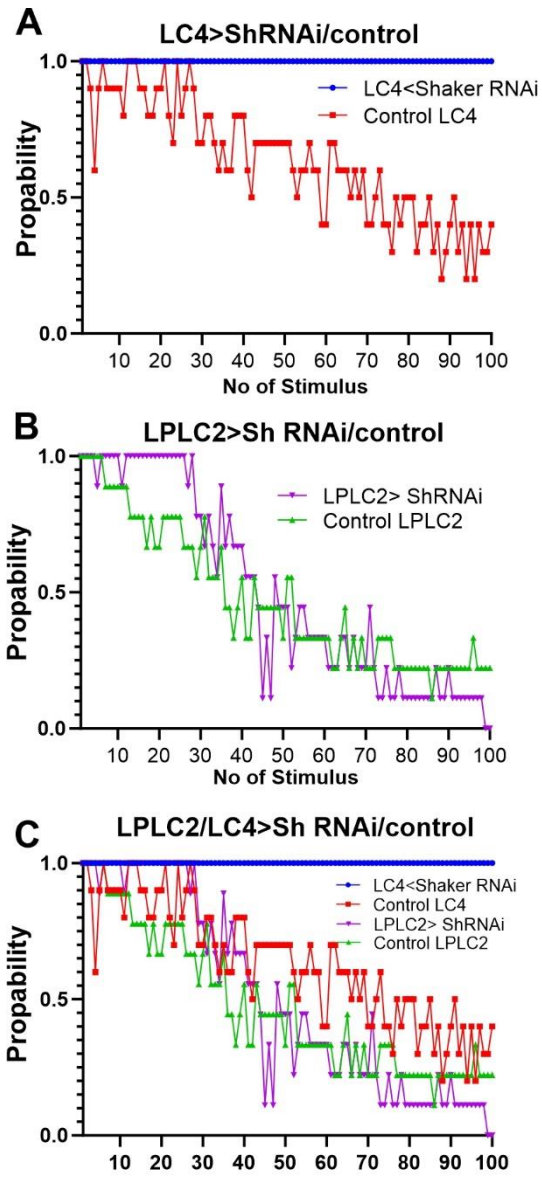

Figure 4. Habituation of LLR response propability decreases dramatically in LC4>ShRNAI flies. Habitation plots for the first 100 stimuli, for LC4>ShRNAi (A), LPLC2>shRNAi (B) and combined (C).

To examine when during stimulation the response fails from one to five consecutive times, we performed LLR recordings by delivering 1000 stimuli with a frequency of 5Hz (Fig. 5A). The stimulus number at which the first failure (F1), the first two failures (F2), the first three (F3), the first four (F4) and the first five consecutive failures (F5) were occurred, were estimated from all recordings for every genotype (Fig. 5B). The plots depicting the mean number of stimuli to attain criteria of one to five consecutive failures, show again that response failure pattern occurs within the first 100 stimuli for LPLC2> ShRNAi and their controls, but with the exception of the first failure, all other failures in LC4>ShRNAi LLR occur after the first 100 stimuli which is highly significant compared to controls (Fig. 5C, D, E). Thus, shaker subunit downregulation

in LC4 neurons substantially delays habituation of LLR. This is a strong indication for LC4 neurons being the locus of habituation of the giant fiber circuit.

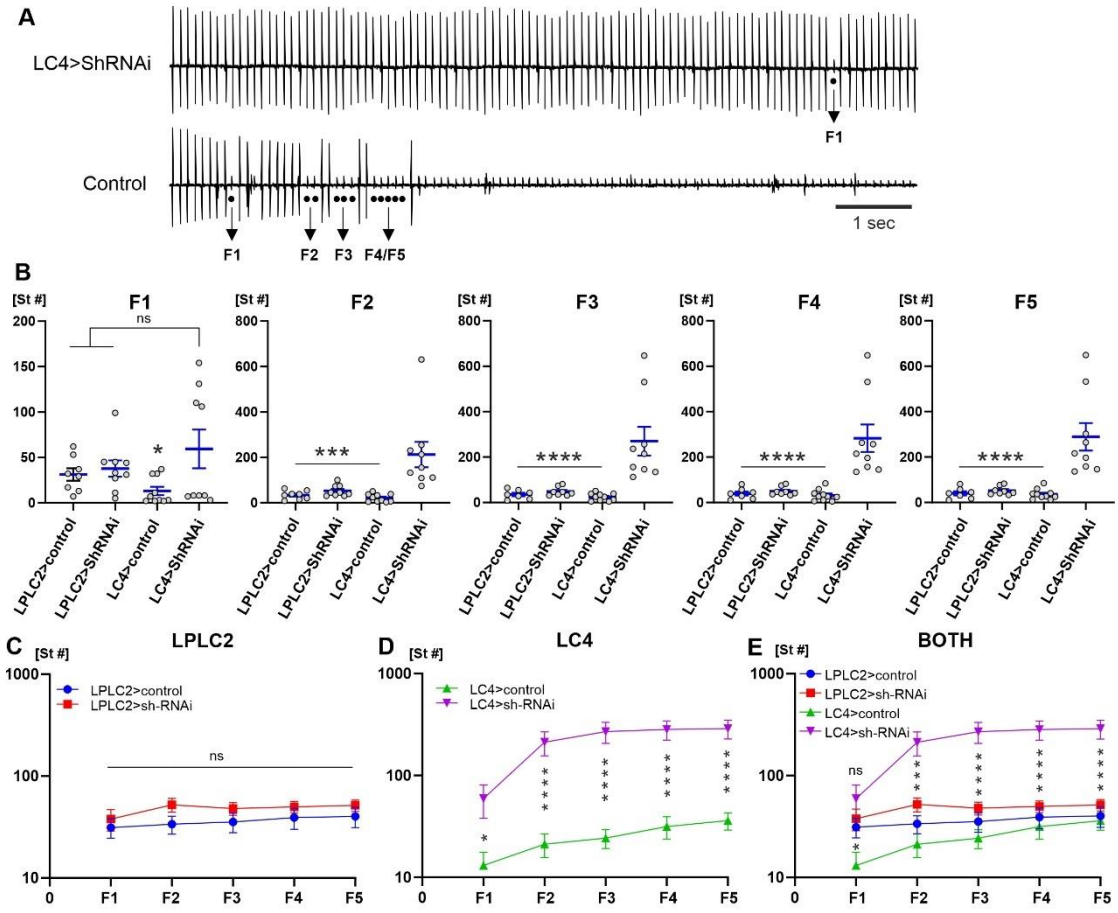

Figure 5. Habituation of LLR in flies, where the *shaker* gene is specifically downregulated in LC4 neurons, is delayed. A. Representative recordings of the first 100 LLRs in LC4>ShRNAi and control flies. Electrical stimuli were given at 5Hz. Note that all failures occur within the first 100 stimuli for the control flies and only the first failure for the LC4>ShRNAi flies. B. C. Statistical analysis (One-way Anova, Tukey’s multiple comparison test) was based on the estimation of the number of stimuli (means ± SEM) to attain one to five consecutive failures (F1 – F5) in long 1000 stimuli trains.

Discussion

In the current study, expression of Shaker channels was downregulated using the GAL4-UAS system with LC4-specific and LPLC2-specific split GAL4 promoters driving expression of ds RNAi for shaker. It was found that shaker downregulation in LC4 neurons, but not in LPLC2 neurons, dramatically delays the habituation of the long latency response. Both LC4 and LPLC2 neurons are the primary direct inputs to the giant fiber interneurons (Ache et al., 2019). Stimulating with a frequency of 5 Hz we examined the rate of habituation for each category of the genetic manipulated neurons (LPLC2, LC4 and controls). The flies with ShRNAi in LPLC2 neurons exhibited a habituation phenotype close to the control, in contrast to the flies with ShRNAi in LC4 neurons, that didn’t habituate during the first 100 stimuli. This finding suggests that LC4 neurons is the locus of habituation. This is in agreement with Engel and Wu statement that the locus of plasticity (habituation) in the giant fiber escape circuit is likely in neurons afferent to the giant fiber in the central brain and does not involve sensory adaptation or motor fatigue (Engel and Wu, 1998).

LPLC2 and LC4 neurons are both required for GF driven escape and these neurons contribute 99.4 % of the GF’s direct input synapses from the optic lobe (Ache et al., 2019). Furthermore, LPLC2 neurons provide a strong input to the LC4 neurons and finally these two neuronal components through their synaptic connections at the GF dendrites evoke the excitatory GF response to looming objects (Ache et al., 2019). Thus, there are two parallel pathways implicated in the signal processing for the initiation of the escape response. Both LC4 and LPLC2, which are part of the lamina columnar neurons, are necessary initiation components for escape. Specifically, LPLC2 neurons are responsible for the size of the looming object and the LC4 for the calculation of the speed of the object (Ache et al., 2019). Normally under repeated stimulation the giant fibers fail to produce action potentials, expressing a non-associative form of memory, EPSPs are not recorded in the TTMns and DLMns and thus jump and flight responses are not initiated. LC4 neurons may be the plastic component of the GFS . However, it remains to be shown to what extend presynaptic neurons to LC4 and LPLC2 participate in visual input processing and habituation of GFS response. Given that the connectome for the fly visual system has been completed and numerous Split-GAL4 lines are available (Ache et al., 2019) we could test all presynaptic to LC4, LPLC2 neuronal populations for GFS habituation.

The habituation decline after downregulating the shaker channels in LC4 neurons contrasts with the slightly enhanced habituation reported in Shaker mutant flies (Engel and Wu, 1998). *Shaker* gene has been knocked-out from every cells of the body, including all neurons of the GFS. Thus, we cannot explain this discrepancy before we examine the role of shaker potassium channels in every neuron and target muscles of the GFS.

Although the LC4 neurons seem to be the locus of GFS habituation, our study does not provide information on the sub-cellular region where habituation occurs. Given that habituation is considered to be a presynaptic mechanism (Ramanathan K et al., 2012; Castellucci and Kandel, 1974; Cohen TE et al., 1997; Simons-Weidenmaier et al., 2012), the synapses of the LC4 axons on GF dendrites may be a likely candidate, but this remain to be shown.

References

1. Ache, J. M. et al. Neural basis for looming size and velocity encoding in the Drosophila giant fiber escape pathway. Curr. Biol. 29, 1073–1081.e4 (2019).
2. Allen MJ, Godenschwege TA, Tanouye MA, Phelan P (2006) Making an escape: development and function of the Drosophila giant fibresystem. Semin Cell Dev Biol 17:31–41.
3. Allen, M.J., Godenschwege, T.A., Tanouye, M.A., Phelan, P., 2006. Making an escape: development and function of the Drosophila giant fibre system. Sem. Cell Dev. Biol. 17, 31e41.
4. Avery SN, McHugo M, Armstrong K, Blackford JU, Woodward ND, Heckers S. Disrupted Habituation in the Early Stage of Psychosis. Biol Psychiatry Cogn Neurosci Neuroimaging. 2019 Nov;4(11):1004-1012. doi: 10.1016/j.bpsc.2019.06.007. Epub 2019 Jun 27. PMID: 31445881; PMCID: PMC6842099.
5. B. Hille, Ionic channels in excitable membranes. Current problems and biophysical approaches, Biophysical Journal, Volume 22, Issue 2, 1978, Pages 283-294, ISSN 0006-3495, https://doi.org/10.1016/S0006-3495(78)85489-7.
6. Carew TJ, Castellucci VF, Kandel ER. An analysis of dishabituation and sensitization of the gill-withdrawal reflex in Aplysia. Int J Neurosci. 1971 Aug;2(2):79-98. doi: 10.3109/00207457109146995. PMID: 4347410.
7. Castellucci VF, Kandel ER. A quantal analysis of the synaptic depression underlying habituation of the gill-withdrawal reflex in Aplysia. Proc Natl Acad Sci U S A. 1974 Dec;71(12):5004-8. doi: 10.1073/pnas.71.12.5004. PMID: 4373738; PMCID: PMC434028.
8. Engel J, Wu CF. Altered habituation of an identified escape circuit in Drosophila memory mutants. J Neurosci 1996;16:3486–3499. [PubMed: 8627381]
9. Engel JE, Wu C-F (1992) Interactions of membrane excitability mutations affecting potassium and sodium currents in the flight and giant fiber escape systems of Drosophila. J Comp Physiol 171:93– 104.
10. Engel JE, Wu CF. Genetic dissection of functional contributions of specific potassium channel subunits in habituation of an escape circuit in Drosophila. J Neurosci. 1998 Mar 15;18(6):2254-67. doi: 10.1523/JNEUROSCI.18-06-02254.1998. PMID: 9482810; PMCID: PMC6792913.
11. Engel JE, Wu CF. Neurogenetic approaches to habituation and dishabituation in Drosophila. Neurobiol Learn Mem. 2009;92(2):166-175. doi:10.1016/j.nlm.2008.08.003

12. Giles AC, Rankin CH. Behavioral and genetic characterization of habituation using Caenorhabditis elegans. Neurobiol Learn Mem. 2009 Sep;92(2):139-46. doi: 10.1016/j.nlm.2008.08.004. Epub 2008 Sep 26. PMID: 18771741.
13. Glanzman DL. Habituation in Aplysia: the Cheshire cat of neurobiology. Neurobiol Learn Mem. 2009 Sep;92(2):147-54. doi: 10.1016/j.nlm.2009.03.005. Epub 2009 Mar 28. PMID: 19332142.
14. Joiner MA, Asztalos Z, Jones CJ, Tully T, Wu CF. Effects of mutant Drosophila K+ channel subunits on habituation of the olfactory jump response. J Neurogenet. 2007;21(1-2):45-58. doi:10.1080/01677060701247375
15. Kadas D, Duch C, Consoulas C. Postnatal Increases in Axonal Conduction Velocity of an Identified Drosophila Interneuron Require Fast Sodium, L-Type Calcium and Shaker Potassium Channels. eNeuro. 2019;6(4):ENEURO.0181-19.2019. Published 2019 Aug 6. doi:10.1523/ENEURO.0181-19.2019
16. Kent CF, Daskalchuk T, Cook L, Sokolowski MB, Greenspan RJ. The Drosophila foraging gene mediates adult plasticity and gene-environment interactions in behaviour, metabolites, and gene expression in response to food deprivation. PLoS Genet. 2009;5(8):e1000609. doi:10.1371/journal.pgen.1000609
17. King, D.G., Wyman, R.J., 1980. Anatomy of the giant fiber pathway in Drosophila I. Three thoracic componentsof the pathway. J. Neurocytol. 9, 753e770.
18. llen, Marcus & Murphey, R. 2007. The chemical component of the mixed GF-TTMn synapse in Drosophila melanogaster uses acetylcholine as its neurotransmitter. The European journal of neuroscience. 26. 439-45. 10.1111/j.1460-9568.2007.05686.x.
19. Martin JR, Ollo R. A new Drosophila Ca2+/calmodulin-dependent protein kinase (Caki) is localized in the central nervous system and implicated in walking speed. EMBO J. 1996 Apr 15;15(8):1865-76. PMID: 8617233; PMCID: PMC450104.
20. Martínez-Padrón M, Ferrús A. Presynaptic recordings from Drosophila: correlation of macroscopic and single-channel K+ currents. J Neurosci. 1997 May 15;17(10):3412-24. doi: 10.1523/JNEUROSCI.17-10-03412.1997. PMID: 9133367; PMCID: PMC6573676.
21. McDiarmid TA, Bernardos AC, Rankin CH. Habituation is altered in neuropsychiatric disorders-A comprehensive review with recommendations for experimental design and analysis. Neurosci Biobehav Rev. 2017 Sep;80:286-305. doi: 10.1016/j.neubiorev.2017.05.028. Epub 2017 Jun 1. PMID: 28579490.
22. Pezier, Adeline & Jezzini, Sami & Bacon, Jonathan & Blagburn, Jonathan. (2016). Shaking B Mediates Synaptic Coupling between Auditory Sensory Neurons and the Giant Fiber of Drosophila melanogaster. PLOS ONE. 11. e0152211. 10.1371/journal.pone.0152211.
23. Phelan, P., Bacon, J.P., Blagburn, J.M., 2017. Neural circuits underlying escape behavior in Drosophila: focus on electrical signaling. In: Jing, J. (Ed.), Network Functions and Plasticity. Elsevier (Chapter 2)

24. Pinsker HM, Hening WA, Carew TJ, Kandel ER. Long-term sensitization of a defensive withdrawal reflex in Aplysia. Science. 1973 Dec 7;182(4116):1039-42. doi: 10.1126/science.182.4116.1039. PMID: 4748675.
25. Power, M.E., 1948. The thoracico-abdominal nervous system of an adult insect. Drosophila melanogaster. J. Comp. Neurol. 88, 347e409.
26. Qiu Y, Davis RL. Genetic dissection of the learning/memory gene dunce of Drosophila melanogaster. Genes Dev. 1993 Jul;7(7B):1447-58. doi: 10.1101/gad.7.7b.1447. PMID: 7687228.
27. Rose JK, Rankin CH. Analyses of habituation in Caenorhabditis elegans. Learn Mem. 2001 Mar-Apr;8(2):63-9. doi: 10.1101/lm.37801. PMID: 11274251.
28. Salkoff, L., Wyman, R. Genetic modification of potassium channels in Drosophila Shaker mutants. Nature 293, 228–230 (1981). https://doi.org/10.1038/293228a0
29. Saur T, Peng IF, Jiang P, et al. K+ channel reorganization and homeostatic plasticity during postembryonic development: biophysical and genetic analyses in acutely dissociated Drosophila central neurons. J Neurogenet. 2016;30(3-4):259-275. doi:10.1080/01677063.2016.1255212
30. Shen Y, Dasgupta S, Navlakha S. Habituation as a neural algorithm for online odor discrimination. Proc Natl Acad Sci U S A. 2020 Jun 2;117(22):12402-12410. doi: 10.1073/pnas.1915252117. Epub 2020 May 19. PMID: 32430320; PMCID: PMC7275754
31. Solc CK, Aldrich RW. Voltage-gated potassium channels in larval CNS neurons of Drosophila. J Neurosci. 1988;8(7):2556-2570. doi:10.1523/JNEUROSCI.08-07-02556.1988
32. Tanouye MA, Wyman R (1980) Motor outputs of giant nerve fiber inDrosophila. J Neurophysiol 44:405–421.
33. Thompson RF, Spencer WA. Habituation: a model phenomenon for the study of neuronal substrates of behavior. Psychol Rev. 1966 Jan;73(1):16-43. doi: 10.1037/h0022681. PMID: 5324565.
34. Z. Guan, L. K. Buhl, W. G. Quinn, and J. T. Littleton, “Altered gene regulation and synaptic morphology in Drosophila learning and memory mutants,” Learn. Mem. , vol. 18, no. 4, pp. 191–206, 2011.

RESEARCH PROPOSAL

Giant Fiber System of Drosophila – A functional readout of habituation mechanisms

1.Summary

Habituation is a form of non-associative learning expressed as a response decrement after repeated stimulation. In Drosophila, the Giant Fiber System (GFS), a sensory-motor circuit that transmits light and wind inputs to giant fiber neuron and sequentially to motoneurons and corresponding jump and flight muscles, underlies the escape behavior of the animal. The sensory part of the GFS (photoreceptors-to-four groups of interneurons-to GF neuron) is amenable to habituation but the locus was unknown. In our study we demonstrate that the population of neurons responsible for habituation is the LC4 (lobula columnar neurons), one of the two groups (LC4, LPLC2) of neurons presynaptic to GF. Because LPLC2 neurons, apart from the GF neuron, are presynaptic to LC2 neurons as well, we will examine to what extend LPLC2 neurons play a role in LC4 habituation. For this we will employ the GAL4/UAS system to either silence LPLC2-LC4 synaptic transmission by overexpressing a temperature-sensitive allele of shibire (shi^{ts1}) in LPLC2 neurons or eliminate LPLC2 neurons by overexpressing tetanus toxin. Furthermore, we will examine whether LPLC2 neurons are the only neurons responsible for GFS habituation. Given that the connectome for the fly visual system has been completed and numerous Split-GAL4 lines are available we will test all presynaptic to LC4, LPLC2 neuronal ensamples for habituation. Finally, we will emphasize on the cellular substrate on which habituation occurs by using two-photon confocal calcium imaging in the dendrites or axonal terminals of LC2 neurons. The complete characterization of the GFS habituation locus and underlying synaptic mechanism will allow to test candidate molecules for habituation, such as the protein D14.3.3ε that has been found to be involved in olfactory habituation.

2. Project description

2.1 Specific Aims

Previous work in the lab and our study suggested that habituation is located in a specific population of neurons. To gain even more insight about the locus of the giant fiber system we will focus on the following objectives:

1st goal: Ensure that the LC4 neurons are the only cells responsible for GFS (Giant Fiber System) habituation

- Tetanus toxin (UAS-TTX) in LC4 neurons and in LPLC2 neurons (separately) will be used to ablate specifically and separately the two populations of neurons that output to GF interneuron.
- The gene of shibiri (UAS-shi^{ts}) will block neurotransmission between LPLC2-to-LC4 neurons in order to examine the contribution of LPLC2 signals to LC4 habituation.

2nd goal: Define the cellular locus of habituation (pre- or post- synaptic)

- Two-photon confocal calcium imaging in the dendrites of LC4 or the dendrites of GF neuron can help us demonstrate whether habituationis located on the giant fiber dendrites (LC4/GF synapses) or on the dendrites of the LC4 neurons (visual interneurons/LC4 synapses).

3rd goal: Examine putative molecular elements responsible for GFS habituation

- Preliminary data has shown that proteins D14.3.3ε may be involved in GFS habituation and and thus it is an excellent candidate molecule for examination

2.2 Introduction and Significance

Habituation is a fundamental form of non associative learning ubiquitous in the animal kingdom and one of the simplest forms of neuronal plasticity (Asztalos Z et al., 2007). It is actually a major mechanism to decrease responsiveness to repetitive or prolonged non-reinforced stimuli (Harris, 1943). To gain more insight about habituation we took advantage of the identified neuronal circuit of the fly that mediates escape response (Allen MJ et al., 2006). Engel and Wu were the first to investigate the role of several genes encoding potassium channels, including the genes encoding Shaker subunits, in this model system and they indicated that these genes have distinct effects on habituation (Engel and Wu, 1998). In this current study, Shaker channels were genetically downregulated in the LPLC2 (lobula plate/ columnar type 2) and LC4 (lobula columnar type 4) neurons using the GAL4-UAS system with RNAi (del Valle Rodríguez A et al., 2011). LPLC2 and LC4 belong to a class of visual projection neurons with intputs from the optic glomeruli in the central brain and have been found to be the primary input to the giant fiber interneurons (Wu M et al., 2016). Engel and Wu demonstrated that the locus of plasticity in the giant fiber escape circuit is likely in neurons upstream to the giant fibers (Engel JE and Wu, 1998). By taking advantage of the habituation observed with repeated electrical stimulation of first order visual neurons, this study shows that downregulation of the gene encoding Shaker subunits in the LC4 neurons, but not in the LPLC2 neurons, leads to a non habituated

phenotype. Thus, these results indicate that habituation of GFS is located in the LC4 neurons.

In an attempt to provide extra information that LC4 neurons are the key mediators of habituation, our work will focus on the whole pathway involved in escape response (Allen MJ et al., 2006). Firstly, it would be useful to ablate specifically and separately LPLC2 and LC4 neurons by inhibiting neurotransmission in these neurons with tetanus toxin light chain (TNT) using again the aforementioned GAL4 drivers. If that is consistent with our findings, ablation of LC4 neurons will lead to a reduced habituated phenotype. Recent data suggest that LPLC2 provide strong input to the LC4 neurons (Ache JM et al., 2019), thus a next step would be to block neurotransmission between LPLC2-to-LC4with the temperature sensitive shibiri (UAS-shi^{ts}). This will ensure that LC4 neurons are the only cells responsible for habituation.

So far the most acceptable theory among research community supports that the underlying mechanism of habituation is located presynaptically (Ramanathan K et al., 2012; Castellucci and Kandel, 1974; Simons-Weidenmaier et al., 2012). Among the first researchers to set the principles for the molecular mechanisms of habituation were Kandel and his colleagues performing a quantal analysis in the gill-withdrawal reflex of Aplysia (Castellucci and Kandel ER, 1973). They demonstrated that the response decrement observed behaviorally in Aplysia is due to decreased neurotransmitter release at the sensorimotor synapse (Castellucci and Kandel, 1974, Cohen TE et al., 1997). In an effort to define the sub-cellular locus of GFS habituation we will perform an analysis of calcium signals using two-photon confocal calcium imaging on the dendritic arbors of LC4 neurons or on the synaptic sites of their axonal terminals on the dendrites of GF neuron.

Finally, Drosophila is a premier system for unravelling the molecular interplay leading to habituation (Acevedo et al., 2007). In an attempt to get the whole picture we will further test candidate molecules known to be involved in habituation. Previous data in our lab (Kadas PhD, 2012) support that D14.3.3ε has profound effects on LLR habituation of the giant fiber system. The study used homozygous mutant lines (D14-3-3ε^{ex4}/D14-3-3ε^{ex4} and D14-3-3ε$^{l(3)j2B10}$/D14-3-3ε$^{l(3)j2B10}$) which exhibited increased habituation, highlighting a protective role upon premature habituation. It is suggested to use the new GAL4 drivers to verify their role in GFS habituation.

2.3 Research Strategy

Neuron-specific ablation

The Drosophila strains needed for our experiments will be obtained by crossing LPLC2-GAL4 and LC4-GAL4 to UAS-TTX. In order to block neurotransmission from LPLC2 to LC4 the gene *shibiri* will be overexpressed in the LPLC2 neurons specifically, thus a

cross LPLC2-GAL4 to UAS-shi^{ts} will be needed. To obtain controls for our experiments, Gal4 driver will be crossed to homozygotes to UAS-shi^{ts} and UAS-TTX. Similarly, UAS-shi^{ts}, UAS-TTX and Gal4 driver homozygotes will be crossed to w^{1118}, to obtain heterozygous controls. Animals able to express the tetanus toxin light chain transgene (UAS-TNT) (Sweeney et al. 1995) will be raised at 18°C until hatching, then they will be placed at 20°–22°C for 2 d prior to testing.

Transgenes that will be used to block neurotransmission are UAS-TNT (Keller et al. 2002) and UAS-shits bearing a temperature-sensitive mutation of the dynamin encoded by the gene shibire (Kitamoto 2001). Tetanus toxin light chain prevents neurotransmitter release by cleaving synaptobrevin, a protein required for synaptic vesicle docking on presynaptic neurons (Humeau et al. 2000). In contrast, inactivation of the temperature-sensitive dynamin Shibire^{ts} is thought to prevent neurotransmitter recycling to the presynaptic neurons, causing their functional depletion (Kitamoto 2001).

Electrophysiological preparation and recordings

The protocol that will be followed for our electrophysiological studies will be the same as in our previous experiments. It is described below:

Flies will be anesthetized (placed them on ice) and glued to a thin metal wire attached to the neck with cyanoacrylate adhesive. In order to stimulate the GF neurons and subsequently the following motorneurons, a pair of electrodes will beinserted in the eyes. A similar electrode will be used to record from the DLM (fibers 5 and 6) or TTM. A fourth tungsten reference electrode will be placed into the scutellum or the abdomen (Tanouye and Wyman, 1980; Engel and Wu, 1992; Allen et al., 2000). Brain or thoracic stimulation will be performed by delivering stimuli (0.15 ms in duration) with a Grass S88 stimulator (Grass Technologies), while DLM or TTM muscle action potentials will be acquired in the frequency range (300 Hz to 10 KHz) and amplified 100X by a differential AC amplifier (model 1700; A-M Systems). Data will be digitized with an analog-to-digital converter (Digidata 1200; Molecular Devices) without filtering and will beanalyzed and displayed with Clampex 8.1 version software (Molecular Devices).

Measuring "latency response" (the time between the stimulation and muscle depolarization) will provide a way to reproducibly and quantitatively assess the functional status of the GFS components, including both central synapses (GF-TTMn, GF-PSI, PSI-DLMn) and the chemical (glutamatergic) neuromuscular junctions (TTMn-TTM and DLMn-DLM). The parameters that will be used in order to assess habituation of the Drosophila’s escape response system will be the SLR (Short Latency Response) and the LLR (Long Latency Response). In general, “latency response” is the time

between the stimulation and the depolarization of the muscle. Specifically short latency response (SLR), is an estimation of the time required for the signal, that is initiated by direct electrical stimulation of the GFs, to activate the target muscle. Five recordings will be acquired to estimate the average response latency of each fly. For long-latency responses (LLRs), GF neurons will beactivated synaptically (indirect GF stimulation). Their activation will beachieved by low strength- compared to short latency response activation- electrical pulses (Engel and Wu, 1996) delivered through electrodes positioned in more external eye layers.

Mimicking the visual stimuli, evoking the giant fiber response which is actually the escape response of the fly, we will stimulate electrically the eyes. Different intensities of electrical stimulation, passed between electrodes in the eyes, can be used to activate afferent elements in the brain (long-latency response) or to bypass them and trigger the thoracic stage (short latency response), enabling the analysis of distinct circuit components with different response properties (Elkins and Ganetzky, 1990; Trimarchi and Schneiderman, 1993; Engel and Wu, 1996; Lin and Nash, 1996). We will also have demonstrate that the long-latency response shows characteristic parameters of habituation (Thompson and Spencer, 1966)

Two-photon confocal calcium imaging

In general two-photon imaging causes less phototoxicity and permits deeper tissue penetration than other fluorescent microscopy techniques, providing high spatial and temporal resolution imaging of calcium influx in neurons (Yuste and Denk, 1995; Wang et al. 2001).

Performing physiology experiments necessitates placing several constraints on the flies. Flies in our holders will have some of their cuticle removed and their brains exposed. We will employ this kind of microscopy in order to record calcium levels in the LC4 neurons stimulating with a frequency of 5 Hz, a frequency used for the habituation protocol. For calcium imaging experiments, the genetically-encoded calcium indicators GCaMP6f/7f or GCaMP6m/7m will be expressed using the Gal4/UAS- system in LC4 driver lines, resulting in the following genotype: LC4>GC6f. Regions of interest (ROIs) will be selected by hand in the dendritic armors of LC4 and in the axon terminals on the GF dendrites outlining the cellular locus of the effect.

Analysis of two-photon imaging data

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

References

1. Acevedo SF, Froudarakis EI, Kanellopoulos A, Skoulakis EM. Protection from premature habituation requires functional mushroom bodies in Drosophila. Learn Mem. 2007;14(5):376-384. Published 2007 May 10. doi:10.1101/lm.566007

2. Ache JM, Polsky J, Alghailani S, Parekh R, Breads P, Peek MY, Bock DD, von Reyn CR, Card GM. Neural Basis for Looming Size and Velocity Encoding in the Drosophila Giant Fiber Escape Pathway. Curr Biol. 2019 Mar 18;29(6):1073-1081.e4. doi: 10.1016/j.cub.2019.01.079. Epub 2019 Feb 28. PMID: 30827912.

3. Allen MJ, Godenschwege TA, Tanouye MA, Phelan P (2006) Making an escape: development and function of the Drosophila giant fibresystem. Semin Cell Dev Biol 17:31–41.

4. Asztalos Z, Arora N, Tully T. Olfactory jump reflex habituation in Drosophila and effects of classical conditioning mutations. J Neurogenet. 2007;21(1-2):1-18. doi:10.1080/01677060701247508

5. Castellucci VF, Kandel ER. A quantal analysis of the synaptic depression underlying habituation of the gill-withdrawal reflex in Aplysia. Proc Natl Acad Sci U S A. 1974 Dec;71(12):5004-8. doi: 10.1073/pnas.71.12.5004. PMID: 4373738; PMCID: PMC434028.
6. Cohen TE, Kaplan SW, Kandel ER, Hawkins RD. A simplified preparation for relating cellular events to behavior: mechanisms contributing to habituation, dishabituation, and sensitization of the Aplysia gill-withdrawal reflex. J Neurosci. 1997 Apr 15;17(8):2886-99. doi: 10.1523/JNEUROSCI.17-08-02886.1997. PMID: 9092610; PMCID: PMC6573098.

7. del Valle Rodríguez A, Didiano D, Desplan C. Power tools for gene expression and clonal analysis in Drosophila. Nat Methods. 2011;9(1):47-55. Published 2011 Dec 28. doi:10.1038/nmeth.1800

8. Engel JE, Wu CF. Genetic dissection of functional contributions of specific potassium channel subunits in habituation of an escape circuit in Drosophila. J Neurosci. 1998 Mar 15;18(6):2254-67. doi: 10.1523/JNEUROSCI.18-06-02254.1998. PMID: 9482810; PMCID: PMC6792913.
9. Foote M, Zhou Y. 14-3-3 proteins in neurological disorders. Int J Biochem Mol Biol. 2012;3(2):152-164.

10. Harris, J. D. (1943). Habituatory response decrement in the intact organism. Psychological Bulletin, 40(6), 385–422. https://doi.org/10.1037/h0053918
11. Keller A., Sweeney S.T., Zars T., O'Kane C.J., Heisenberg M. Targeted expression of tetanus neurotoxin interferes with behavioral responses to sensory input in *Drosophila*. J. Neurobiol. 2002;50:221–233.

12. Kitamoto T. Conditional modification of behavior in *Drosophila* by targeted expression of a temperature-sensitive *shibire* allele in defined neurons. J. Neurobiol. 2001;47:81–92.

13. Ramanathan K, Ning N, Dhanasekar D, Li G, Shi L, Vadakkepat P. Presynaptic learning and memory with a persistent firing neuron and a habituating synapse: a model of short term persistent habituation. Int J Neural Syst. 2012 Aug;22(4):1250015. doi: 10.1142/S0129065712500153. PMID: 22830965.

14. Simons-Weidenmaier, N.S., Weber, M., Plappert, C.F. et al. Synaptic depression and short-term habituation are located in the sensory part of the mammalian startle pathway. BMC Neurosci **7,** 38 (2006). https://doi.org/10.1186/1471-2202-7-38

15. Svoboda, K., Denk, W., Kleinfeld, D. et al. In vivo dendritic calcium dynamics in neocortical pyramidal neurons. Nature **385,** 161–165 (1997). https://doi.org/10.1038/385161a0

16. Sweeney S.T.B., Keane K., Niemann J., O'Kane C.J. Targeted expression of tetanus toxin light chain in *Drosophila* specifically eliminates synaptic transmission and causes behavioral defects. Neuron. 1995;14:341–351.

17. Wu M, Nern A, Williamson WR, et al. Visual projection neurons in the Drosophila lobula link feature detection to distinct behavioral programs. Elife. 2016;5:e21022. Published 2016 Dec 28. doi:10.7554/eLife.21022

18. Yuste, R., Denk, W. Dendritic spines as basic functional units of neuronal integration. Nature **375,** 682–684 (1995). https://doi.org/10.1038/375682a0

19. Zhu Y. The Drosophila visual system: From neural circuits to behavior. Cell Adh Migr. 2013;7(4):333-344. doi:10.4161/cam.25521

BUDGET		
Category		**Total in €**
Direct Costs Personnel		
Post-Doc Researcher(s)		**55.000**
PhD Candidate(s)		**36.000**
	Total Direct costs for Personnel	
Other Direct Costs	**Justification**	
6.1.2 Consumables	Fly food, antibodies etc	**15000**
6.1.3 Travel	International Conference	**3000**
6.1.4 Dissemination	3 papers	**5000**
6.1.5 Use and/or Access to equipment etc.	Use of 2 photon confocal	**10000**
6.1.6 Equipment	incubators	**7000**
6.1.7 Other Costs		
6.1.8 Purchase of animals	Bloomington, Vienna (RNAi) strains	**2000**
	Total “other direct costs”	**135.000**
	Total Direct Costs	
	Indirect Costs (Institution overhead, 10%)	
	Total Budget	**200000**

Curriculum Vitae

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Research

Diploma thesis

- **Title:** Investigation of expression of genes involved in sudden unexpected death-SUDEP and epilepsy
- **Diploma thesis conducted**: University of Ioannina, Department of Medicine, Medical genetics and assisted reproduction laboratory

Master thesis

- **Title:** Investigation of the synaptic locus of habituation in the giant fiber system in Drosophila
- **Diploma thesis conducted**: University of Athens, Medical School, Department of experimental Physiology

Publications

Dimitriadou A, Chatzianastasi N, Zacharaki PI, et al. Adult Movement Defects Associated with a CORL Mutation in Drosophila Display Behavioral Plasticity. G3 (Bethesda). 2020;10(5):1697-1706. Published 2020 May 4. doi:10.1534/g3.120.400648