A Genetic Screen for Olfactory Habituation Mutations in Drosophila: Analysis of Novel Foraging Alleles and an Underlying Neural Circuit

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Introduction

Habituation is a fundamental behavior that is often overlooked by researchers, yet its prevalence in the animal kingdom suggests it is essential for survival [1]. Habituation is an active process of progressive decline of reaction to a harmless stimulus [2,3]. Habituation allows animals to ignore inconsequential stimuli and may serve as a building block for more complex forms of attention [4]. An inability to habituate has been linked to schizophrenia [5,6], autism [7,8] and fetal alcohol syndrome [9,10]. Despite the biological and clinical importance of habituation, its behavioral simplicity and its first description over 100 years ago [11], few genes that govern habituation have been described to date.

Our understanding of the neural basis of habituation is most extensive in the sea snail Aplysia californica [12,13], whose defensive gill-withdrawal reflex habituates to repeated mechanical stimulation [14–16]. Early work showed that habituation in this sensory-neuron to motor-neuron circuit is due to a presynaptic decrease in excitatory neurotransmission, likely due to the active silencing of presynaptic release [12]. Although this decrease in presynaptic release, termed homosynaptic depression, is a common mechanism of habituation, potentiation of inhibitory connections can also achieve the same behavioral output [17,18].

A variety of paradigms have been used to study habituation in Drosophila including the gustatory-based proboscis extension reflex (PER) and several olfactory-mediated behaviors, such as the jump reflex or startle response [19]. Using reverse genetics, several well studied genes and pathways have been identified as important regulators of habituation in Drosophila. These include K⁺ channels, NMDA and GABAA receptors, as well as the cAMP and cGMP second messenger systems [18,19]. An unbiased forward genetic approach can be useful in identifying novel genes and pathways that regulate habituation. However, due to the labor-intensive nature of many habituation assays, this approach has only sparsely been used [20,21].

Our laboratory has previously described a simple and efficient paradigm to study olfactory startle habituation (OSH) in freely moving adult Drosophila [22]. In this assay, the flies’ gradual decline of a locomotor startle response to short exposures of vaporized ethanol is measured using an automated video tracking system [22]. The organization of the Drosophila olfactory system shows remarkable similarities to that of vertebrates [23,24], suggesting the principal genes, circuits and mechanisms of olfactory habituation maybe conserved. In flies, odors are detected by the olfactory receptor neurons (ORNs), most of which reside in the antennae and project to the antennal lobe (AL) where they synapse with glomerulus-specific projection neurons (PNs) and local interneurons (LNs) [24]. Both excitatory and inhibitory LNs are present in the AL and make intra- and inter-glomerular...
connections with the PNs, shaping the neural representation of odors from the 1st to 2nd order neurons [25–27]. The PNs project to the mushroom body (MB) and lateral horn. Importantly, the MB is critical for habituation [22,28], associative olfactory learning [29], and modulating locomotor responses [30]. Recently, much progress has been made in deciphering the neural circuits and identifying several genes mediating olfactory habituation in Drosophila [17,18,20,31,32].

In this report we describe the results of a genetic screen using our OSH paradigm [22] and identify 26 mutations affecting OSH. We also further characterize two hypomorphic mutations in the gene foraging (for), which encodes a cGMP-dependent kinase (PKG). These new for mutations decrease the expression of a specific isoform of for, for-T1. We show that for-T1 normally functions to inhibit OSH in a subset of neurons that include ORNs and the MB. We also show that overexpression of for-T1 in ORNs, but not the MB, reduces OSH, suggesting that for-T1 principally functions in ORNs to regulate OSH. Finally, we show that synaptic transmission of ORNs is required to promote startle habituation. Taken together, our results raise the possibility that for-T1 may inhibit OSH by decreasing synaptic release in ORNs after their exposure to ethanol vapor.

Materials and Methods

Fly Strains

All flies were maintained on standard cornmeal molasses agar at 25°C and 70% humidity under constant dim light. The P element collection screened was a collective effort generated internally in the Heberlein Lab. NP2614 was obtained from GETDB (Drosophila Genetic Resource Centre in Kyoto Institute of Technology). Our two control strains, 4.59 and 16.57, have P elements inserted just 5' of CG5630 and in Soc36E respectively, genes with no known association with habituation or PKG. UAS-TetX and UAS-TetXm strains were obtained from Sean Sweeney, pdf-GAL4 flies were obtained from Paul Tagert, and Orco-GAL4 flies were obtained from Marla Sokolowski. OK-107-GAL4, UAS-GFP-CD8 and the septime Junction P elements were from the Bloomington Stock Centre. All strains, except the for polymorphisms, were backcrossed for at least five generations to a w1118 Berlin stock.

Habituation Assay

The habituation assay is essentially the same as described in [22], except that we used the “booz-o-mat” [66] which allows simultaneous video recording of eight individual genotypes. Films were recorded using Adobe Premiere (Adobe Systems, San Jose, CA). To measure the locomotor tracking response to ethanol, films were analyzed with a modified version of DIAS 3.2 (Solltech, Oakdale, IA) that was controlled by the OneClick 2.0 scripting language (Westcode Software, San Diego, CA). Briefly, for each genotype, 20 2-4 day-old male flies were collected under CO2 anesthesia and kept in fresh food vials for 2 days. Flies were placed into a 16×125 mm cylindrical tube with perforations clustered at the rounded base. Flies were left to acclimate for 7 min before the start of video recording. After a further two minutes the flies were administered the first 30-second pulse of vaporized ethanol (P1); subsequent 30-second pulses of ethanol vapor were administered every 5 minutes. One minute after the forth pulse (P4) the flies were dishabituated with a sudden mechanical shock (banging the apparatus). A final pulse of ethanol vapor was administered 4 minutes after the dishabitation. Ethanol vapor was produced with an evaporator [22,66] and the concentration controlled by a flow meter (Cole Parmer). Mixtures of ethanol and air vapor are noted as ratios. For screening purposes, a ratio of ethanol/air of 65/77 was used. All subsequent testing was carried out at an ethanol/air ratio of 80/60, where 80 units of flux is equivalent to 2.7 liters/min. Habituation assays were repeated on 2 to 3 different days with new flies to incorporate the day-to-day variations in behavior. In all Figures, n corresponds to the number of experiments performed on an independent group of 20 flies.

Calculations and Statistics

The total movement travelled during odor exposures was calculated as the area under the pulse curve, i.e., summing the velocities measured during the 30-second exposure, at 5-second intervals and multiplying the sum by 5 seconds. The habituation index (HI) was calculated as I-P÷P1, where P4 and P1 are the areas under the locomotor activity curve for the 4th and 1st pulse respectively, such that a HI of 1 indicates complete habituation and a HI of 0 indicates no habituation. In order to more easily compare the extent of habituation in all graphs the total movement was normalized to the magnitude of the first startle. Significance was established by one-way-ANOVA with post-hoc Newmans-Keuls comparisons. Error bars in all experiments represent standard error of the mean (SEM). Statistical significance was achieved where p<0.05.

Genetic Screen

A total of 874 P-element insertion strains were initially screened in the habituation assay, (n = 2–4). The habituation index (HI) for each strain was calculated and ranged from 0.92 to –0.42. A frequency distribution of the habituation indices showed a near normal distribution with a mean of 0.58, median of 0.64 and mode of 0.65. From 874 strains screened, 93 were identified, 63 had pronounced habituation (with an HI >0.8) and 30 failed to habituate (with an HI <0.2). These strains were backcrossed for 5 generations to our w1118 Berlin genetic background to eliminate unlinked mutations. After retesting in the habituation assay (n = 6), 26 strains maintained their habituation phenotype: 25 exhibited enhanced habituation (HI >0.8) and 1 was a non-habituator (HI <0.2). All mutant strains were considered to be within the normal range of a locomotor startle response as none were significantly different than at least one of the control strains (see below). Further, all of these mutant strains appeared healthy, fertile and viable. Two representative backcrossed strains, 4.59 and 16.57, that had a normal HI similar to the screen median and mode (0.49 and 0.5, respectively) and initial startle (36.7 mm/fly and 29.5 mm/fly, respectively) were chosen as controls and used throughout the behavioral experiments, although only one control strain is shown.

Molecular Characterization of for Alleles

The location of the insertions was determined by inverse PCR. (The 11.247 P element is located in the first intron of for, 994 bp downstream of exon 1, and the NP2614 insertion is located in the same intron, 666 bp downstream of exon 1. Imprecise excision strains of 11.247 were generated through remobilization of the PGawB element by introduction of a stable transposase source. Several phenotypic revertants were obtained. The excision strains were screened by PCR on genomic DNA using primers 5’-ACTACGCTACGCTGGCAGAAAC-3’ and 5’-AACACGGAACGGAAGATTGG-3’, and several were found to be precise excisions.
RNA Analysis

Total RNA was extracted from adult flies using Trizol Reagent (Invitrogen). Poly A+ RNA was purified from total RNA using the Oligotex system (Qiagen). Probes for the Northern blot were generated by PCR using genomic DNA as a template. For the for-T1 probe, the primers used were 5'-ATCTGGTGGGTG-GATTGTTAG-3' and 5'-ACTCCTTGTGCTTATTTGGGAA-3'. For the for-T2, the primers were 5'-AGGAACAGAACTG-GAAG-3' and 5'-GATAAGAAAACCTGCCCGTTA-3'. As a control for RNA loading, a tubulin 84B gene probe was amplified using primers 5'-ACAGCCGTCTCTAGCTCCG-3' and 5'-CATCACCTCGGCCACGGTCTG-3'. Northern blots were performed using mRNA isolated from 2–4 day old adult heads and bodies (or heads only) and probed with 32P labeled probes.

PKG Enzymatic Activity Assay and Immunohistochemistry

PKG enzyme assays and immunohistochemistry were performed as previously described [44].

Results

A Behavioral Screen for Mutations Affecting Olfactory Startle Habituation

In *Drosophila*, exposure to a high concentration of ethanol vapor provokes a transient olfaction-dependent increase in locomotor activity termed the olfactory startle response [22]. Subsequent pulses of ethanol vapor result in a reduced startle that shows characteristics of habituation, including dishabituation following a novel stimulus (Fig. 1A; [22]). In order to identify genes that regulate OSH, we screened 874 fly strains, each harboring a randomly inserted P element in the genome, for strains with altered OSH. As a simple measure for habituation we calculated a habituation index (HI), defined as the ratio between the magnitudes of the fourth and first startle response (see Methods). Using inverse PCR and DNA sequencing followed by genomic database searches (www.flybase.org) we mapped the location of the transposon insertions and identified the candidate genes disrupted (Table 1). Classified by their molecular function, the largest categories were those including genes with predicted or unknown molecular function (lama, ehu, hehe, CG1896, CG8221, CG3967, CG42697, CG11337) and genes with functions related to nucleic acids (HmgD, tara, Camta, heph, snp). Smaller categories included genes involved in cell signaling (for, gish, wun, PXUTS), the regulation of the cytoskeleton (kl-2, Rtm1) or cell junctions (pyd, cora).

Several Olfactory Startle Habituation Mutations are Associated with Septate Junctions

One candidate gene we identified in our screen, *oracle* (*ora*), encodes an integral component of septate junctions [33]. In the insect nervous system, septate junctions are known to seal neighboring glial cells together to protect axons from the high K+ environment of the hemolymph [34]. Septate junctions are found in the fly’s blood-brain barrier, between perineurial and peripheral glia, and also between peripheral glia and axons [35]. An analogous structure in mammals is the paranodal junction found at the nodes of Ranvier, which enables rapid saltatory conduction of action potentials [36–38]. Interestingly, a parallel screen for OSH mutations identified *glutacin* (*glb*) (B. Cho and U.H, unpublished data), another component of the septate junction [39,40]. The identification of two septate junction genes in our screens suggested that the structure and/or function of the septate junction might be important for OSH. To test this hypothesis, we selected fifteen P element mutations in eight known

![Figure 1](https://example.com/figure1.png)

**Figure 1. *Drosophila* habituates to an ethanol-induced olfactory startle. A) The olfactory startle attenuates with the characteristics of habituation. Ethanol naïve flies exposed to a 30-sec pulse of ethanol vapor showed an olfactory mediated startle response, characterized by a transient increase in locomotor activity. In subsequent pulses of ethanol vapor the olfactory startle increasingly attenuated. To demonstrate habituation (and not sensory adaptation or fatigue) flies were dishabituated (arrow) with a mechanical stimulus before the final pulse of ethanol. B) The frequency distribution of habituation indices (HI) of all strains tested in the genetic screen. The habituation index was calculated as a ratio of the total movement in the fourth pulse (P4) and first pulse (P1) (HI = 1-P4/P1; a HI of 1 indicates complete habituation while a HI of 0 or lower indicate no habituation or sensitization, respectively). A frequency distribution of the habituation indices showed a near normal distribution with a mean of 0.58, median of 0.64 and mode of 0.65. A HI of 0.8 or higher was used as the cut-off for enhanced habituation, while a HI of 0.2 or lower was the cut-off for failure to habituate normally. Strains with low and high HIs were selected for further analysis.**

doi:10.1371/journal.pone.0051684.g001
genes whose products localize to the septate junctions, normalized their genetic background and tested their OSH response. Of these, seven strains, representing five of the eight genes tested (cora, dig, fas3, gli, nrx-IV), showed an abnormal OSH (Table S1). Further, one strain (EP809, inserted in nrx-IV) failed to habituate, a phenotype rarely seen in the original screen. Therefore, septate junctions may be an important regulator OSH, though future work will be necessary to reveal its specific function in modulating this form of behavioral plasticity.

Foraging Regulates Olfactory Startle Habituation

In *Drosophila*, foraging (*for/dg2*) encodes a cGMP-dependent kinase (PKG) that regulates habituation of the giant-fiber neurons (involved in an escape reflex) [41] and the PER [42]. We identified strain 11.247, carrying a P element insertion in the 5’ region of the *for* locus (from hereon called *for*11.247), that exhibited enhanced habituation (Fig. 2A, 2B). This phenotype is robust as it was maintained in two different genetic backgrounds (Fig. S1A). Moreover, normal habituation was restored upon precise excision of the P element in *for*11.247 (Fig. 2C), indicating that the insertion is responsible for the mutant phenotype. We also identified an additional strain that carries a P element insertion near *for*11.247, NP2614 (called *for*2614; see Fig. 3A), which also enhanced OSH (Fig. 2A, 2B). In both of these *for* alleles, the magnitude of the initial startle was normal (Fig. S1B). Therefore, we conclude that *for* regulates OSH.

Molecular Characterization of for Alleles that Disrupt Olfactory Startle Habituation

The *for* locus produces 11 transcripts that encode four protein isoforms [43]. Of these transcripts, nine encode the three major isoforms *FOR-T1, T2 and T3* (Fig. 3A; see [43] for alternative nomenclature). To determine the molecular nature of our *for* alleles, we performed Northern blots using mRNA derived from adult flies. In control flies we observed two bands using probes specific for the *for-T1* and *for-T3* transcripts (Fig. 3B, top panel). The more intense, larger molecular weight band corresponds to the three *for-T1* transcripts, *for-R1/R2/RH*, while the lower molecular weight less intense band corresponds to *for-T3* (or *for-RB*). With a probe specific to *for-T2* transcripts we detected a doublet corresponding to *for-RD/RF* and *for-RC/RG/RK* (Fig. 3B, middle panel).

In both *for* alleles we observed a reduced intensity of the *for-T1* band and did not detect a change in either the *for-T3* or *for-T2* band. Additionally, the relative expression in *for-RB* was neither increased nor decreased compared to control flies.

### Table 1. OSH mutants isolated from genetic screen.

| Strain   | Initial Startle (mm/fly) | Habitation Index | P-element orientation and candidate gene affected                                      | Molecular class | Nucleotide Insertion |
|----------|-------------------------|------------------|---------------------------------------------------------------------------------------|-----------------|----------------------|
| 9.181    | 35.0                    | 0.85             | => in *caskin*                                                                        | CGd             | 10850830             |
| 9.189    | 26.6                    | 0.82             | => in *hephaestus*                                                                   | RB              | 27811472             |
| 9.197    | 32.8                    | 0.83             | => in *gilgamesh*                                                                    | CS              | 12106609             |
| 10.66    | 41.5                    | 0.86             | => in *High mobility group protein D*                                                | DB              | 17601579             |
| 11.158   | 36.3                    | 0.83             | => 5’ of *snap*                                                                       | RB              | 17948455             |
| 11.244   | 23.3                    | 0.83             | => in *taranis*                                                                       | DB              | 12056400             |
| 11.247   | 34.1                    | 0.84             | => in *foraging*                                                                     | CS              | 3655713              |
| 11.272   | 24.0                    | 0.80             | => in *polychaetoid*                                                                  | CA              | 4720698              |
| 12.112   | 31.5                    | 0.82             | => in *wunen*                                                                        | CS              | 5297595              |
| 12.132   | 31.6                    | 0.05             | => in *Calmodulin-binding transcription factor*                                       | DB              | 5339712              |
| 12.19    | 31.3                    | 0.84             | => 5’ of *PNUTS*                                                                      | CS              | 870364               |
| 12.82    | 31.5                    | 0.83             | => 5’ *CG1806*                                                                        | CG              | 11901097             |
| 12.95    | 37.5                    | 0.83             | => 5’ of *ade5*                                                                       | M               | 12654602             |
| 12.167   | 38.1                    | 0.86             | => 5’ of *coracle*                                                                   | CA              | 15116495             |
| 12.171   | 40.0                    | 0.85             | => in *CG8321*                                                                        | CG              | 7922908              |
| 12.172   | 39.3                    | 0.83             | => in *Rtn1*                                                                         | CY              | 5001033              |
| 12.222   | 34.5                    | 0.86             | => in *CG3967, 5’ of astray*                                                          | CG, CS          | 9416260              |
| 14.29    | 32.6                    | 0.82             | => in *male fertility factor kl2*                                                     | CY              | 1322704              |
| 14.86    | 27.1                    | 0.83             | => in *CG42697*                                                                      | CG              | 14499267             |
| 18.56    | 36.6                    | 0.87             | => in *Puromycin sensitive aminopeptidase*                                           | PP              | 1517272              |
| 18.94    | 27.0                    | 0.82             | => 5’ of *Peroxiredoxin 2540-1*                                                        | CGd             | 6310865              |
| 18.104   | 31.7                    | 0.86             | => in *hephaestus*                                                                   | RB              | 27811472             |
| 19.28    | 30.2                    | 0.82             | => in *starvin*                                                                      | PP              | 13473388             |
| 19.47    | 26.4                    | 0.85             | => in *CG11357*                                                                      | CGd             | 4542807              |
| 19.70    | 40.9                    | 0.85             | => in *lamina ancestor*                                                              | CG              | 5348461              |
| 21.28    | 40.2                    | 0.85             | => 5’ of *hebe*                                                                       | CG              | 5724097              |

Initial startle: distance moved per fly during first 30-second startle. Arrows represent direction of the P element. Molecular classes: cell signalling (CS), DNA binding (DB), RNA binding (RB), cell adhesion (CA), cytoskeleton (CY), metabolism (M), proteases (PP), annotated genes unknown molecular function without homology (CG), and those annotated genes with conserved structural domains (CGd). Information current to FlyBase release: FB2012_05; Sept 7th, 2012.

doi:10.1371/journal.pone.0051684.t001
transcripts (Fig. 3B, 3C). Therefore, both for11.247 and for2614 have specifically reduced expression of for-T1 transcripts. The precise excision of the P element in for11.247 that showed normal habituation (Fig. 2C) also restored for-T1 transcripts to control levels (Fig. 3B, 3C, top panel). Therefore, our data suggest that for-T1 functions to inhibit OSH.

Using a FOR antibody [44], we next determined levels of FOR-T1 and FOR-T3 in the adult heads of flies carrying these for alleles; we were unable to determine FOR-T2 levels with this antibody. We observed a significant reduction in FOR-T1 protein expression in for11.247 and for2614 (Fig. 3C), while levels of FOR-T3 appeared normal (Fig. S1C). Therefore, consistent with the Northern blot, we conclude that, both for11.247 and for2614 have reduced expression of FOR-T1 in the adult head.

We next attempted to measure the level of PKG activity in the heads of for11.247 and for2614 flies. However, the levels of PKG activity in our P element control strain and genetic background control were significantly different (Fig. S2A), precluding any definitive conclusions about relative levels of PKG activity in for11.247 and for2614 (which were also significantly different from each other). We also failed to see a difference in OSH in the natural variants of for (forR, forL, and forS) that do subtly but significantly differ in PKG activity [44,45] (Fig. S2B). Therefore, for reasons we do not currently understand, we were unable to find a correlation between PKG activity, levels of FOR-T1 and OSH. In summary, we conclude that for11.247 and for2614 have reduced levels of for-T1 in the adult head suggesting that for-T1 functions to inhibit OSH.

for11.247-GAL4 Expression Partially Recapitulates the FOR Expression Pattern

The neuronal expression pattern of all FOR isoforms has been reported previously and includes specific neuroanatomical loci, namely the ellipsoid body (EB), mushroom body (MB), dorsal posterior cells (DPC) and clusters of neurons situated laterally [44,46]. Since the P elements in the for alleles drive GAL4 expression [47], its insertion in/near the 5’ end of for-T1 may capture the endogenous for-T1 expression pattern. Expression of GFP with for11.247-GAL4 or for2614-GAL4 (in flies of genotype for-GAL4;UAS-GFP/+ or for-GAL4;UAS-GFP/+ for11.247-GAL4;UAS-GFP/+;TeTx-in flies of genotype for-GAL4;UAS-GFP/+;TeTx-in) revealed expression in the aristae (AR), a subset of ORNs of the 3rd antennal segment, discrete glomeruli of the antennal lobe (AL), the pars intercerebralis (PIs), and very weakly in the MB and lateral cell (LC) (Fig. 4A, 4B, data not shown). In for11.247 homozygotes (flies of genotype for-GAL4;UAS-GFP/+;TeTx+/+) we observed stronger GFP expression in the MB, as well as additional expression in the EB and the pigment-dispersing factor (PDF)-expressing ventral lateral neurons (LNv) (Figs. 4C, S3A–C).

This GFP expression pattern partially overlaps, in the MB and LN, with that observed with the FOR antibody [44]. We did not, however, detect GFP expression in the DPCs, which stain with a FOR antibody (Fig. S3D); thus DPCs may not express the FOR-T1 isoform of FOR. Expression of GFP was also observed in regions not labeled by the FOR antibody, including the ORNs, LNv, and PI neurons. However, our behavioral data suggests that FOR is likely expressed in ORNs (see below). Furthermore, mammalian PKG is expressed in ORNs and the suprachiasmatic nucleus [48]. Therefore, FOR may not be expressed at levels detectable by this FOR antibody in the ORNs, LNv, and some neurons of the PI.

for-T1 Functions in for11.247-GAL4 Neurons to Inhibit Olfactory Startle Habituation

In order to test if for-T1 functions in the neurons defined by for11.247-GAL4, we attempted to rescue the enhanced habituation of for11.247 by expressing for-T1 with for11.247-GAL4. Indeed, expressing for-T1 in homozygous for11.247-GAL4 flies restored normal OSH (Fig. 5A, 5B). We conclude that the enhanced OSH of for11.247 flies is due to reduced for-T1 expression and that for-T1 function in for11.247-GAL4 neurons is sufficient for flies to show normal habituation.

Synaptic Silencing of ORNs Inhibits Olfactory Startle Habituation

We next investigated whether the activity of neurons expressing for11.247-GAL4 directly regulates OSH. To test this, we first blocked synaptic release in for11.247-GAL4+/+ expressing neurons with tetanus toxin light chain (TeTx) [49]. Blocking synaptic release by expressing TeTx in for11.247-GAL4+/+ expressing neurons reduced OSH (Fig. 6A), without affecting the initial startle (Fig. S4A). Further, expressing an inactive form of TeTx (TeTx–) in for11.247-GAL4+/+ expressing neurons did not alter OSH (Fig. 6A).
These data suggest that synaptic activity of the ORNs, a few MB neurons and/or PI neurons promotes habituation. To further define the neurons regulating OSH, we next silenced specific subsets of for11.247-GAL4/+ expressing neurons. As blocking synaptic release in the MB is already known to regulate OSH [22], we focused on other neurons of the for11.247-GAL4-expression pattern, specifically the ORNs and LNvs.

To test if the activity of the ORNs promotes habituation, we silenced them by expressing TeTx with the Odorant receptor co-receptor-GAL4 (Orco-GAL4) driver, which is expressed in ~80% of ORNs [50]. Like for11.247-GAL4/+ expressing neurons, synaptic silencing of the ORNs also significantly suppressed OSH (Fig. 6B), without affecting the initial startle (Fig. S4B). Therefore, neuronal transmission in the ORNs defined by Orco-GAL4 is required to promote OSH.

Since the PDF-expressing LNvs are also labeled by for11.247-GAL4 (Fig. S3A-C), we next tested if synaptic activity of the LNv neurons regulates OSH. However, silencing neurotransmission in the LNvs, by expressing TeTx with Pdfl-GAL4 [51], did not affect OSH (Fig. S4C). Therefore, our data suggest that LNv neurons do not regulate OSH.

Figure 3. Molecular characterization of for alleles. A) Schematic of the for transcription unit, with insertion sites of for11.247 and for2614. Blue bars represent translation start/stop sites, gray bars represent region probed for for-T1/T3 and for-T2 transcripts. The 3 major for isoforms, collectively called for-T1/T2/T3 have a total of nine splice forms, all encoding a common kinase domain at the 3' end. FOR-T1 is a 1088 amino acid (aa) protein encoded by for-RA/RI, FOR-T2 is a 894 aa protein encoded by for-RC/RD/RG/RK, and FOR-T3 is a 742 aa protein encoded by for-RB. B) Northern blot of adult fly mRNA using probes specific to for-T1/T3 or for-T2 transcripts. B, top panel) In the control strain (wBerlin) we detected two bands with the for-T1/T3 probe. Based on its size, the upper, more intense, band corresponds to for-T1 transcripts, while the lower, less intense band, to the for-T3 transcript. Compared to wBerlin and for11.247 (a precise excision of for11.247), a reduced intensity of for-T1, but not for-T3 transcripts, was seen in for11.247 and for2614. B, middle panel) Using a for-T2 probe we detected no differences in levels of for-T2 transcripts in either for11.247 or for2614. B, bottom panel) A tubulin probe was used to compare total mRNA levels. C) Quantification of Northern Blot showing reduced for-T1, but not for-T2 or for-T3, in for11.247 and for2614. Levels were calculated as a ratio between for and tubulin band intensity. D) Quantification and representative Western blot of extracts from adult heads analyzed with an antibody that recognizes FOR-T1. Compared to controls, we saw a reduction of FOR-T1 in both for11.247 and for2614 (p<0.001; n = 3). doi:10.1371/journal.pone.0051684.g003
Figure 4. Expression pattern of for11.247-GAL4. A, B) Expression of for11.247-GAL4/+;UAS-GFP/+ flies. A) In the antenna, GFP (green) was expressed in the arista (AR) and a sub-population of olfactory receptor neurons (ORNs) in the third antennal segment. B) In the CNS, GFP was expressed in specific glomeruli in the antennal lobe (AL), par intercerebals (PI) neurons, and weakly in the mushroom body (MB) and lateral cells (LC). C) In for11.247-GAL4/+;UAS-GFP/+ flies strong GFP expression was seen in MB, PIs, LC and sub-oesophageal ganglion (SOG), as well as the ventral lateral neurons (LNvs), the giant dorsal interneuron (DGI), parts of the antennal lobe (AL) and ellipsoid body (EB). D) Higher magnification of for11.247-GAL4/UAS-GFP/+ flies showing partial co-localization with a FOR antibody (red) in the MB and LC, but not in DGI.

doi:10.1371/journal.pone.0051684.g004

Figure 5. for11.247-GAL4 flies expressing for-T1 have normal olfactory startle habituation. A) Habituation profile of functional rescue of for11.247-GAL4 by expressing UAS-for-T1. No significant difference in distances travelled were seen between for11.247-GAL4;UAS-for-T1/+ and either Ctrl or UAS-for-T1/+ at pulse 2, 3 and 4. A significant difference was only seen between for11.247-GAL4;UAS-for-T1/+ and for11.247 (p<0.01; n = 12). B) HI of for11.247 rescue. No significant differences were seen between for11.247-GAL4;UAS-for-T1/+ and either Ctrl or UAS-for-T1/, but were observed between for11.247-GAL4;UAS-for-T1/+ and for11.247 (p<0.01; n = 12).

doi:10.1371/journal.pone.0051684.g005
Figure 6. Analysis of neuronal circuitry implicated in olfactory startle habituation. A) Blocking synaptic activity in for11.247-GAL4 neurons reduces OSH. Heterozygous for11.247-GAL4 flies expressing tetanus toxin (TeTx) had reduced OSH. Significant differences were seen between for11.247-GAL4/+ or UAS-TeTx/+ and for11.247-GAL4/+UAS-TeTx/+ (p<0.001; n = 9). No significant difference was seen in flies expressing inactive TeTx (TeTx0) with for11.247-GAL4/+ (p>0.05; n = 9). B) Synaptic silencing of ORNs inhibits OSH. Expressing UAS-TeTx with Orco-GAL4 significantly reduced OSH. Differences were observed between Orco-GAL4/+ or UAS-TeTx/+ and Orco-GAL4/+UAS-TeTx/+ (p<0.01; n = 6). No effect was seen upon expressing UAS-TeTx with Orco-GAL4/+ (p>0.05; n = 9). C) for-T1 overexpression in ORNs inhibits OSH. Expressing UAS-for-T1 with Orco-GAL4, but not with MB driver OK107-GAL4, reduced OSH. Significant differences were observed between Orco-GAL4/+ or UAS-for-T1/+ and Orco-GAL4/+UAS-for-T1/+ (p<0.001; n = 14), but not between controls and OK107-GAL4/+UAS-for-T1/+ (p>0.05; n = 8).

doi:10.1371/journal.pone.0051684.g006

not regulate OSH. In summary, our data indicate that synaptic activity of ORNs promotes OSH.

FOR-T1 Overexpression in the ORNs Inhibits Olfactory Startle Habituation

We have shown that for-T1 inhibits OSH (Fig. 5) and that blocking synaptic release in ORNs, which likely express for-T1 (Fig. 4A), also reduces OSH (Fig. 6B). Therefore, it is possible that for-T1 inhibits OSH by decreasing synaptic release in ORNs. If this was the case, increasing levels of for-T1 in ORNs should reduce OSH. Indeed, similar to the effect of silencing ORNs with TeTx, overexpression of for-T1 with Orco-GAL4 significantly reduced OSH (Fig. 6C). Therefore, for-T1 may inhibit OSH by reducing synaptic release in ORNs. We also tested whether for-T1 overexpression in MB affects OSH. However, flies expressing for-T1 with the pan-MB driver OK107-GAL4 had a normal OSH (Fig. 6C), suggesting that in the MB for-T1 may not regulate OSH.

To conclude, our data suggest that for-T1 may act primarily in ORNs to inhibit OSH and a possible FOR-T1 function here is reduction of synaptic release after an initial exposure to ethanol vapor.

Discussion

We describe the isolation of Drosophila mutants that disrupt olfactory startle habituation (OSH); of these 26 mutants, the majority showed enhanced OSH. Additional targeted analysis also identified several strains carrying mutations in genes that play a role in septate junctions thus implicating this structure in regulating OSH. We characterized two mutations in for that enhanced OSH due to reduced expression of a specific for product, FOR-T1. We show that for-T1 limits OSH by function in a subset of neurons that include ORNs and the MB. Our data further map for-T1 function primarily to ORNs, implying that OSH can occur in the sensory neurons of the olfactory circuit.

for encodes several isoforms of protein kinase G (PKG), a cGMP-dependent serine/threonine kinase that regulates neuronal excitability [52] and leaning and memory [46]. With respect to habituation, the natural variant (for) with reduced PKG activity [45] also has reduced habituation of the giant-fiber system, which mediates escape responses to visual stimuli [41] and the gustatory-based PER [42], implying that for limits these behaviors. We now show that for also limits OSH; thus for appears to be a central suppressor of habituation, regardless of sensory modality. A question remains as to whether for isoforms and their function is similar in these separate neuronal populations. Interestingly, the mammalian PKG with highest homology to for, PRKG1, [53] has been associated with Attention Deficit/Hyperactivity Disorder [54], a condition characterized by a persistent lack of attention possibly due to a failure to habituate to large amounts of information received from the environment [55].

Ethanol activates several olfactory receptors (ORs): OR7a, OR22a, OR35a, OR85b (http://neuro.uni-konstanz.de/DoOR). Although, curiously, activity of the ORNs expressing these ORs does not appear to be needed for flies to initially sense the smell of ethanol, as the magnitude of the initial startle response was unaffected by synaptic silencing using Orco-GAL4. Interestingly, one glomerulus that appeared labeled in for11.247-GAL4 heterozygotes is VC31, which expresses OR35a, the OR most strongly activated by acute ethanol. Therefore, VC31 maybe a glomerulus mediating ethanol-induced OSH. It is also worth noting that, in addition to activating particular ORs, ethanol is also a known GABA_A receptor agonist [56] and may also act on GABA_A receptors expressed in LNs and PNs that promote OSH [18,32,57].

How might for-T1 function in ORNs to limit OSH? Since for-T1 overexpression in ORNs, or their synaptic silencing, reduced OSH, for-T1 may limit OSH by decreasing synaptic release. Indeed, cultured neurons of for flies with reduced PKG activity [45] exhibit increased excitability, resulting in increased spontaneous and evoked activity [52]. for-T1 may achieve decreased synaptic release by modulating cAMP levels, as PKG does in mammalian ORNs [38,59]. Alternatively, as in the mammalian neurons, it may phosphorylate a number of possible substrates
including: TRPC channels, which regulate Ca\(^{2+}\) influx [60], SEPTIN3, a regulator of vesicle targeting or tethering [61,62], or transporters of serotonin [63,64], a neurotransmitter implicated in presynaptic inhibition in the AL [37].

Finally, our data suggest that olfactory habituation can occur in the 1st order neurons of the olfactory circuit (the ORNs), while several recent papers demonstrate that the 2nd order neurons of the olfactory circuit (the LNs and PNs) are key players in olfactory habituation [17,18,19,27,32]. MB silencing and ablation experiments also suggest that these 3rd order neurons are also involved [20,22]. Indeed, studies in the rat show that olfactory cortex and not peripheral circuits, regulate olfactory habituation [65]. Therefore, the capacity to habituate to olfactory cues appears to be distributed throughout the olfactory circuit. Indeed, synaptic silencing of either the ORNs (this study) or the MB [22] did not completely block OSH, as one might expect if habituation occurred at a singular point in the circuit. This distributed mechanism of habituation may allow the fruit fly a greater flexibility in the interplay between its innate responses and learnt experience.

Supporting Information

Figure S1 for\(^{11.247}\) has enhanced OSH in two genetic backgrounds. A) for\(^{11.247}\) in the uBerlin background has enhanced OSH (p<0.001; n = 7, Unpaired t-test). for\(^{11.247}\) in the 2202U isogenic background has enhanced OSH (p>0.0134; n = 7, Unpaired t-test). B) for alleles have a normal initial startle response. Total movement during the first ethanol pulse was similar between Ctrl, for\(^{11.247}\) and for\(^{2614}\) (p>0.05; n = 8). C) FOR-T3 are unaffected in for\(^{11.247}\) and for\(^{2614}\). Representative Western blot of adult heads using an antibody that recognizes FOR-T3.

Figure S2 PKG activity levels do not correlate with OSH or for\(^{T7}\) levels. A) Levels of PKG activity levels were significantly different between control strains uBerlin and Ctrl (p<0.001; n = 5), precluding informative conclusions about PKG activity in for\(^{11.247}\) and for\(^{2614}\), which were also significantly different from each other (p<0.001; n = 5). B) for\(^{5}\), for\(^{6}\) and for\(^{7}\) did not show significant differences in OSH (p>0.05; n = 6–9).

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Figure S3 for\(^{11.247}\)-GAL4 is expressed in PDF expressing neurons, but not DPC neurons. A) Expression of GFP (green) in for\(^{11.247}\)-GAL4 flies revealed expression in the lateral ventral neurons (LNv, s), identified in (B) by a PDF antibody (red). B) Co-localization of GFP and PDF in for\(^{11.247}\)-GAL4/+/UAS-GFP/+ flies. D) Co-staining of for\(^{11.247}\)-GAL4/+/UAS-GFP/+ flies with FOR antibody (red), revealed no co-localization in the dorsal posterior cells (DPCs).

Figure S4 for\(^{11.247}\)-GALA and Orco-GALA neurons expressing TeTx have a normal initial startle. A) No difference in total movement in the initial startle was seen between for\(^{11.247}\)-GAL4/+;UAS-TeTx/+ and for\(^{11.247}\)-GAL4/+ and UAS-TeTx/+ (p>0.05; n = 9). B) No difference in total movement in the initial startle was seen between Orco-GAL4/+;UAS-TeTx/+ and UAS-TeTx/+ (p>0.05; n = 6). C) Expressing Tetanus Toxin in PDF neurons did not alter OSH. No significant difference in HI was seen between Ppdf-GAL4/+;UAS-TeTx/+ and Ppdf-GAL4/+;UAS-TeTx/+ (p>0.05; n = 8–12).

Table S1 Habituation Index of P elements inserted in or 5’ to septate junction genes.

Acknowledgments

We thank Fred Wolf, Anita Devineni, Karla Kaun and the anonymous reviewers for helpful comments that improved this manuscript.

Author Contributions

Conceived and designed the experiments: ME UH. Performed the experiments: ME ATB. Analyzed the data: ME ATB. Contributed reagents/materials/analysis tools: ME ATB MBS UH. Wrote the paper: ME UH.
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