Γενετική και Μοριακή Διερεύνηση της Εξοικείωσης στη *D. melanogaster*

Διδακτορική Διατριβή

Ουρανία Σεμελίδου

Πανεπιστήμιο Κρήτης

Ιατρική Σχολή

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Ourania Semelidou

University of Crete

Medical School

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Τριμελής Συμβουλευτική Επιτροπή

Σαββάκης Χαράλαμπος, Ομότιμος Καθηγητής, Πανεπιστήμιο Κρήτης

Σκουλάκης Ευθύμιος, Ερευνητής ΕΚΕΒΕ «Αλέξανδρος Φλέμινγκ»

Ταβερναράκης Νεκτάριος, Ερευνητής ΙΜΒΒ

Επταμελής Εξεταστική Επιτροπή

Δελιδάκης Χρήστος, Ερευνητής ΙΜΒΒ, Καθηγητής Πανεπιστήμιο Κρήτης

Καραγωγέως Δόμνα, Ερευνήτρια ΙΜΒΒ, Καθηγήτρια, Πανεπιστήμιο Κρήτης

Ποϊράζη Παναγιώτα, Ερευνήτρια ΙΜΒΒ

Ζέρβας Χρήστος, Ερευνητής ΙΒΕΑΑ

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Περίληψη

Όλοι οι οργανισμοί εκτίθενται καθημερινά σε πληθώρα ερεθισμάτων τα οποία αξιολογούν και στα οποία εν συνεχεία αποκρίνονται. Η εξοικείωση αποτελεί τη βάση της επιλεκτικής προσοχής, καθώς επιτρέπει τη βαθμιαία προσαρμογή της απόκρισης σε μη σημαντικά, επαναλαμβανόμενα ερεθίσματα. Πρόκειται για έναν τύπο μη συνειρμικής μάθησης απαραίτητο για την επιβίωση και ως εκ τούτου συντηρημένο κατά την εξέλιξη. Επιπλέον, η εξοικείωση συχνά περιγράφεται ως η βάση της συνειρμικής μάθησης, καθώς η διατήρηση της απόκρισης σε ένα ερέθισμα είναι απαραίτητη για την σύνδεσή του με άλλα ερεθίσματα του περιβάλλοντος.

Στον άνθρωπο, διαταραχές στην εξοικείωση έχουν συνδεθεί με γνωσιακές δυσλειτουργίες καθώς και με παθήσεις όπως η σχιζοφρένεια, οι διαταραχές του αυτιστικού φάσματος, η Διαταραχή Ελλειμματικής Προσοχής – Υπερκινητικότητας, η οριακή (μεταιχμιακή) διαταραχή προσωπικότητας και οι ημικρανίες. Τα εύρος των ασθενειών που παρουσιάζουν διαταραχές στην εξοικείωση καταδεικνύει τη σημασία αλλά και την πολυπλοκότητα της διεργασίας αυτής, ενώ η αντιμετώπιση των προαναφερθέντων ασθενειών χρήζει βαθύτερης κατανόησης της εξοικείωσης. Ωστόσο, μέχρι σήμερα, δεν έχουν ταυτοποιηθεί οι μηχανισμοί που διέπουν την μείωση της απόκρισης σε μη σημαντικά ερεθίσματα.

Έχοντας ως στόχο την μελέτη των νευρωνικών υποσυνόλων που εμπλέκονται στην εξοικείωση ανέπτυξα ένα νέο παράδειγμα για τη μελέτη της εξοικείωσης σε οσφρητικά ερεθίσματα στη *Drosophila melanogaster*. Χρησιμοποιώντας το παράδειγμα αυτό, δείχθηκε ότι η συνεχής ή επαναλαμβανόμενη έκθεση τόσο σε απωθητικές όσο και σε ελκτικές οσμές για τέσσερα λεπτά είναι επαρκής για να οδηγήσει σε μείωση της αρχικής απόκρισης. Η προσαρμογή της συμπεριφοράς που παρατηρήθηκε παρουσιάζει όλα τα χαρακτηριστικά της εξοικείωσης, όπως αυθόρμητη ανάκαμψη της αρχικής απόκρισης μετά από απομάκρυνση του ερεθίσματος (spontaneous recovery), μείωση της απόκρισης μόνο στο επαναλαμβανόμενο ερέθισμα και όχι σε άλλα νευρωνικά ερεθίσματα (γενίκευση – generalization), πιο γρήγορη μείωση της απόκρισης σε λιγότερο δυνατά ερεθίσματα, και απεξοικείωση (dishabituation). Επιπλέον, η χρήση αυτού του παραδείγματος επέτρεψε την κατηγοριοποίηση της απόκρισης των ατόμων σε δύο στάδια και τον περαιτέρω χαρακτηρισμό τους μέσω χρήσης γενετικών εργαλείων.

Η μελέτη των δύο διακριτών σταδίων απόκρισης κατέδειξε ότι ο οργανισμός επιστρατεύει διαφορετικά νευρωνικά υποσύνολα ανάλογα με τη διάρκεια του ερεθίσματος, και η ενεργοποίηση των νευρώνων αυτών οδηγεί σε διαφορετική απόκριση. Αρχικά η απόκριση διατηρείται για ένα χρονικό διάστημα το οποίο ονομάζεται λανθάνουσα περίοδος (habituation latency). Κατά τη λανθάνουσα περίοδο απαιτείται η ενεργοποίηση των ανασταλτικών τοπικών διάμεσων νευρώνων και των διεγερτικών νευρώνων προβολής των οσφρητικών λοβών, όπως επίσης και η ενεργοποίηση των αβ και α'β' λοβών των μισχοειδών σωματίων. Επιπλέον, απαραίτητη για τη διατήρηση της απόκρισης κατά τη λανθάνουσα περίοδο είναι η έκφραση της αδενυλικής κυκλάσης Rutabaga στους ανασταλτικούς τοπικούς διάμεσους νευρώνες των οσφρητικών λοβών. Αντίθετα, η περίοδος εξοικείωσης, η οποία ακολουθεί της λανθάνουσας περιόδου μετά από επαρκή έκθεση στο οσφρητικό ερέθισμα, απαιτεί την ενεργοποίηση των ανασταλτικών νευρώνων προβολής των οσφρητικών λοβών, οι οποίοι επιστρατεύονται μετά από συνεχόμενη ενεργοποίηση των διεγερτικών νευρώνων προβολής. Τα αποτελέσματα των πειραμάτων εξοικείωσης καταδεικνύουν έναν πολύ σημαντικό ρόλο για το πλάγιο κέρας (lateral horn), μίας περιοχής του εγκεφάλου απαραίτητης για τις εγγενείς συμπεριφορές στη Drosophila, στη ρύθμιση της απόκρισης σε οσφρητικά ερεθίσματα. Πιο συγκεκριμένα, η ενεργοποίηση της περιοχής αυτής από τα μισχοειδή σωμάτια και τους διεγερτικούς νευρώνες προβολής είναι απαραίτητη για την αποφυγή της πρώιμης εξοικείωσης και τη διατήρηση της απόκρισης κατά τη λανθάνουσα περίοδο, ενώ η καταστολή του πλάγιου κέρατος από τους ανασταλτικούς νευρώνες προβολής μετά από επαρκή έκθεση στο ερέθισμα οδηγεί σε εξοικείωση. Επιπλέον, πειράματα στα οποία πραγματοποιήθηκε εκτεταμένη έκθεση στην οσμή (τριάντα λεπτά), κατέδειξαν ότι η διάρκεια έκθεσης στο ερέθισμα οδηγεί σε διαφορετικά είδη εξοικείωσης. Η μείωση της απόκρισης μετά από εκτεταμένη έκθεση σε μία οσμή απαιτεί διαφορετικά ερεθίσματα για την επίτευξη απεξοικείωσης, εξαρτάται από την έκφραση διαφορετικών γονιδίων και διέπεται από διαφορετικά νευρωνικά υποσύνολα. Πιο συγκεκριμένα, η αυξημένη διάρκεια της έκθεσης στο ερέθισμα οδηγεί σε εξοικείωση εξαρτώμενη από τους ανασταλτικούς νευρώνες προβολής του οσφρητικού λοβού και όχι από τους ανασταλτικούς νευρώνες προβολής, ενώ η έκφραση της αδενυλικής κυκλάσης Rutabaga που είναι επουσιώδης κατά την εξοικείωση μετά από μικρό διάστημα έκθεσης στο ερέθισμα, είναι απαραίτητη για τη μείωση της απόκρισης που ακολουθεί την εκτεταμένη έκθεση στην οσμή.

Παράλληλα με τα πειράματα μελέτης της εξοικείωσης, πραγματοποιήθηκαν πειράματα απεξοικείωσης ώστε να εξεταστούν τα νευρωνικά υποσύνολα που διέπουν αυτήν την διεργασία. Η απεξοικείωση, η οποία αποτελεί τη στιγμιαία επανάκτηση της αρχικής απόκρισης μετά από έκθεση του ατόμου σε ένα νέο, ανεξάρτητο ερέθισμα, είναι μία διεργασία ελάχιστα μελετημένη και λίγα είναι γνωστά για τους μηχανισμούς που εμπλέκονται σε αυτήν. Χρησιμοποιώντας το παράδειγμα της εξοικείωσης σε οσφρητικά ερεθίσματα, πραγματοποιήσαμε απεξοικείωση της απόκρισης μετά από έκθεση σε μηχανικά ερεθίσματα και με τη χρήση γενετικών εργαλείων μελετήσαμε τα νευρωνικά υποσύνολα που είναι απαραίτητα για την επανάκτηση της αρχικής απόκρισης στην οσμή. Τα πειράματα αυτά κατέδειξαν ότι η ενεργοποίηση δύο νευρωνικών υποσυνόλων, των ντοπαμινεργικών PPL1 νευρώνων και των οκτοπαμινεργικών πρόσθιων συζευγμένων πλευρικών (APL) νευρώνων είναι απαραίτητη και επαρκής για τη φυσιολογική απεξοικείωση. Οι νευρώνες αυτοί δημιουργούν συνάψεις με τους νευρώνες των μισχοειδών σωματίων οι οποίοι μεταφέρουν περαιτέρω την πληροφορία στους νευρώνες του πλάγιου κέρατος, καταδεικνύοντας το βασικό ρόλο της ενεργοποίησης αυτών των περιοχών στην επανάκτηση της απόκρισης στο ερέθισμα που πραγματοποιείται κατά την απεξοικείωση.

Τέλος, μελετήθηκε ο ρόλος των σεροτονεργικών υποδοχέων στην εξοικείωση με πειράματα εξοικείωσης σε ηλεκτρικό ερέθισμα. Τα αποτελέσματα κατέδειξαν το ρόλο της σεροτονίνης στη διεργασία αυτή, καθώς μεταλλάξεις σε σεροτονεργικούς υποδοχείς προκαλούν ελλειμματική εξοικείωση στο ηλεκτρικό ερέθισμα.

Συγκεντρωτικά, τα αποτελέσματα αυτής της μελέτης αποσαφηνίζουν το ρόλο διαφορετικών νευρωνικών υποσυνόλων στην προσαρμογή της απόκρισης σε ένα ερέθισμα κατά την εξοικείωση και την απεξοικείωση, και παρέχουν το υπόβαθρο για περαιτέρω μελέτες με στόχο την ταυτοποίηση των μοριακών μηχανισμών που διέπουν τις διεργασίες αυτές.

Abstract

Habituation is the process that enables salience filtering, precipitating perceptual changes that alter the value of environmental stimuli. To discern the neuronal circuits underlying habituation to brief inconsequential stimuli, we developed a novel olfactory habituation paradigm, identifying two phases of the response that engage distinct neuronal circuits. Responsiveness to the continuous odor stimulus is maintained initially, a phase we term habituation latency, and requires Rutabaga Adenylyl-Cyclase-depended neurotransmission from GABAergic Antennal Lobe Interneurons and activation of excitatory Projection Neurons (PNs) and the Mushroom Bodies. In contrast, habituation depends on the inhibitory PNs of the middle Antenno-Cerebral Track, requires inner Antenno-Cerebral Track PN activation and defines a temporally distinct phase. Collectively, our data support the involvement of Lateral Horn excitatory and inhibitory stimulation in habituation.

In parallel, we investigated the neuronal subsets that underlie dishabituation, the instant recovery of naive response after exposure to a novel stimulus. We have demonstrated that exposure to mechanical stimuli results in dishabituation of the olfactory response and we identified two neuronal subsets, the dopaminergic PPL1 and the octopaminergic anterior paired lateral (APL) neurons, which are necessary for response recovery after electric shock stimulation. Innervation of the Mushroom bodies by these neurons and further activation of the Lateral Horn underlines the role of these neuropils in modulation of the response and proposes a novel role for them in dishabituation.

Finally, we examined the role of serotonin, which has been linked to various disorders, including schizophrenia and anxiety disorders, in habituation to electric shock. We have demonstrated that serotonin is necessary for normal habituation, and mutations on the serotonergic receptors disrupt this process.

Collectively, our results provide essential cellular substrates for future analyses of the molecular mechanisms that govern the duration and transition between the distinct temporal habituation phases, and dishabituation.

Introduction

1. Habituation

Animals perceive a multitude of stimuli that undergo evaluation every day. Habituation is the behavioral modification whereby responses to repetitive or continuous stimuli not associated with other salient stimuli or events are attenuated (Harris 1943). This behavioral response decrement does not involve sensory adaptation, sensory fatigue or motor fatigue and is a form of non-associative learning, where the subject learns about the quality of a stimulus (Rankin, Abrams et al. 2009).

The ability of an organism to evaluate every stimulus is not only extraordinary, but of great significance as well. Habituation has been observed in an wide range of species, from motile single-celled organisms such as the amoeba (Groves and Thompson 1970) and *Stentor coeruleus* (Jennings 1906), to sea slugs (Gagliano, Renton et al. 2014) and humans (Pellegrino, Sinding et al. 2017). The evolutionary conservation of the process indicates its significant role in survival. Habituation allows information filtering, thus enabling responses to potentially important stimuli while the animal ignores the insignificant ones. Proper evaluation of stimuli affects associative learning as well. Since habituation results in stimulus devaluation, preventing its premature onset is essential to maintain information content and salience long enough to allow association with other stimuli or events. This led to the notion that habituation is a “building block for associative learning” and deficient habituation may be connected with cognitive dysfunction (Schmid, Wilson et al. 2014). Understanding the relationship between non-associative and associative learning is necessary to verify the possible role of abnormal habituation in cognitive dysfunction.

In humans, habituation paradigms have been used to assess cognitive abilities (Chard, Roulin et al. 2014) and recent studies indicated that genes involved in intellectual disability are linked to impaired habituation (Lugtenberg, Reijnders et al. 2016, Stessman, Willemsen et al. 2016). Habituation deficiencies have been linked to various disorders, such as schizophrenia (Akdag, Nestor et al. 2003, Ludewig, Geyer et al. 2003), migraines (Kalita, Bhoi et al. 2014, Kropp, Wallasch et al. 2015), attention-deficit/hyperactivity disorder (Jansiewicz,

Newschaffer et al. 2004, Massa and O'Desky 2012) and autism spectrum disorders (Bruno, Garrett et al. 2014, Lovelace, Wen et al. 2016, Tam, King et al. 2017). The implication of habituation in multiple cognitive disorders and its potential effects on associative learning highlight the significance of understanding the molecular mechanisms and neuronal circuitry that govern it.

Because habituation is a well-conserved process, its features and parameters have been studied in various organisms. In 1966, Thompson and Spencer first described the parametric characteristics of habituation (Thompson and Spencer 1966).

1.1 The characteristics of habituation

In 1966, Thompson and Spenser reviewed the habituation literature with emphasis on electrophysiological studies, concluding that habituation is a central neural process which conforms to specific characteristics. These characteristics were further described as nine parametric relations for stimulus and training variables common in the habituated response of various intact organisms. *Rankin et al*. revised the parameters of habituation in 2009, based on the forty years of studies that followed Thompson and Spenser's review (Rankin, Abrams et al. 2009).

The characteristics of habituation are applied in a variety of habituation paradigms assaying different responses, studied in numerous organisms and are described below.

1. 'Given that a particular stimulus elicits a response, repeated applications of the stimulus result in decreased response (habituation). The decrease is usually a negative exponential function of the number of stimulus presentations.'

All behavioural studies of a regularly presented stimulus show response attenuation after certain repetitions. Behavioral responses that undergo habituation may include any output of the nervous system, from simple reflexes such as pupillary responses, sweating, and muscle contraction to more intricate ones, such as stimulus avoidance. Repeated application of a stimulus results in a progressive decrease of the response to an asymptotic level and that decrement is usually exponential, but it may also be linear. This decrement may refer to a number of the response parameters, such as frequency, magnitude or duration of the response (Rankin, Abrams et al. 2009).

2. ‘If the stimulus is withheld, the response tends to recover over time (spontaneous recovery).’

A habituated response should spontaneously recover after stimulus withdrawal and this recovery might be full or partial (Rankin, Abrams et al. 2009). The time interval necessary for spontaneous recovery of the response to a particular stimulus varies among different paradigms, depending on the protocol used and the response studied. Importantly, many variables influence the time course of spontaneous recovery to a particular response. An enlightening example is the spontaneous recovery of the habituated startle response to sound in intact rats. Depending on testing parameters, this habituated response may recover in 10 minutes (Prosser and Hunter 1936) or fail to recover even after 24 hours (Thompson 2009).

3. ‘If repeated series of habituation training and spontaneous recovery are given, habituation becomes successively more rapid (this might be called potentiation of habituation).’

Potentiation of habituation was first described on turtle leg withdrawal to shell tap (Humphrey 1933). Later on, studies in many animals described this phenomenon. Potentiation of habituation in humans was studied with EEG recordings of individual visual evoked potentials, where a statistically significant linear declining trend in the magnitude of habituation was observed over the course of the experiment (Wastell and Kleinman 1980). Potentiation of habituation suggests that the response attenuation after repetitive stimulation is not merely the outcome of an established refractory period, but that habituation shows plasticity and is a dynamic process in which evidence of long-term storage of neuronal modifications can be discerned.

4. ‘Other things being equal, the more rapid the frequency of stimulation, the more rapid and/or more pronounced is habituation.’

This phenomenon was observed even in the first reflex studies (Harris 1943) and occurs within certain limits in terms of number of trials as well. Furthermore, spontaneous recovery becomes more rapid after more frequent stimulation, as shown in various organisms including depression at *Aplysia* sensory-motor synapses, *C. elegans* tap habituation and rats startle response (Rankin, Abrams et al. 2009). In mechanosensory tap habituation in *C.*

elegans the rate of spontaneous recovery depends on the frequency of the applied stimulus during training rather than the level of habituation achieved, the number of stimuli delivered or the number of missed stimuli before the recovery test (Rankin and Broster 1992).

5. 'The weaker the stimulus, the more rapid and/or more pronounced is habituation. Strong stimuli may yield no significant habituation.'

This stimulus strength-habituation relationship is characteristic of most types of responses, ranging from simple reflexes (Harris 1943) to complex exploratory behaviour (Welker 1961). The inability to habituate to strong stimuli is probably beneficial to the animals, since such stimuli are potentially harmful. In contrast, fast habituation to weak inconsequential stimuli enables the animals to shift their attention to other, possibly more salient stimuli. Interestingly, a study on the habituation of the startle response in the rat indicated that a stimulus of gradually increasing intensity results in stronger habituation than a constant loud stimulus, while a constant stimulus of intermediate intensity shows the weakest habituation when tested against a loud intensity stimulus (Davis and Wagner 1969).

6. 'The effects of habituation training may proceed beyond the zero or asymptotic response level.'

Repetition of a stimulus after attenuation of the response is cumulative and can alter subsequent behavior, such as the onset of spontaneous recovery. Experiments on mechanosensory tap habituation in *C. elegans* showed that increased number of habituating stimuli leads to delayed spontaneous recovery onset. However, the number of stimuli did not affect the rate of spontaneous recovery, which remained the same once recovery started (Rankin and Broster 1992).

7. 'Habituation of response to a given stimulus exhibits stimulus generalization to other stimuli.'

The generalization characteristic described by Thompson and Spencer in 1966 was significantly revised by Rankin et al, since additional studies demonstrated that generalization occurs only in the case of stimuli within the same modality (Rankin, Abrams et al. 2009). However, it was later shown that habituation can be specific even between stimuli of the same modality (e.g. olfactory stimuli) that are encoded by different neuronal subsets in the brain (Das, Sadanandappa et al. 2011).

To test for stimulus specificity/stimulus generalization, a second, novel stimulus is presented, and a comparison is made between the changes in the response to the habituated stimulus and the novel stimulus. This characteristic is of great importance, because while stimulus generalization can lead to habituation between similar, innocuous stimuli, it is essential for the animal to maintain the ability to respond to novel stimuli. Furthermore, this property differentiates habituation with general sensory adaptation or fatigue, since cross-habituation/generalization can be observed only within similar stimuli.

8. ‘Presentation of another (usually strong) stimulus results in recovery of the habituated response (dishabituation).’

Dishabituation is the phenomenon whereby presentation of a different stimulus after habituation results in recovery of the initial response. The dishabituating stimulus (or dishabituator) by itself need not even trigger a response on its own and naïve animals should not change their response to the initial stimulus after exposure to the dishabituating stimulus. It was traditionally thought that the dishabituating stimulus must be a strong one, but a study in *Aplysia* showed that a touch or a weak electric shock produced better dishabituation than a strong shock did (Marcus, Nolen et al. 1988). Dishabituation has been commonly used to demonstrate that habituation has occurred and to distinguish this process from fatigue, while various stimuli has been used as dishabituators.

9. ‘Upon repeated application of the dishabituating stimulus, the amount of dishabituation produced habituates (this might be called habituation of dishabituation).’

Habituation of dishabituation follows a negative exponential course, as was shown in rats, cats, and humans (Rankin, Abrams et al. 2009).

10. ‘Some stimulus repetition protocols may result in properties of the response decrement (e.g. more rapid rehabituation than baseline, smaller initial responses than baseline, smaller mean responses than baseline, less frequent responses than baseline) that last hours, days or weeks.’

Habituation that lasts hours or days is termed ‘long-term habituation’ and has been shown in a number of systems, including *Aplysia* (Castellucci, Carew and Kandel, 1978; Ezzadine and Glanzman 2003), *C. elegans* (Rose , Kaun, Chen and Rankin, 2003), *D.*

melanogaster (Acevedo, Froudarakis et al. 2007, Das, Sadanandappa et al. 2011), rats (Bhatnagar, Huber, Nowak and Trotter, 2002), and humans (Maschke, Drepper, Kindsvater, Kolb, Diener and Timmann, 2000). Depending on the protocol used, habituation comes in at least two forms, short-term habituation and long-term habituation. The distinction is the duration of the effects of training and the demonstrated changes in protein synthesis that underlie long-term habituation. In Drosophila, for example, olfactory long term habituation depends on long-lasting potentiation of inhibitory synapses through CREB and synaptic growth-dependent processes (Das, Sadanandappa et al. 2011).

Studies of habituation may also measure cellular or molecular responses or neuronal activity, including population activity, such as measured with EEG or functional imaging. These responses at the molecular, cellular or population levels may be monitored in an effort to identify underlying mechanisms or they may be used as indices of habituation.

1.2 Habituation Studies

Habituation was first studied at the end of the nineteenth century and by the beginning of the twentieth century the process was thought as an 'instance of elementary learning', a central phenomenon for all organisms with a nervous system (Thompson 2009). In 1956, Jasper and Sharpless studied habituation of arousal focusing on the shift of electroencephalographic frequency (EEG arousal) of sleeping cats. They observed a stimulus specific decrement of response which recovers spontaneously after withdrawal of the stimulus or when the animals experienced a different, strong stimulus (dishabituator) (Jasper and Sharpless 1956). Sharpless and Jasper's interesting findings revived the interest in habituation as a fundamental form of behavioral plasticity. Further experiments similar to the EEG arousal in the cat were conducted in human, focusing on the alpha blocking response. The alpha rhythm of the EEG disappears or shows decreased amplitude when attention is focused on a stimulus (Berger 1929), a phenomenon called alpha blocking response. Interestingly, studies demonstrated that repetitive tactile, auditory and visual stimulation result in habituation of the human alpha blocking response (Sokolov 1960). Later on, Glickman and Feldman's experiments in cats revealed that habituation depends on the central nervous

system, since peripheral receptors are probably not involved in habituation of EEG arousal to sensory stimulation (Glickman and Feldman 1961). Following these studies, habituation was investigated using various types of stimuli at most levels of the CNS, from first-order sensory nuclei to the cerebral cortex. Thompson and Spencer studied habituation of the spinal flexion reflex, showing that skin receptors, cutaneous afferent nerve terminals and motor neurons are not the loci underlying the decreased response, confirming that the process takes place in the central nervous system (Thompson and Spencer 1966).

1.2.1 Habituation in *Aplysia californica*

Aplysia californica has been extensively used in learning and memory studies because of the reflexive withdrawal of its gill and siphon in response to weak or moderate tactile stimulation of its skin (Figure 1). Habituation of this withdrawal reflex after repetitive stimulation was first described in 1970 (Pinsker, Kupfermann et al. 1970) and is mainly caused by the depression of transmission at the monosynaptic connection between sensory and motor neurons. Short-term habituation lasts for less than 30 minutes and is mediated solely by presynaptic depression at the sensorimotor synapse, due to silencing of release sites and not vesicle depletion (Gover, Jiang et al. 2002). In contrast, long-term habituation of this withdrawal reflex requires spaced training, lasts for more than a day and involves postsynaptic activation of AMPA- and NMDA-type receptors, the activity of protein phosphatases 1, 2A, and 2B, as well as activity of voltage-dependent Ca^{2+} channels. Long-term habituation results in decreased number of terminal varicosities (sites of presynaptic release) on the axonal branches of sensory neurons, less neurotransmitter vesicles, and decreased number and area of active zones postsynaptically. Studies of the sensorimotor synapse in dissociated cell culture showed long-term homosynaptic depression (LTD) that requires elevated intracellular Ca^{2+} within the motor neuron, whereas isolated motor neurons in culture demonstrated LTD of the glutamate response ("hemisynaptic" LTD) that depends on AMPA- and NMDA-type receptor activity and activation of group I and II metabotropic glutamate receptors (mGluRs) (Glanzman 2009).

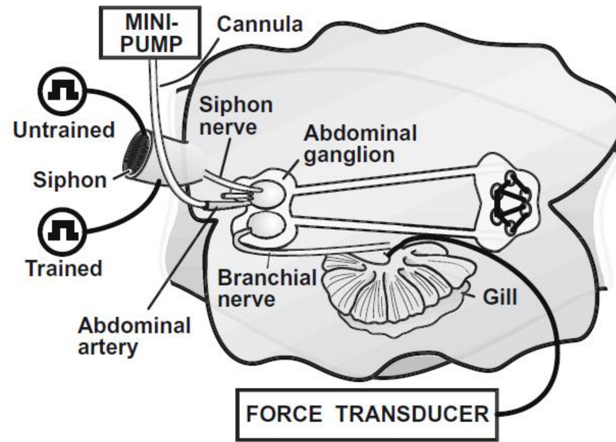


Figure 1. Aplysia californica reduced preparation for the study of withrawal reflex habituation (Ezzeddine and Glanzman 2003).

1.2.2 Habituation in Caenorhabditis elegans

Habituation in C. elegans was first described in 1990, when repetitive stimulation resulted in attenuation of the reverse response elicited by a mechanical tap (tap withdrawal response) (Rankin, Beck et al. 1990). Tap withdrawal habituation is mediated by multiple mechanisms differentially activated by different stimulation parameters (Rankin and Broster 1992) and is mediated by five mechanosensory neurons and their synaptic connections onto the interneurons responsible for the tap response.

Substantial work has been done concerning the genes implicated in tap withdrawal habituation, using various mutations available in *C. elegans* (Giles and Rankin 2009). The abnormal, faster habituation of *eat-4* vesicular glutamate transporter mutants suggested a role for glutamatergic neurotransmission in the process while dopamine is another neurotransmitter investigated for its function in habituation. Mutations in the D1-like dopamine receptor homolog *dop-1* affect habituation differentially depending on the inter-stimulus intervals (ISI) applied. Animals with mutated *dop-1* show more rapid habituation when 10-s ISI is applied, but not when the ISIs are longer. This phenotype was further validated in tyrosine hydroxylase *cat-2* mutants, which have significantly decreased dopamine levels since tyrosine hydroxylase catalyzes the rate-limiting step of dopamine synthesis. In contrast, *dat-1* knockout mutants who lack the gene encoding for the dopamine reuptake transporter and therefore have stronger and more sustained dopamine neurotransmission,

present slower habituation compared to wild type animals, suggesting that the level of dopamine neurotransmission is crucial for normal habituation.

Since DOP-1 is a G-protein coupled receptor, various downstream effectors of the receptor were tested for their role in habituation (Giles and Rankin 2009). Mutations in the G protein alpha subunit G_q EGL-30 or its common effector, the phospholipidase-C_b (PLC_b) EGL-8 which produces the second messengers diacyl glycerol (DAG) and inositol triphosphate (IP3), showed similar phenotypes to *dop-1* and *cat-2* mutants. DAG and IP3 are known to influence intracellular calcium signaling by activating enzyme like protein kinase C (PKC) and calcium channels on the endoplasmic reticulum. As expected, *pkc-1* mutants show more rapid habituation than wildtype animals. Further studies demonstrated that mechanical stimulation results in a large calcium influx concentration in the anterior mechanosensory neurons that is gradually decreased with repeated stimulation, and this decrement occurs faster in *dop-1, egl-8,* and *pkc-1* mutants.

Apart from short-term habituation, *C. elegans* also exhibits long-term habituation after spatial training, a process that depends on the interstimulus interval duration, lasts at least one day and requires new protein synthesis (Giles and Rankin 2009). Long-term habituation relies on glutamatergic neurotransmission, with both EAT-4, the vesicular glutamate transporter, and GLR-1, the ionotropic glutamate receptor subunit homologous to the mammalian AMPA-type glutamate receptor subunit GluR1, being essential for long-term attenuation of the response.

1.2.3 Habituation in rodents

A. Startle response habituation

The mammalian startle response is a protective response that results in the contraction of skeletal and facial muscles when animals are exposed to sudden acoustic, tactile or vestibular stimuli. Short-term habituation of the startle response occurs upon repeated presentation of a stimulus and presents spontaneous recovery within several minutes (Pilz and Schnitzler 1996).

Habituation of the startle response in mice is located within the primary startle pathway (Koch 1999) (Figure 2), at the sensory synapses on giant neurons in the caudal

pontine reticular nucleus (PnC) (Lingenhohl and Friauf 1994, Pilz and Schnitzler 1996). Short trains of action potentials in sensory afferent fibers induce depression of synaptic responses in PnC giant neurons, a phenomenon that has been proposed as the cellular correlate for short-term habituation. These relatively few giant neurons (Lee, Lopez et al. 1996) receive multiple sensory inputs converging from auditory, trigeminal and vestibular afferent pathways (Koch and Schnitzler 1997) and their axons project directly onto motoneurons that innervate the facial and skeletal muscles (Lingenhohl and Friauf 1994).

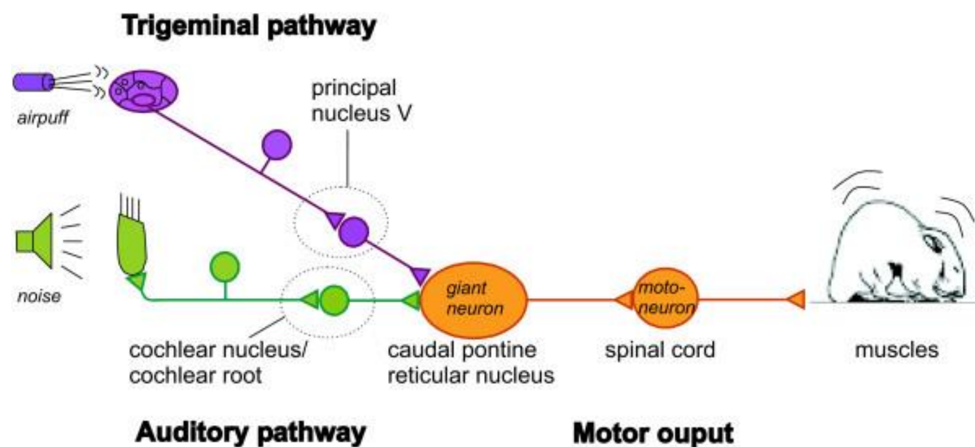


Figure 2. Scheme of the primary startle pathway in rats and mice. Acoustic or tactile information is conveyed by only two serial types of sensory neurons in each pathway to the caudal pontine reticular nucleus (PnC). Synapses of secondary sensory neurons converge onto giant neurons that form the sensorimotor interface of the startle response. Axons of the PnC giant neurons directly project onto motorneurons in the facial nucleus or spinal cord (Simons-Weidenmaier, Weber et al. 2006).

Electrophysiological and behavioral experiments on acoustic startle response in mice indicated that synaptic depression in the PnC and behavioral short-term habituation are located presynaptically, at the sensory part of the startle pathway, and prior to multimodal signal integration (Simons-Weidenmaier, Weber et al. 2006). In addition, the large conductance voltage- and calcium-activated potassium (BK) channel, which was previously found necessary for habituation in *Drosophila melanogaster* (Engel and Wu 1998), is essential for normal short-term habituation to acoustic startle response in mice (Typlt, Mirkowski et al. 2013). BK channels are densely expressed in synaptic terminals in cerebral cortex,

hippocampus, hypothalamus, brainstem, and spinal cord (Tseng-Crank, Foster et al. 1994, Sausbier, Sausbier et al. 2006) and are activated in response to Ca^{2+} influx and membrane depolarization (Lang and Ritchie 1987). These channels control neurotransmitter release by shortening the duration of presynaptic action potentials (Martire, Barrese et al. 2010, Griguoli, Sgritta et al. 2016) and are well suited to mediate synaptic depression. Interestingly, BK channels are necessary in short-term habituation of startle response, but in not motivated behavior habituation (exploratory locomotor behavior in the open field box) or long-term habituation (Typlt, Mirkowski et al. 2013). In addition, haplo-insufficiency in the gene *KCNMA1* which encodes for a BK channel, has been detected in an ASD subject and was further confirmed in 116 people with autism, suggesting a possible association between the BK channel-dependent deficient synaptic neurotransmission and autistic disorder (Laumonnier, Roger et al. 2006).

The crucial role of BK channels in short-term habituation was also confirmed in an independent study in rats, where these channels are localized predominantly on glutamatergic terminals synapsing onto giant neurons in the PnC and regulate synaptic transmission. BK channel activation and its subsequent phosphorylation upon strong synaptic stimulation by various kinases including CaMKII was found to be critical for synaptic depression within the startle pathway, underlying short-term startle response habituation in rats (Zaman, De Oliveira et al. 2017).

These results confirm the notion that even though there are common key players necessary in habituation, there is no universal mechanism underlying this process, but a variety of different mechanisms that contribute to the overall behavioral response, dependent on the sensory pathway studied, the time course of stimulation, and the behavioral/electrophysiological read out. Habituation is a process occurring in every synapse, but the molecular mechanisms that different neurons utilize to receive and transmit signals may diverge, and thus different neurons may recruit distinct mechanisms to achieve attenuation of the response.

B. Olfactory habituation

The olfactory system has a relatively simple, well-described anatomy, with strong reciprocal connections to the limbic system and an important role in reflexive and motivated

behaviors – characteristics that render it as a great substrate to study habituation. Indeed, in the rodent olfactory system, repeated odor stimulation was shown to evoke habituation of both autonomic and behavioral responses (Wilson and Linster 2008).

Studies on rodent olfactory habituation have identified two fundamentally different types of habituation, operant at different timescales, encoding memories of different duration and specificity, depended on distinct glutamatergic systems and residing in different olfactory structures. Short-exposure (short-timescale) habituation is odor-specific, while long-exposure (long-timescale) habituation presents generalization between odors. Studies on the molecular components of these two habituation types indicated that mGluR is necessary for habituation after short exposure to the stimulus, while N-methyl-D-aspartate (NMDA) receptor is required for response attenuation after exposure of a longer timescale (McNamara, Magidson et al. 2008). In addition, the short and long timescale odor habituation apparently are mediated by different neuronal subsets. Short timescale habituation is mediated within the anterior piriform cortex, and more specifically the piriform cortical pyramidal cells, where adaptation is associated with mGluR II/III-mediated depression of the glutamatergic mitral-pyramidal cell synapse (Wilson 1998, Wilson 1998, Best, Thompson et al. 2005, Yadon and Wilson 2005). In contrast, habituation to longer timescales depends on the olfactory bulb (McNamara, Magidson et al. 2008). The olfactory bulb mitral cells were shown to adapt to odorants depending on stimulus duration and intertrial interval parameters as well as on functioning NMDA receptors (Chaudhury, Manella et al. 2010). These results highlight the importance of stimulus duration in habituation and demonstrate how the same behavioral output – response attenuation – can be the outcome of different molecular mechanisms and neuronal subsets, depending on the stimulation parameters.

1.2.4 Habituation in human

A. Olfactory habituation

Experiments on human subjects have shown an exponential decline of the perceived odor intensity after continuous or repetitive exposure to the odorant (Cain 1977, Dalton and Wysocki 1996, Hummel, Knecht et al. 1996, Wang, Walker et al. 2002, Jacob, Fraser et al. 2003, Stuck, Fadel et al. 2014). The degree of habituation is influenced by the concentration

of the odorant, with a lower concentration usually resulting in more rapid and pronounced habituation (Cain 1977, Jacob, Fraser et al. 2003, Stuck, Fadel et al. 2014). As shown for habituation in animals, in most cases the response is not completely attenuated (Ekman, Berglund et al. 1967). Habituation shows spontaneous recovery, which depends on the duration and concentration of the stimulus, as well as the subjects' age and mood (Ekman, Berglund et al. 1967, Cain 1977). In addition, cross-habituation between two odorants (also called cross-adaptation) has been observed, especially between structurally similar odorants or unfamiliar odors that are less discriminable (Pierce, Zeng et al. 1995, Pierce, Wysocki et al. 1996). Interestingly, cross-adaptation is not reciprocal, and its effect is always weaker than habituation to only one odorant. Humans also present long-term habituation, as shown by a study where subjects were exposed to an odorant for six hours per day for the interval of two weeks. Recovery of long-term habituation was slower, with only half of the individuals presenting complete recovery after two weeks. Interestingly, in a study evaluating habituation to 32 different odorants, activation of the trigeminal nerve, which is associated with pain, appeared as a factor that strongly reduces habituation. Other factors that affect olfactory habituation are some of the physicochemical characteristics of the odor, like high vapor pressure, small molecular weight, and low number of double bonds (Sinding, Valadier et al. 2017).

Aiming to investigate the contribution of the peripheral and central nervous system in human habituation, an early study used a mono-rhinal odor exposure protocol, measuring neuronal adaptation at the exposed and the contralateral nostril. This study revealed a role for the cerebral neurons in habituation, without excluding the implication of the peripheral system (Cain 1977). Further investigation of the role of peripheral neurons had been conducted using the electro-olfactogram (EOG), a validated technique that represents the summated generator potentials of olfactory receptor neurons in response to an olfactory stimulus (Getchell and Shepherd 1978, Lapid and Hummel 2013). This technique allows the recording of neuronal input from the peripheral olfactory system during adaptation while simultaneously obtaining psychophysical responses in awake humans. EOG experiments showed that recordings from peripheral neurons were obtained even after behavioral habituation was observed. Perceived odor intensities decrease more quickly than electrical peripheral recordings, suggesting that peripheral neurons don’t mediate rapid adaptation,

and thus proposing a crucial role for the central nervous system in the process (Hummel, Knecht et al. 1996, Hummel, Mojet et al. 2006).

In agreement with work in rodents focusing on the neurons underlying olfactory habituation, functional magnetic resonance (fMRI) experiments in humans showed that habituation occurs in the primary olfactory cortex after 30-60 s of odor stimulation (Sobel, Prabhakaran et al. 2000, Poellinger, Thomas et al. 2001). A similar adaptation during prolonged odor exposure was shown for the hippocampus and anterior insula, suggesting a close interaction between these areas and the primary olfactory cortex, while the orbitofrontal cortex exhibited a sustained increase in activation (Poellinger, Thomas et al. 2001). The significant activation of the orbitofrontal cortex throughout stimulation suggested a role for these neurons in controlling the activity of piriform cortex, likely through inhibitory connections (Poellinger, Thomas et al. 2001). Further studies on human habituation using electroencephalography (EEG) recorded the olfactory event-related potential (OERP) in response to odors embedded in a constant air-flow. These studies suggested that olfactory adaptation is more rapid at the perceptual level (~ 2.5 s) than the electrophysiological (4–10 s). In addition, even though the OERP remained at about 50% with increasing pulse duration, perceived intensities completely adapted to zero (Wang, Walker et al. 2002). Interestingly, another study demonstrated that behavioral habituation began after the initial decrease in OERP responses at the central level (Boesveldt, Haehner et al. 2007), verifying the crucial role of the central nervous system in habituation.

B. Auditory and visual habituation

Habituation of auditory or visual stimuli has been studied using functional neuroimaging methods, indicating a major role for the amygdala in the process. An fMRI study on human auditory habituation proposed an amygdalocortical network as the potential basis of affective habituation in humans. Continuous auditory stimulation (with piano melodies for 43 minutes) linked long-term habituation with attenuated responses in the laterobasal amygdala, associated cortical areas and areas important for valence and arousal ratings. In contrast, decreased responses in the primary auditory cortex were recorded within seconds, indicating that short-term and long-term habituation reside in different neuronal structures (Mutschler, Wieckhorst et al. 2010). Repeated presentations of emotional facial expressions

resulted in decreased activation of the left dorsolateral prefrontal and premotor cortex, and the right amygdala (Phillips, Medford et al. 2001, Wright, Fischer et al. 2001). The amygdala was shown to be preferentially activated in response to emotional stimuli (fearful or happy faces versus neutral ones), while repeated presentation results in rapid habituation in this area (Breiter, Etcoff et al. 1996), underlining the important role of the central nervous system in habituation.

1.3 Habituation links with human disorders

Abnormalities in habituation have been reported in a variety of neuropsychiatric disorders, with the severity of habituation deficits predicting the symptom severity across these disorders. More specifically, abnormal habituation has been linked with Autism Spectrum Disorder, Fragile X syndrome, Schizophrenia, Parkinson's Disease, Huntington's Disease, Attention Deficit Hyperactivity Disorder, Tourette's Syndrome, and Migraines. The role of abnormal habituation in some of these diseases will be further discussed in the following chapters.

1.3.1 Habituation in Autism Spectrum Disorder

Autism spectrum disorder (ASD) refers to a number of neurodevelopmental disorders characterized by three core deficits: impaired communication, impaired reciprocal social interaction and restricted, repetitive and stereotyped patterns of behaviors or interests. Genetic and non-genetic risk factors are thought to contribute to ASD, with deficits in synaptogenesis and axon motility playing a potentially crucial role in the manifestation of the disorder. Neuropathological and neuroimaging studies have demonstrated that the amygdala, a major component of the limbic system and the affective loop of the cortico-striatothalamo-cortical circuit, is implicated both in cognition and ASD.

Amygdala dysfunction has been proposed as a critical component in the social impairment observed in autism spectrum disorders. A study on children and adolescents with ASD has shown decreased amygdala habituation to sad and neutral faces and suggested a role for the ventromedial prefrontal cortex-depended modulation of amygdala in the process (Swartz, Wiggins et al. 2013), while another study demonstrated differential amygdala

habituation in response to fearful faces between control and ASD individuals (Kleinhans, Richards et al. 2016). In addition, children with ASD showed decreased habituation of the autonomic arousal responses after repeated exposure to facial stimuli, specifically concerning their responses to direct gaze (Kaartinen, Puura et al. 2016). Importantly, the level of behavioral and amygdala habituation has been related to the severity of the disorder, associating severe social impairment with lower levels of habituation to face stimuli (Kleinhans, Johnson et al. 2009, Kaartinen, Puura et al. 2016).

Deficient amygdala habituation in response to social stimuli in ASD was found to be genetically influenced. The serotonin transporter-linked polymorphic region variant (5-HTTLPR) was shown to impact the severity of the disorder, since individuals with ASD and low expressing 5-HTTLPR genotypes exhibit worse social symptoms (Brune, Kim et al. 2006). In addition, 5-HTTLPR was shown to impact changes in amygdala response to repeated sad face presentation differently in individuals with ASD compared to controls. More specifically, individuals with ASD and low expressing 5-HTTLPR genotypes presented disrupted amygdala habituation to sad faces and displayed a statistical trend toward sensitization, or increase in activation over time, in contrast to controls or individuals with ASD and higher expressing genotypes (Wiggins, Swartz et al. 2014). Besides the amygdala, disrupted serotonergic innervation of the nucleus accumbens, a part of the reward system, is also considered as a mechanism underlying the social behavior deficits observed in ASD (Walsh, Christoffel et al. 2018).

Apart from the social impairment, inability or slower rate of habituation was also suggested to mediate the atypical sensory behaviors of autism. Autistic individuals exhibit a slower rate of short-term habituation to startle response (Ornitz, Lane et al. 1993), while infants with high risk for developing autism present deficient habituation to repeated sounds (Guiraud, Kushnerenko et al. 2011). In addition, children with ASD exhibit impaired responses and habituation to tactile stimulation, suggesting a deficit in the somatosensory inhibitory system (Puts, Wodka et al. 2014).

1.3.2 Habituation in Schizophrenia

Schizophrenia is a complex, heterogeneous behavioural and cognitive syndrome that begins in young adult years and lasts a lifetime. Prodromal symptoms often precede acute psychosis, including cognitive dysfunction and negative symptoms such as social withdrawal, self-neglect, loss of motivation and initiative and emotional blunting. Schizophrenia has long been associated with abnormal stimulus processing and aberrant patterns of arousal that are thought to reflect disturbances in the reticular-activating system of the brain. Schizophrenic patients exhibit sensorimotor gating and habituation deficits that prevent them from screening out trivial, distracting stimuli to attend to and process salient stimuli in the environment (Braff, Swerdlow et al. 1995).

Prepulse inhibition (PPI), the normal suppression of the startle reflex when an intense startling stimulus is preceded by a barely detectable prepulse, and acoustic startle reflex habituation have both been proposed as sensitive intermediate phenotypic markers for information-processing deficits in schizophrenic patients (Ludewig, Geyer et al. 2003). Indeed, schizophrenic patients exhibit extensive deficits in both intramodal and cross-modal sensorimotor gating, with deficient prepulse inhibition of startle amplitude when an acoustic prepulse stimulus precedes either an acoustic or a tactile startle stimulus (Braff, Grillon et al. 1992). Schizophrenic subjects also demonstrate significantly reduced rate of eye blink habituation (Akdag, Nestor et al. 2003) as well as enhanced sensitization, presumably due to an abnormal arousal modulation (Meincke, Light et al. 2004).

Functional MRI studies have shown that patients with schizophrenia exhibit reduced habituation of the hippocampus and visual cortex, and a lack of neural discrimination between old and new images in the hippocampus; a function that correlates with memory performance, further suggesting that reduced habituation may contribute to the memory deficits commonly observed in schizophrenia (Williams, Blackford et al. 2013). Another study has validated the implication of defective hippocampus habituation in schizophrenia, describing impaired right hippocampal habituation in response to fearful faces in schizophrenic individuals (Holt, Weiss et al. 2005).

Patients with schizophrenia also present impaired habituation of autonomic nervous system responses, such as abnormal habituation of skin conductance activity (ectodermal activity), a phenotype that is proposed as a behavioral marker of genetic predisposition

to schizophrenia. An increasing level of genetic risk for schizophrenia has been related to impaired habituation of autonomic nervous system activity over time. Individuals with two schizophrenia-spectrum parents presented no habituation, those with one spectrum parent presented some habituation, and those with normal parents habituated rapidly. In addition, subjects who developed schizophrenia in adulthood presented significant deficits in habitation as adolescents (Hollister, Mednick et al. 1994).

1.3.3. Attention Deficit Hyperactivity Disorder

Attention Deficit Hyperactivity Disorder (ADHD) is one of the most common childhood disorders, affecting 3-7% of the population, while the main symptoms of the disorder are retained well into adulthood. ADHD is characterized by a persistent pattern of inattention and/or hyperactivity-impulsivity. Structural and functional imaging data suggest that children, adolescents, and adults with ADHD have decreased volume and abnormal activation of the frontal lobe brain regions, as well as decreased activation and volume of the anterior cingulate cortex (Schulz, Fan et al. 2004, Seidman, Valera et al. 2006, Semrud-Clikeman, Pliszka et al. 2006, Schneider, Krick et al. 2010). The results obtained from habituation studies on ADHD patients have been contradictory, with subjects presenting deficient, enhanced, or normal habituation.

Habituation plays an important role in attention and the frontal cortex was found to mediate selective attention via habituation to the insignificant peripheral stimuli (Mennemeier, Chatterjee et al. 1994). These results propose that impaired habituation and inability to filter out irrelevant stimuli might lead to deficient attention. Indeed, adults with ADHD exhibit decreased habituation rate to peripheral stimuli in comparison with control subjects (Massa and O'Desky 2012). In addition, children with ADHD exhibit impaired visual habituation, suggesting that abnormal habituation contributes to off-task behavior in children with the disorder (Jansiewicz, Newschaffer et al. 2004).

In contrast to these reports, participants with ADHD showed enhanced habituation in experimental paradigms where the task required active decisions and sustained attention. Children with ADHD displayed faster heart rate habituation to reward, but in contrast to the control group, they did not present increased skin conductance levels when the reward was

removed (Iaboni, Douglas et al. 1997). Another study showed that children with ADHD, Oppositional defiant disorder (ODD) and Conduct disorder (CD) showed increased habituation of skin conductance responses to signal stimuli but not to passive stimuli (Zahn and Kruesi 1993). However, ADHD children showed reduced basal skin conductance responses and more rapid habituation to passive, but not to active acoustic stimuli in another study (Shibagaki, Yamanaka et al. 1993). Importantly, the rapid habituation of ADHD individuals may underlie the performance deficits they present in tasks requiring sustained attention and associative memory, since enhanced habituation may contribute to the devaluation of stimuli that act as reinforcers in memory formation (Lloyd, Medina et al. 2014).

In conclusion, both reduced and enhanced habituation were suggested to contribute to the impairments in attention and associative learning observed in ADHD, and additional studies are necessary to clarify the link between habituation and attention.

1.4 Habituation of the unconditioned stimulus in associative learning

Habituation has been often described as a ‘building block’ for associative learning because of its fundamental role in stimulus evaluation. In classical conditioning, a conditioned stimulus (CS) acquires the ability to trigger a new response after being paired with an unconditioned stimulus (US), which is capable of triggering an innate response. Hence, the signal value of both the conditioned and the unconditioned stimulus is important for associative learning. Habituation to one of the two stimuli would reduce its value leading to a weaker association with the second stimulus. Indeed, when animals are pre-exposed to the CS before conditioning the strength of association is decreased. Similarly, the US efficacy is known to decrease with extended associative training (Cevik and Erden 2012). In 2013, *Lloyd et al* proposed that the unconditioned stimulus used as a reinforcer during associative learning presents habituation, a process they named habituation of reinforcer effectiveness (HRE). Repeated presentation of a reinforcing stimulus decreases its effectiveness, while treatment with stimulant drugs that increase dopamine neurotransmission disrupts the slow-rate HRE. The authors proposed that abnormal HRE due genetic and/or environmental factors may underlie particular behavioral disorders, such as ADHD, that might reflect a condition of accelerated HRE (Lloyd, Medina et al. 2014).

1.5 The role of dopamine in habituation

Dopamine is cardinal in motor coordination, motivation, reward, addiction, learning, and memory. Dopamine release in the nucleus accumbens has been linked to the efficacy of unconditioned rewards, while dopaminergic neurotransmission at the terminal fields of the nigrostriatal, mesolimbic and mesocortical dopamine systems, but not that the nucleus accumbens, is crucial to attach motivational importance to otherwise neutral environmental stimuli during learning and memory formation (Wise 2004). In addition, changes in dopaminergic signaling were shown in a number of human neurologic and psychiatric disorders. Abnormal dopaminergic transmission is implicated in schizophrenia, attention deficit hyperactivity disorder, and drug addiction.

The role of dopamine in plasticity has been demonstrated in *C. elegans*, where mutations in dopamine receptors disrupt habituation. Dopamine signaling via the D1-like dopamine receptor DOP-4 resulted in slower backward locomotion habituation (Ardiel, Giles et al. 2016), while mutations in the D1-like dopamine receptor gene *dop-1* or in the tyrosine hydroxylase gene *cat-2* resulted in faster habituation to non-localized mechanical stimulation (Sanyal, Wintle et al. 2004).

However, the role of dopamine in mouse habituation is not as straightforward as in *C. elegans*. Hyperdopaminergic mutant mice show hyperactivity and impaired habituation in novel environments but treating DAT knockdown mice with indirect or direct dopamine receptor agonists inhibits their locomotor activity. These results highlight the importance of the balance between dopamine receptor activation pre- and postsynaptically, providing insight for the possible therapeutic effect of psychostimulants that interfere with dopaminergic neurotransmission in attention deficit hyperactivity disorder (Zhuang, Oosting et al. 2001).

In rats, studies have demonstrated that taste stimuli increase dopamine neurotransmission in different dopaminergic terminal areas of the brain, such as the nucleus accumbens (NAc) shell and core and the medial prefrontal cortex (mPFC). However, only the NAc shell displays habituation after a single trial. Enhanced dopamine release after repeated administration of morphine (morphine sensitization) was shown to abolish habituation of

dopaminergic responsiveness in NAc shell, while it induced habituation in mPFC. Interestingly, NAc shell habituation is dependent upon an intact dopaminergic input to the mPFC (Bimpisidis, De Luca et al. 2013, De Luca 2014).

Studies in humans have demonstrated an important role for dopaminergic neurotransmission in habituation to painful stimuli. Treatment with haloperidol, a typical antipsychotic drug that acts as a dopamine receptor antagonist, blocks habituation to painful stimuli and the neuronal adaptation that occurs in the left postcentral gyrus and midcingulate cortex in the placebo condition (Bauch, Andreou et al. 2017).

1.6 The role of serotonin in habituation

Serotonin is implicated in mood, anxiety, stress, aggression, feeding, sexual behavior and cognition (Olivier 2015). Pharmacological disruption of serotonin neurotransmission facilitates memory formation in cerebral regions that are under dopamine modulation, such as the striatum and the PFC, while increased serotonin neurotransmission appears to have a detrimental effect on cognitive functions integrated in these structures. Drugs that modify dopamine or serotonin neurotransmission may exert beneficial effects on the cognitive functions of individuals with neurological and psychiatric diseases, which evolve with cognitive dysfunctions (Olvera-Cortes, Anguiano-Rodriguez et al. 2008). The abnormal habituation of schizophrenic patients was shown to be ameliorated after treatment with atypical antipsychotic drugs, that target serotonin, norepinephrine, and dopamine receptors (Oranje, Van Oel et al. 2002). However, the role of serotonin in habituation is not well studied. The impact of serotonergic neurotransmission in habituation was studied in rats, where decreased serotonin concentration abolishes habituation of exploratory motor activity in the open field (Bidzinski, Siemiatkowski et al. 1998). This study further proposed a functional interaction between brain serotonin and GABA systems in a behavioral and biochemical level. Additional studies on serotonin and habituation are necessary, to determine the role of serotonergic neurotransmission in the process.

2. Drosophila melanogaster

Drosophila are small flies in the order of Diptera and the family of Drosophilidae, commonly known as fruit or vinegar flies. *Drosophila melanogaster* in particular, is one of the organisms studied by geneticists in the 20th century and its technical advantages soon made it one of the models widely used in biomedical research to study a broad range of phenomena. *D. melanogaster* are easy and inexpensive to culture in laboratory conditions since their maintenance doesn't require sophisticated equipment, they have a short life cycle that lasts 60-80 days, produce large numbers of externally laid embryos and they can be genetically modified in numerous ways. Furthermore, there are fewer ethical issues involved in experimental Drosophila research compared with studies in laboratory rodents, or primates.

It has been more than a hundred years since William Castle's group at Harvard used the first time *Drosophila melanogaster* in the laboratory. However, it was Thomas Hunt Morgan who established Drosophila as a major model organism in genetics. Morgan refined the theory of inheritance first proposed by Gregor Mendel, defining genes and establishing that they were found within the chromosomes long before it was even established that DNA is the genetic material. Morgan won the Nobel Prize in Physiology or Medicine in 1933 "*for his discoveries concerning the role played by the chromosome in heredity*" while one of Morgan's protégés, Hermann Muller, won the Nobel Prize in Physiology or Medicine in 1946 "*for the discovery of the production of mutations by means of x-ray irradiation*". Using *D. melanogaster* in the 1920s, Muller demonstrated that x-rays cause a massive increase in gene mutations with the offspring of irradiated flies frequently showing the effects of mutation. This led to the realization that radiation causes harmful genetic defects in the offspring of exposed humans too – a timely observation given that this was at the advent of man's attempts to harness and exploit nuclear fission.

Drosophila has been since used as a model organism in many fields, such as cell and developmental biology, neurobiology and behavior, molecular biology, evolutionary and population genetics, and other fields. Studies using *Drosophila* led to major discoveries, establishing that many of the underlying building blocks and fundamental biological mechanisms are conserved across evolution between flies and human.

Additional evidence for the evolutionary relationship between D*rosophila* and humans was provided in the beginning of 2000, when the *Drosophila* genome was fully sequenced. Comparison between the *Drosophila* and human genome revealed that approximately 75% of the known human disease genes have a recognizable match in the genome of fruit flies, thus consolidating its legitimacy as a model organism for medical research. The homology between fly and human genes can widely vary and many fly genes have more than one human homolog. The fly genome contains approximately 16,000 genes, ∼13,000 of which encode proteins. Of the latter, more than 60% have human homologs and can be subdivided based on whether the fly homolog has multiple or single human homologs. However, the evolutionary relationships can be more complex in some cases, with multiple fly genes sometimes having a single human homolog, or multiple fly genes being homologous to multiple human genes (Wangler, Yamamoto et al. 2015).

2.1 The life cycle of *Drosophila melanogaster*

The *Drosophila* life cycle can be divided in four stages; egg, larva, pupa, and adult fly (Figure 3). *Drosophila* belongs to ectothermic species, and its developmental period varies with temperature. The shortest development time (from egg to adult) is 7 days, and it is achieved at 28C, while development times increase at higher temperatures (11 days at 30C) due to heat stress. Under ideal conditions, the development time at 25C is 10 days, while it takes 20 days at 18C. Furthermore, crowded conditions also affect development time, as well as the size of the progeny. Each female fly can lay up to ∼100 eggs per day, for up to 20 days. Once fertilized, the embryo develops in the egg for around one day (at 25C) before hatching as a larva. The larva grows over five days while molting twice into second- and third-instar larvae, at about 24 and 48 h after hatching, until it pupates and undergoes metamorphosis into the adult fly over the course of four days. During metamorphosis, most of the embryonic and larval tissue is destroyed. The adult tissues (e.g., wing, leg, eye) develop from groups of cells known as “imaginal discs” that have been set-aside since early embryonic development.

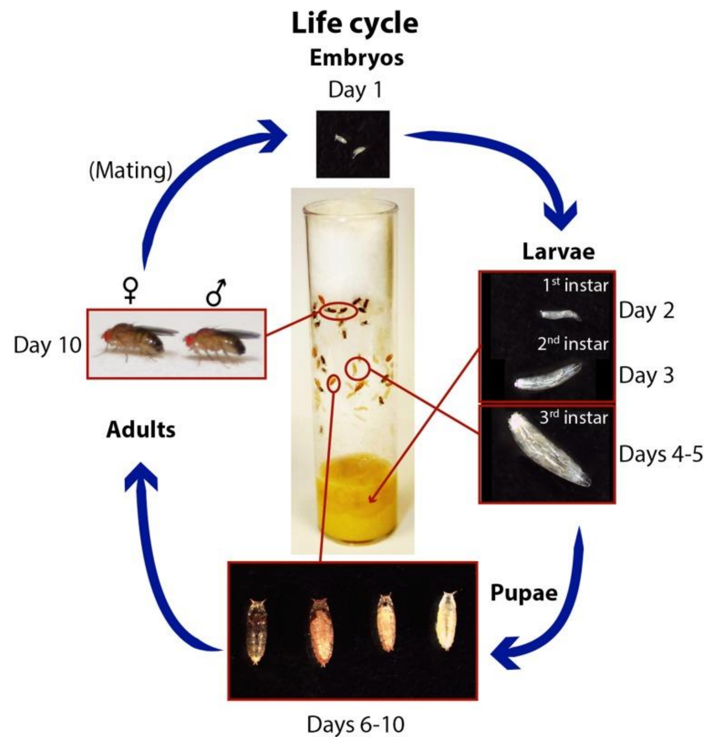


Figure 3. Life cycle of Drosophila melanogaster (Hales, Korey et al. 2015).

2.2 Genetic tools in *Drosophila melanogaster*

Genetic analysis is essential to understand the role of individual factors in a given biological process. One of the advantages of *D. melanogaster* is the range of genetic tools that have been developed throughout the years, which surpass those available in any other multicellular organism.

2.2.1 Mutations

Mutations have been widely used as the first approach to determine the role of a gene in a certain biological process. In *Drosophila,* there is a vast assortment of strains containing mutations, publicly available. Traditional ways of mutagenesis, such as chemical mutagens added to food or ionizing radiation such as X-rays have been used to trigger a variety of mutations, from point mutations to larger chromosomal changes (Greenspan 1997).

The identification of transposable elements provided an additional tool to alter genetic sequences through the mobilization of genetic elements throughout the genome.

More specifically, the identification and development of the *P*-element (Bingham, Kidwell et al. 1982, Rubin, Kidwell et al. 1982) as a germ-line transformation vector led to the production of mutations that were subsequently used in forward screens to identify the role of the mutated genes. Furthermore, by replacing the internal transposase gene with a gene of interest, the P-element provided an ideal system for inserting DNA into the fly genome (Rubin and Spradling 1982). Later on, the Gene Disruption Project used different transposable elements to achieve a broad coverage of insertions and to generate a variety of useful types of insertions. However, this approach has not completely saturated the genome, due to the particular idiosyncrasies of the elements (Bellen, Levis et al. 2004, Bellen, Levis et al. 2011, Venken, Schulze et al. 2011). Transposable element insertions have also been used to create small deletions through imprecise transposon excision (Voelker, Greenleaf et al. 1984), mutations in nearby genes through "local hopping" of the element (Tower, Karpen et al. 1993), and defined sets of isogenic deletions that cover the genome through the excision of DNA between elements at defined insertion points (Parks, Cook et al. 2004, Ryder, Blows et al. 2004, Thibault, Singer et al. 2004).

2.2.2. The UAS/GAL4 expression system and the TARGET system

The use of the *P*-element transformation vector to create a gene expression system was a major breakthrough in the *Drosophila* field, since it allowed selective activation of any cloned gene in a wide variety of tissue- and cell-specific patterns. The UAS/Gal4 system took advantage of the yeast transcription mechanism, using the gene encoding the yeast transcription activator GAL4. This gene was cloned into a *P*-element vector and got randomly inserted into the Drosophila genome. In this way, GAL4 expression was driven by one of a diverse array of genomic enhancers, acting as an enhancer trap. The subsequent introduction of a corresponding *P*-element vector, pUAST, containing the upstream activating sequences to which GAL4 protein binds followed by the gene of interest, resulted in the expression of this gene only in the cells where GAL4 is expressed. These UAS sequences were connected to a general *Drosophila* promoter and a cloning site to allow for the insertion of any gene of interest (Brand and Perrimon 1993).

The tissue-specific expression of a transgene using the UAS/Gal4 system requires two fly lines; one carrying the “driver”, where the gene encoding for the yeast Gal4 transcription factor is placed 3’ of a tissue-specific promoter/enhancer and the other carrying the gene of interest, placed 3’ of the upstream activated sequence (UAS) where Gal4 binds. Progeny of these two lines will carry both constructs and the Gal4 transcriptional factor expressed in a particular tissue will bind to the UAS, inducing the expression of the gene of interest only in specific cells (Figure 4).

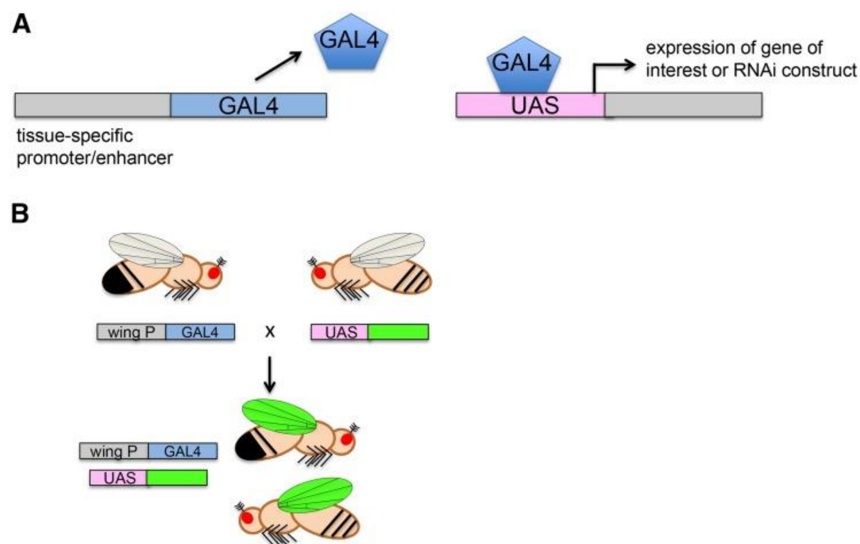


Figure 4. The UAS/Gal4 system. A Gal4 line that drives expression in the wings and a gene of interest that is indicated with the green color are used as an example (Hales, Korey et al. 2015).

This bipartite system can drive the expression of a gene in a defined way, allowing researchers to perform rescue experiments and other manipulations, such as silencing the expression of specific gene by the expression of a microRNA, small interfering RNA or hairpin RNA. Such constructs use the RNAi pathway and guide the target mRNAi to be degraded by the RNA-induced silencing complex (RISC) (Agrawal, Dasaradhi et al. 2003) only in the tissue where the Gal4 transcription factor is expressed. This temporary “knock down” enables researchers to elucidate the function of particular genes in a specific tissue.

The random insertion of both constructs in the UAS/Gal4 system leads to drivers with unpredictable expression levels but can also affect endogenous genes’ function. In addition,

the local transcriptional/chromatin environment may reduce expression of either the GAL4 transcription factor or the genes associated with the UAS elements. To overcome this issues, a new transformation system was developed using the serine recombinase protein from the PhiC31 bacteriophage to enable site-specific recombination using the attB and attP DNA sequences (Groth, Fish et al. 2004). The series of attP landing sites that were created at distinct points throughout the genome provided molecularly defined positions for the high-efficiency creation of transgenic flies using transformation vectors with an incorporated attB sequence (Venken, He et al. 2006). The characterization of these sites for the levels of expression of the inserted transgene allows for a fine tuned expression of a transgene as well as the comparison of different transgenes at the same chromosomal location having ensured that same levels of expression (Ni, Markstein et al. 2008).

An additional advancement of the Gal4 system came with further restriction of gene expression, with the development of split-Gal4 lines (Luan, Peabody et al. 2006, Pfeiffer, Ngo et al. 2010). These lines express either the DNA-binding domain or the transcription-activation domain of Gal4 in distinct spatial patterns. Because both domains must bind together to form a functional Gal4, transcription is driven only at the intersection of expression patterns of complementary split-Gal4 lines. A further restriction to the number of cells labeled in each transgenic line is ideal for several reasons. First, it facilitates the characterization of wiring and function of specific cells. If fewer cells are labeled, a cell of interest can be more precisely traced in morphology and reliably targeted with electrodes. Second, it accelerates the discovery of neurons driving the behavior. If cells of mixed types are manipulated together in a Gal4 line that elicits an interesting behavior, it is difficult to ascribe the phenotype to particular neurons. However, with the use of sparsely labeled lines the interpretation of the behavioral results becomes straight forward, although sometimes perturbing the activity of just a few neurons may not induce measurable phenotypes.

A powerful extension of this approach is to tag two sets of cells in a brain separately such that the activity of one set of cells is monitored while certain properties of the other are altered. This necessitates the use of another binary expression system that functions independently of the Gal4/UAS system. Such expression frameworks like the lexA/lexAop and Q systems have recently been engineered (Lai and Lee 2006, Potter, Tasic et al. 2010) and are broadly used to manipulate more than one specific neuronal subsets.

Finally, a substantial addition to the UAS/Gal4 expression system was the expression of the temperature-sensitive form of Gal80 (Gal80^{ts}) that enabled the temporal control of expression of the gene of interest. Gal80^{ts} represses GAL4 transcriptional activity at a permissive temperature (18C), while elevation of the temperature to 30C leads to degradation of Gal80 and allows Gal4 to bind on UAS again, thus driving the expression of the gene of interest (Figure 5). This system, called the TARGET system, provides temporal and regional gene expression, and allows the investigation of the role of genes in specific time-points in the animal's life. In addition, it provides a way to avoid potential developmental contributions of various genes, and focus only on their function in adulthood (McGuire, Mao et al. 2004).

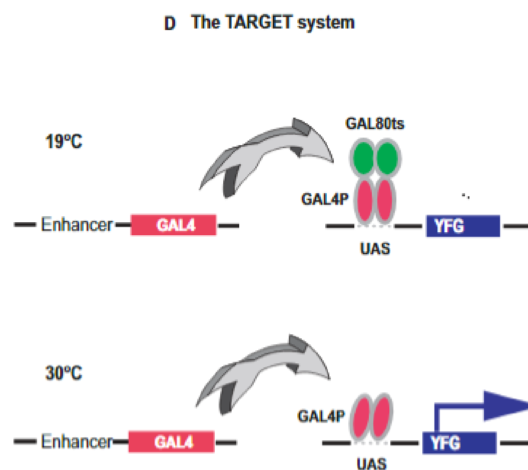


Figure 5. The TARGET system for spatial and temporal control of transgene expression (McGuire, Mao et al. 2004).

2.2.3. Transgenes used to dissect neuronal function.

Manipulation of certain cell subsets is necessary to understand their function. In neurobiology, inhibition or activation of specific neurons and neuronal subsets has been essential in the identification of their role in a defined behavior and provided insight to the connectivity and dynamics of neural circuits. To this end, various transgenes that inhibit or activate individual circuit elements have been developed. Using these transgenes, it is possible to determine the necessity of a neuronal subset in a specific behavior. Expression of transgenes that functionally silence the neurons necessary for a specific process would lead

to disruption of this process. On the other hand, transgenes used in neuronal activation have proved the sufficiency of the neurons of interest in a particular process.

A. Inhibiting neuronal activity

A variety of transgenes has been developed to functionally silence specific neuronal subsets. Expression of toxins, including diphtheria toxin A from bacteria (Lin, Auld et al. 1995) and ricin A from castor plant seeds (Hidalgo and Brand 1997), induce cell death by inhibiting protein synthesis, while proapoptotic genes such as *reaper*, *grim*, and *head involution defective* induce programed cell death by activating caspases (Zhou, Schnitzler et al. 1997, Wing, Zhou et al. 1998). However, despite their effectiveness in earlier stages, when these genes are expressed only in adulthood they act slowly or even fail to cause behavioral phenotypes in some cells (Thum, Knapek et al. 2006).

The most effective way to inhibit neuronal function is to target synaptic transmission. The tetanus toxin light chain cleaves synaptobrevin, a component of the vesicular release machinery, abolishing action potential-evoked neurotransmitter exocytosis. Expression of tetanus toxin light chain in *Drosophila* embryonic neurons removes detectable synaptobrevin and eliminates evoked, but not spontaneous, synaptic vesicle release (Sweeney, Broadie et al. 1995). To overcome the irreversible effect of tetanus toxin, a thermo-sensitive transgene, *UAS-shibire*^{ts1} (*Shi*^{ts1}), was developed to block synaptic transmission reversibly by disabling vesicular endocytosis only at a restrictive temperature (Kitamoto 2001). Therefore, overexpression of Shi^{ts} allows temporal control of neuronal silencing, which is restored approximately after fifteen minutes in permissive temperature. Shi^{ts1} has been extensively used in behavioral experiments to define the neuronal circuits that underlie a process, such as olfactory learning and memory. Other transgenes used to inhibit synaptic neurotransmission include a human inward-rectifying potassium channel Kir2.1 which leads to membrane depolarization (Baines, Uhler et al. 2001, Paradis, Sweeney et al. 2001), a *Drosophila* truncated open-rectifier potassium channel dORK-ΔC (Nitabach, Blau et al. 2002), and a modified *Drosophila* Shaker potassium channel EKO (White, Osterwalder et al. 2001).

However, these methods do not eliminate neuronal communication through electrical synapses. To target the electrical synapses *shak-B* mutants or RNAi expressed specifically in

the neurons of interest have been used, disrupting the assembly and/or maintenance of functional gap junctions at electrical synapses (Phelan, Nakagawa et al. 1996, Das, Trona et al. 2017).

B. Enhancing neuronal activity

Artificial neuronal activation has been effectively used to identify neurons whose activation triggers a behavior. The *Drosophila* transient receptor potential channel *dTrpA1*, a thermosensitive cation channel which opens above a certain temperature, leads to neuronal activation and has been widely used to define the sufficiency of a neuronal subset in a process (Hamada, Rosenzweig et al. 2008).

Another way to excite specific neuronal subsets is via overexpression of sodium channels, such as NaChBac, a voltage-dependent bacterial sodium channel whose measured property suggested its contribution to increase the excitability of the cell (Nitabach, Wu et al. 2006). A different method of neuronal activation has used optically gated ion channels, like the ionotropic purinoceptor gated by adenosine triphosphate, that evoke action potentials in response to laser light (Lima and Miesenbock 2005).

A breakthrough in neuronal activation was provided by the blue-light-sensitive cation channel Channelrhodopsin-2 (ChR2), which opens upon application of light and closes with its termination. Neuronal-specific expression of transgenic ChR2 has been used in various studies to excite neurons with high temporal resolution (Nagel, Szellas et al. 2003, Fenno, Yizhar et al. 2011, Packer, Roska et al. 2013). However, due to the poor penetration of blue light through the fly cuticle, it has been difficult to excite central neurons of the brain by ChR2 activation. To overcome this obstacle, new mutations in the ChR2 gene were developed, such as the D156C mutant termed ChR2-XXL for extra high expression and long open state. This mutation displays increased expression, improved subcellular localization, elevated retinal affinity, an extended open-state lifetime, and photocurrent amplitudes greatly exceeding those of all heretofore published ChR2 variants. In addition, the red-light-sensitive channelrhodopsin ReaChR (Lin, Sann et al. 2013, Inagaki, Jung et al. 2014) and Chrimson (Klapoetke, Murata et al. 2014) have been used, since fly cuticle is more transparent to red than blue light (Inagaki, Jung et al. 2014).

2.3 *Drosophila melanogaster* in Systems Neuroscience

The variety of genetic tools available in *Drosophila* aids the elucidation of the neuronal subset organization and how they communicate to orchestrate behaviors. Systems neuroscience focuses on the study of the neurons and circuits that underlie a certain behavior, as well as the mechanisms within these neurons mediating behavior and neural computations.

Different neuronal subsets can be identified by their location, morphology, physiology, gene expression, lineage, or any other attributes. The study of neuronal subsets is especially facilitated in *Drosophila,* because of the small number of overall neurons (100,000 neurons in the adult *Drosophila* brain) (Ito, Masuda et al. 2013) and the genetic tractability available through the Gal4 lines that label different neuronal subsets and collectively cover a large portion of the fly brain (Manseau, Baradaran et al. 1997, Hayashi, Ito et al. 2002, Rodan, Kiger et al. 2002, Pfeiffer, Jenett et al. 2008, Pfeiffer, Ngo et al. 2010, von Philipsborn, Liu et al. 2011, Jenett, Rubin et al. 2012).

Studying the connectivity among different neuronal subsets is necessary to gain insight into the mechanisms that confer specific functions to a circuit. The most precise and exhaustive method to obtain a neuronal wiring diagram is via serial electron microscopy, an approach recently used to reconstruct the complete electron microscopy volume of the brain of adult *Drosophila,* aiming to map synaptic connectivity (Figure 6) (Zheng, Lauritzen et al. 2018). Previous studies have used confocal microscopy and computational methods to construct fly brain connectivity maps that provided valuable information about the interaction among brain regions (Jefferis, Potter et al. 2007, Lin, Lai et al. 2007, Yu, Kanai et al. 2010, Chiang, Lin et al. 2011).

The identifiable and genetically tractable neurons of *Drosophila melanogaster* render the fly an exceptional tool to study the mechanisms underlying behavior at the level of genes, cells, and circuits. Neuronal responses to sensory stimuli have been measured with imaging techniques (Ng, Roorda et al. 2002, Wang, Chiang et al. 2003), while the application of the whole-cell patch-clamp technique to central neurons *in vivo* (Wilson, Turner et al. 2004) and the physiological recordings in behaving adult animals (Maimon, Straw et al. 2010) marked a

new era for fly systems neuroscience. In addition, genetic tools that allow manipulation of neuronal activation have been widely used to identify the necessary and sufficient neurons in various behaviors. These powerful approaches provide the opportunity to study the mechanisms and computations that support behavior at cellular resolution.

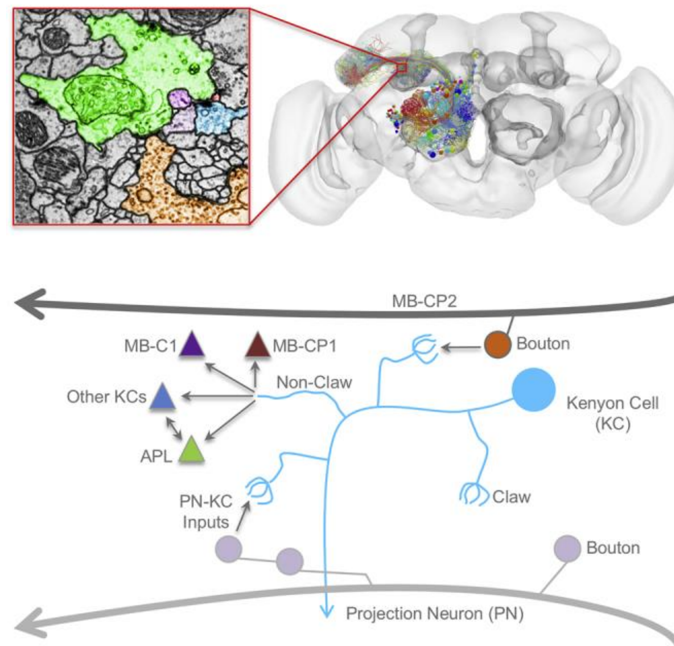


Figure 6. Brain-spanning circuit mapping with electron microscopy (Zheng, Lauritzen et al. 2018)

2.3.1 The olfactory system of Drosophila melanogaster.

Olfactory stimuli are of a major importance for survival, since they convey information about food and predators, and are necessary for reproduction. For these reasons, olfactory systems have evolved a great sensitivity and discriminatory power and are evolutionary conserved. The functional organization of the olfactory system is remarkably similar in organisms ranging from insects to mammals and the principles elucidated through studies in one experimental organism often apply to many others.

In both insects and mammals, odorants bind to receptors in the cilia or dendrites of the olfactory receptor neurons (ORNs), each of which expresses one or a small number of different receptor types. The large number of diverse odor receptors allows the detection of

a vast number of odors, while the subsequent combinatorial coding and the circuit-level interactions enable stimulus discrimination. ORNs that express the same odor receptor send their axons to the same glomeruli, the spherical neuropil elements consisting of ORN axon terminals and the dendrites of second order neurons. These glomeruli form the antennal lobe or olfactory bulb, in insects and mammals respectively, where the olfactory signals are processed and relayed to higher centers of the brain. Analysis of the insect antennal lobe, the mammalian olfactory bulb, and higher brain regions has led to a better understanding of how olfactory signaling is shaped by the circuit-level interactions between neurons.

A. The glomerular organization of the olfactory system in *Drosophila melanogaster*

Drosophila melanogaster is capable of detecting a large number of volatile chemical stimuli through the approximately 1300 olfactory receptors (ORs) that are localized on two olfactory sensory organs, the antenna and the maxillary palp (Stocker 1994). Drosophila olfactory receptors have selective but broad ligand-binding properties, with a given OR activated by multiple odors and a given odor activating multiple ORs. There are ~50 types of olfactory receptor neurons (ORN) and each one of them expresses only one of the ~62 olfactory receptor proteins that have been identified, together with a putative co-receptor or chaperone protein, Or83b (Elmore, Ignell et al. 2003, Couto, Alenius et al. 2005, Benton, Vannice et al. 2009).

The *Drosophila* olfactory receptor neurons (ORNs) project their axons to the antennal lobe (AL), and segregate in different glomeruli depending on their receptor type (Gao, Yuan et al. 2000, Jefferis, Marin et al. 2001). The antennal lobe glomeruli are synapse-dense, spherical neuropil structures that contribute to the interpretation of signals from the ORNs. ORNs expressing the same receptor can be distributed around the periphery of the antennae and maxillary palps, but they project their axons to the same glomerulus. Each odorant activates a specific set of glomeruli, creating a characteristic pattern of activation in the antennal lobe and subsequently, in higher order structures of the brain. In the glomeruli, ORNs form synapses with projection neurons (PN) and local excitatory or inhibitory interneurons, which modulate the output of projection neurons onto the higher neuronal structures.

Local interneurons are in their majority inhibitory (iLNs) (Okada R, Awasaki T et al. 2009), although a small subset of excitatory LNs (eLNs) has been identified as well (Shang Y 2007). Local interneurons are necessary to tune the PN response and can be activated both by ORNs and PNs, are multiglomerular, and their axons cover the expanse of the antennal lobe. Inhibitory LNs were proposed to increase the resolution of odor coding via lateral inhibition and they may also provide a negative feedback loop which generates oscillatory activity in the output neurons (Okada R, Awasaki T et al. 2009). GABA neurotransmission from the iLNs inhibits the PNs by binding on both $GABA_A$ and $GABA_B$ receptors. $GABA_A$ receptors shape PN odor responses during the early phase of olfactory responsiveness, while $GABA_B$ receptors mediate odor-evoked inhibition on longer time scales. iLNs display broad but diverse morphologies and odor preferences, suggesting a cellular basis for odor- and glomerulus-dependent patterns of inhibition. Odors elicit stimulus-specific spatial patterns of GABA release, and as a result, GABAergic inhibition increases the degree of difference between the neural representations of different odors (Wilson and Laurent 2005).

After being processed in the glomeruli, olfactory information is conveyed via three distinct antennocerebral tracks formed by PN axons to higher order neurons of the brain: The Mushroom Bodies (MBs), known for their role in learning and memory, and the Lateral Horn (LH), which is implicated in innate responses and memory retrieval (Figure 7).

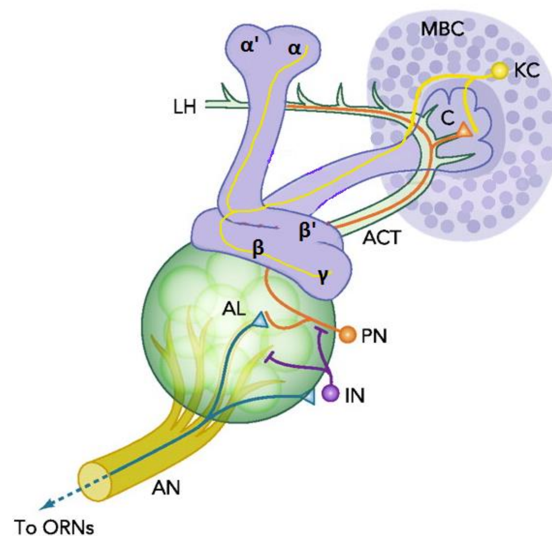


Figure 7. The neuronal subsets of the Drosophila olfactory system. (Modified from (Busto, Cervantes-Sandoval et al. 2010)

B. The olfactory antennocerebral tracks

There are three distinct ascending tracks, the inner, the medial and the outer antennocerebral track, formed by approximately 150 PNs, that connect the antennal lobe to the Mushroom Bodies (MB) and the Lateral Horn (LH). In contrast to the olfactory receptor neurons, the second-order neurons display broader tuning and more complex responses (Wilson, Turner et al. 2004), with a dense network of lateral connections distributing odor-evoked excitation between PNs of different glomeruli (Olsen, Bhandawat et al. 2007). Projection neurons that innervate the same glomerulus reveal stereotypical axon branching patterns and terminal fields in the lateral horn, whereas PNs from different glomeruli exhibit very different patterns of projections in the protocerebrum (Wang, Gong et al. 2014).

Projection neurons of the inner antennocerebral tract (iACT – also known as medial antennal lobe tract, mALT) innervate both the calyx of the MBs, where the dendrites of these neurons reside, and the lateral horn, while the medial (mACT – also known as mediolateral antennal lobe tract, mlALT) and outer (oACT - also known as lateral antennal lobe tract, lALT) antennocerebral tracts bypass the MBs and innervate only the lateral horn (Figure 8). This circuit organization allows the MBs to receive only feedforward excitation from cholinergic excitatory PNs (ePNs), whereas the LH receives parallel excitatory and inhibitory inputs via ePNs and multiglomerular GABAergic inhibitory PNs (iPNs) (Jefferis, Marin et al. 2001, Lai, Awasaki et al. 2008, Okada R, Awasaki T et al. 2009, Tanaka, Endo et al. 2012).

The iACT (or mALT) is formed by the majority of the cholinergic PNs that arise from only one glomerulus (uniglomerular neurons). Their axons emerge from the posterior dorsomedial area of the AL, run dorso-posteriorly along the posterior surface of the fan-shaped body, turn laterally in front of the protocerebral bridge, pass in front of the MB calyx, and terminate in the LH (Tanaka, Endo et al. 2012).

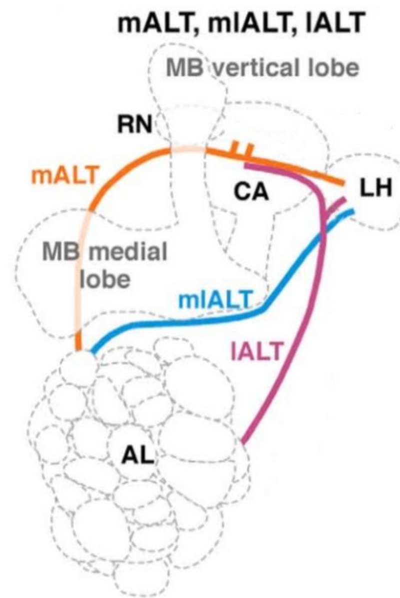


Figure 8. Projection neurons connect to the higher brain structures through three distinct tracks, the inner, the medial and the outer antennocerebral track (iACT, mACT, and oACT, respectively), also known as medial, mediolateral, and lateral antennal-lobe tracts (mALT, mlALT, and lALT, respectively) (modified by (Tanaka, Endo et al. 2012)

The mACT neurons (or mlALT neurons) also arise from the posterior dorsomedial AL and share the same root with the iACT, but they connect the AL directly to the LH, bypassing the MB calyx (Tanaka, Endo et al. 2012). Whereas many mACT neurons terminate in the LH, some of them additionally send their projections from the LH to the ring neuropil (Marin, Jefferis et al. 2002, Wong, Wang et al. 2002). In contrast to the iACT neurons, the mACT PNs are in their majority GABAergic and extend their dendrites into multiple glomeruli (Lai, Awasaki et al. 2008, Tanaka, Endo et al. 2012). The mACT inhibitory PNs (iPNs) were shown to be subdivided into two morphological groups, encoding either positive hedonic valence or intensity information and convey this information into separate domains in the LH. Interestingly, silencing the iPNs diminished attraction behavior (Strutz, Soelter et al. 2014). Furthermore, iPNs were shown to selectively suppress food-related odorant responses, but spare signal transmission from pheromone channels using a parallel inhibition motif. In this way, they provide specificity in inhibition and funnel specific olfactory information, such as food and pheromone, into distinct downstream circuits in the LH (Liang, Li et al. 2013). In the

LH, the inhibitory PNs (iPNs) of the mACT regulate the transmission characteristics of ePN terminals, providing presynaptic inhibition and improving innate performance when the distance between two odor representations is small (Parnas, Lin et al. 2013). The arrangement of parallel ePN and iPN projections to the LH appears to result in a tunable filter whose transmission characteristics adjust to the level of activity in the olfactory system. It is possible that iPNs are necessary to increase the odor detection sensitivity when low levels of ORN input result in weak ePN activation, while after high levels of ORN input, the iPNs may provide a high-pass filter to enhance discrimination. In addition, iPNs connect via chemical synapses to the ePNs within the antennal lobe, proposing additional roles for these neurons in the olfactory process (Shimizu and Stopfer 2017).

The oACT PNs (or lALT neurons), unlike the iACT and mACT PNs, emerge from the posterior ventral part of the AL, at the horizontal level of the top of the V glomerulus, run posteriorly, turn dorsolaterally, and then through the ventrolateral protocerebrum to project to the LH (Stocker, Lienhard et al. 1990, Tanaka, Endo et al. 2012).

C. The higher order neurons of the olfactory system

Interestingly, the spatial stimulus representation map in the higher brain centers presents major differences compared to this of the antennal lobe. In contrast to the tight convergence of primary sensory axons, whose arbors are often restricted to the small 5–10 μm spherical glomeruli, axon arbors in the protocerebrum are diffuse and extensive, often covering the entire dorsal-ventral dimension of the brain hemisphere (Wong, Wang et al. 2002).

The terminal fields of PN axons in the lateral horn are stereotyped according to PN class, and LH neurons arborizations are constrained within a zone defined by the endings of PNs from a specific subset of glomeruli. Unless there are indirect inter-zone interactions mediated by local neurons, each group of LH neurons cannot alone read olfactory information from more antennal lobe glomeruli than the ones represented in their branching domain (Tanaka, Endo et al. 2012). LH neurons can be categorized in the broadtuned (type I) neurons that receive input from several antennal lobe glomeruli and the narrow-tuned (type II) neurons that respond to information from approximately one glomerulus and have distinctive

post-LH connectivity (Fisek and Wilson 2014). The axon projection patterns of PNs largely maintain the information of their glomerular class, and therefore the odorant receptors activated. In the LH, the third neurons link segregated subgroups of PNs exclusively with specific brain areas. (Marin, Jefferis et al. 2002).

In contrast to the lateral horn, the mushroom bodies do not consist of functional subunits that connect with specific PN types. A single mushroom body neuron (Kenyon Cell – KC) can receive input from PNs representing either one class of glomeruli or various combinations of them. In this way, each mushroom body lobe integrates information from the entire antennal lobe (Tanaka, Awasaki et al. 2004). Kenyon cell responses to odors are highly selective and sparse compared to the PN activation. Different odors activate distinct patterns of MB neurons without any particular spatial organization depending on response probability, odor tuning, anatomic features, or developmental origins. Moreover, different classes of KCs do not appear to preferentially integrate inputs from specific combinations of glomeruli. The degree of sparseness is consistent across a wide range of stimuli, from monomolecular odors to artificial blends and even complex natural smells (Honegger, Campbell et al. 2011, Caron, Ruta et al. 2013). This transformation of the signal depends on three contributing factors: rapid decay of excitatory synaptic potentials from the PNs that curtail temporal integration, low PN convergence onto individual KCs (10 PNs per KC on average), and high KC firing thresholds that require all inputs of projection neurons to be coactive to spike (Turner, Bazhenov et al. 2008, Gruntman and Turner 2013). The sparseness of MB activation makes the Kenyon cells an important site of integration in the fly olfactory system and indicates that the fundamental feature of odor processing in the MBs is to create sparse stimulus representations in a format that facilitates arbitrary associations between odor and punishment or reward. When associated with another stimulus, the valence of a particular odor can be increased or decreased, based on modulation of the activation of a particular KC subgroup.

It was initially believed that the MBs and the LH have distinct functions, limited to experience-dependent and innate behaviors, respectively. Indeed, the LH is a key center for experience-independent responses to olfactory information, and coordinates behavioral responses to novel or frequently encountered olfactory stimuli (de Belle and Heisenberg 1994, Heimbeck, Bugnon et al. 2001). LH neurons encode valence and modulate behavioral

responses to innate aversive and appetitive odorants (Wang et al., 2003), and are necessary for the pheromone-depend male and female mating behavior (Kurtovic, Widmer et al. 2007). The two streams of information from the AL to the LH neurons – an excitatory across-PN pattern corresponding to the glomerular pattern, and a summed inhibitory signal – and the spatial arrangement of LH activity that reflects the spatial organization in the AL, render the LH an excellent center for stimulus evaluation. When two odors are compared for their valence, this comparison is highly correlated with the similarity of their across-glomerular patterns (Parnas, Lin et al. 2013). Each glomerular channel from the AL is attributed a value along a valence scale and the LH computes a global valence, that is binary (either 'good' or 'bad') as is the behavioral response of an animal, which is mostly unidimensional – either approach (positive) or withdraw (negative). It is rather interesting that the odor valence can change in different situations. In the hungry state, food odors might be more important than water, but in a thirsty state that situation is reversed. Odor valence shifts according to sexual arousal, need to oviposit, hunger, thirst, attention, stress from a predator, etc. It is possible that peptidergic and/or modulatory control might select the effective connectivity matrix in the LH, in order to switch from one readout axis to another (Galizia 2014).

Interestingly, a learned odor is attributed a specific valence after learning and accumulating evidence supports a role for the LH in learned behavioral responses through cross-talk with the MB. It has been proposed that while the mushroom bodies perform odor identification, the lateral horn neurons perform odor evaluation both in learned and innate responses (Galizia 2014). It is possible that, while innate responses to odors with a particular biological value and valence are mediated through the LH, the final behavioral response will be based upon the positive or negative valence given to that particular odor from previous encounters, a task mediated through the MB. Crosstalk between the LH and the MBs will then determine the behavioral output based on the current circumstances, allowing animals to respond appropriately to the same stimulus in different situations.

Most PN axon bundles relay olfactory information via the iACT/mALT, first traversing the MB prior to final connectivity in the LH and while odor memory formation is mediated by the MB (Heisenberg 2003, Thum, Jenett et al. 2007, Liu, Placais et al. 2012), but memory traces are also formed in the PNs leading to the LH (Thum, Jenett et al. 2007). The MB output neurons (MBONs) respond to odors and encode odor valence to affect the response to a

learned stimulus. MBONs override the presumed innate LH-mediated response towards odors (Aso Y 2014, Hige, Aso et al. 2015) and significantly, were found to communicate with the LH neurons. More specifically, the MB-V2 output neurons, necessary in aversive olfactory memory retrieval, were shown to form synaptic connections in the LH (Sejourne, Placais et al. 2011). Silencing these cholinergic MB-V2 neurons during memory retrieval in the odor-shock association paradigm resulted in strong aversive memory impairment. Olfactory stimuli activate the MB-V2 neurons in naïve flies while odor conditioning resulted in reduced MB-V2 activation. These output neurons are cholinergic and project near the GABAergic LH neurons. This suggests that decreased MB-V2 signaling after aversive memory formation leads to decreased activation of these GABAergic neurons, which no longer repress the innate LH avoidance circuit, thereby enhancing olfactory avoidance (Sejourne, Placais et al. 2011). Another recent study on memory retrieval confirmed the role of the LH neurons in the process, identifying two excitatory LH cell types (PD2a1 and PD2b1), which receive input both from food odor-encoding neurons and from the MB-V2 neuron. These neurons are crucial both for innate attraction and aversive memory retrieval and their activation is decreased after aversive odor conditioning, leading to decreased attraction to the trained odor (Figure 9) (Dolan, Belliart-Guerin et al. 2018).

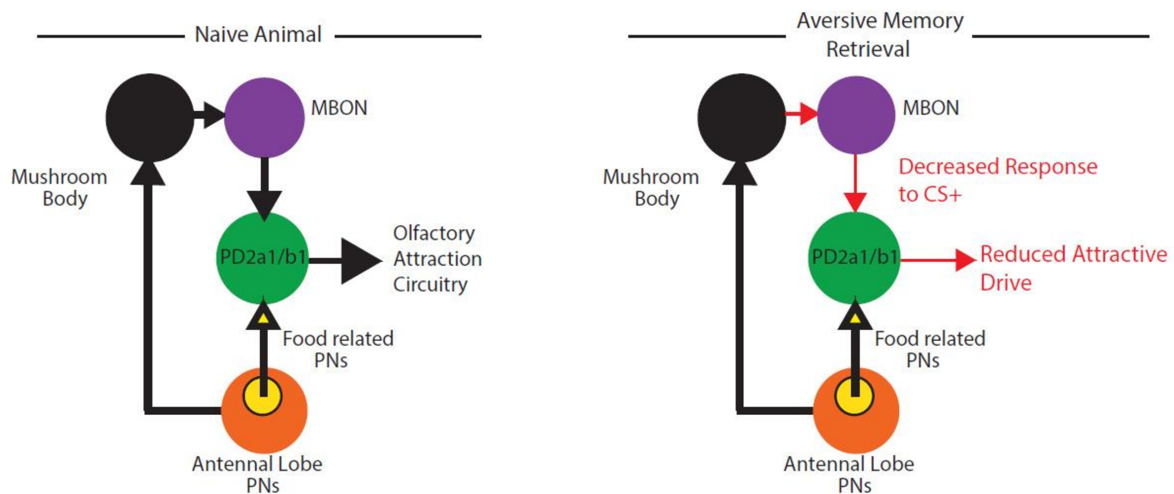


Figure 9. Lateral Horn neurons mediate innate olfactory attraction, leading to a model of aversive memory retrieval (Dolan, Belliart-Guerin et al. 2018).

2.4 *Drosophila melanogaster* as a model system in Behavioral Neuroscience.

D. melanogaster shows sophisticated behaviors that require complex neuronal computations. Intricate behaviors, such as learning and memory, courtship, and aggression, involve multimodal sensory integration, decision-making, and exquisite motor control (Dickson 2008, Villella and Hall 2008, Zwarts, Versteven et al. 2012). A courting male assesses the condition of females by integrating olfactory and gustatory cues. Fighting males and females also use multiple sensory modalities to decide which action to select from a variety of agonistic behaviors. Males were even shown to establish dominance depending on their fighting records. In addition, flies show circadian rhythmicity, with activity – rest cycles that are similar to the sleep – wake cycles of mammals (Allada, White et al. 1998, Tataroglu and Emery 2014). Furthermore, different feeding-related behaviors have been observed in *Drosophila*; some move about sampling food from various sources, whereas others feed locally (Venkatachalam, Weinberg et al. 1996). Moreover, females show a simple form of maternal behavior by choosing an appropriate site to lay their eggs.

Fly behavior presents sensitivity and tolerance to addictive drugs, such as alcohol and cocaine and shows drug-related behaviors, such as shaking and turning. Interestingly, flies can learn and remember what they have been taught for a significant percentage of their lives, presenting all the basic characteristics of mammalian learning and memory (Busto, Cervantes-Sandoval et al. 2010, Kahsai and Zars 2011).

Studies on *Drosophila* behavior have focused on the molecular mechanisms that underlie a lot of the behaviors mentioned above, identifying the genes, proteins, biochemical pathways and neuronal circuits implicated in these processes. These findings played an important role in the subsequent study on mammalian behavior, leading to the discovery of homologous genes in mammals. *Drosophila* is established as an ideal model to precede mammalian behavior studies that present increased complexity, since the basic components of the mechanisms underlying behavior are often conserved.

2.4.1 Olfactory learning and memory

Drosophila's ability to learn and remember, combined with the advanced available genetic tools, make the fruitfly an excellent animal model for the in-depth study of learning

and memory. To understand the behavioral, neuronal and genetic bases of learning and memory, a wide array of paradigms has been developed. Fruit flies can be trained to memorize odors, visual patterns and tactile/spatial cues through classical and/or operant learning. In classical conditioning an animal is trained to associate a conditioned stimulus (CS) with a rewarding or punishing unconditioned stimulus (US) (Pavlov 1927). In operant conditioning, a certain behavior is either rewarded or punished (Skinner 1950).

Adult *Drosophila* can form olfactory memory through classical conditioning, using aversive electric shock punishment or appetitive sugar reward as the US (Quinn, Harris et al. 1974, Tempel, Bonini et al. 1983, Heisenberg, Borst et al. 1985, Tully and Quinn 1985). In the most widely used paradigm for testing aversive olfactory conditioning flies are trained to associate an odor (CS) with electric shock punishment (US) and then get exposed to a second odor, without simultaneously experiencing the US (Tully and Quinn 1985). After a period of rest (from minutes for short-term memory to a day for long-term memory), the flies are forced to choose between the two odors in a T-maze choice point (Figure 10). Importantly, the experiment is done in a reciprocal fashion to rule out non-associative changes leading to altered odor preferences. To achieve that, two groups of flies are trained in parallel, with one group associating odor A with the US, while the other associates odor B with it. The number of flies choosing either odor are counted and used to generate one half of a performance index (PI) score (i.e., the number of flies choosing the US-associated odor is subtracted from the number choosing the other odor, and this number is divided by the total number of flies). The half-PIs from each US-associated odor are then averaged to make a single PI measure. The principles of appetitive olfactory conditioning are similar, except that instead of electric footshock, the flies are starved and exposed to a sugar reward simultaneously with the odor (Tempel, Bonini et al. 1983, Schwaerzel, Monastirioti et al. 2003).

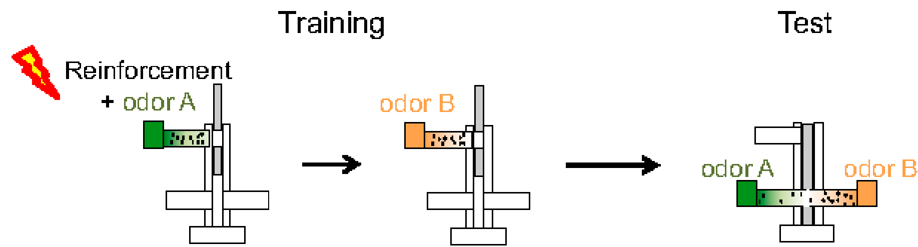


Figure 10. The T-maze aversive olfactory conditioning (modified by (Galili, Dylla et al. 2014))

The classical olfactory conditioning paradigm has been widely used to elucidate the genetic background of learning and memory. *Dunce*, a gene that encodes for a phosphodiesterase, was the first gene identified for its role in learning. Mutations in this gene lead to abnormally high cAMP levels, proposing a role for the cAMP pathway in learning (Dudai, Jan et al. 1976). To date, the number of genes identified for their role in learning or memory approaches forty, while more information is available about the neuronal subsets and the molecular pathways underlying these processes. Although most of the studies about memory formation mechanisms focus on the cAMP/PKA-signaling pathway, several genes that function outside of this cascade have been identified, providing starting points to investigate novel memory-critical signaling mechanisms (Kahsai and Zars 2011).

The studies focused on the genetic background of learning and memory led to increasing evidence proposing a role for the mushroom bodies in olfactory-based shock-avoidance learning and memory formation in Drosophila. Chemical ablation of the MBs, abolishes shock-avoidance olfactory learning (de Belle and Heisenberg 1994), while mutants with structural abnormalities in these neurons show olfactory learning defects (Heisenberg, Borst et al. 1985). In addition, various genes associated with learning and memory-related biochemical cascades have elevated expression in MB neurons (Nighorn, Healy et al. 1991, Han, Levin et al. 1992, Skoulakis, Kalderon et al. 1993, Han, Millar et al. 1996, Skoulakis and Davis 1996, Muller 1997, Grotewiel, Beck et al. 1998). Some of these genes encode molecules that are involved in cyclic AMP (cAMP) signaling, including *rutabaga* (*rut*), which encodes a Ca^{2+}/calmodulin-dependent adenylyl cyclase (AC) that converts ATP to cAMP, *dunce* (*dnc*) that encodes a phosphodiesterase involved in degrading cAMP, and *dco*, encoding for a protein kinase A catalytic subunit. Additional genes of the cAMP pathway with a role in

learning and memory include G-protein subunits, regulatory subunits of protein kinases, and the cAMP-responsive transcription factor CREB (Waddell and Quinn 2001, Davis 2005). Since the mushroom bodies play a crucial role in learning and memory, many studies have focused on genes preferentially expressed in *Drosophila* MBs, like *leonardo* (*leo,* 14–3–3), *volado*, and *fasciclin II* and showed that mutations in these genes result in learning and memory defects (Davis 2005, Turrel, Goguel et al. 2018).

The MBs are composed of three distinct classes of neurons – alpha/beta (α/β), alpha’/beta’ (α’/β’), and gamma (γ) – which differentiate in a strict developmental order (Lee, Lee et al. 1999) and exhibit varied expression levels for several genes preferentially expressed in the MBs (Crittenden, Skoulakis et al. 1998). The axons of the α/β and the α’/β’ MB neurons split into two separate branches, while the axons of the γ MB neurons are unbranched. These three distinct Kenyon cell populations are involved differentially in short- and long-term aversive olfactory memory. The Kenyon cells of the γ lobe are playing an important role in short-term memory, the α’β’ neurons are necessary for memory consolidation, and the αβ neurons have a crucial role in memory retrieval and their requirement becomes more pronounced as time passes (Zars, Fischer et al. 2000, Krashes, Keene et al. 2007, Blum, Li et al. 2009).

2.4.2 Habituation in Drosophila melanogaster

The powerful genetic tools, advanced imaging techniques, electrophysiology, and unbiased behavioural assays available in *Drosophila* render the fruitfly an exceptional organism for the in-depth analysis of habituation, aiming at the elucidation of its mechanisms in a molecular, cellular and systems level. The extended range of behavioural responses in *D. melanogaster* led to the development of a plethora of different habituation paradigms. Using these paradigms, many studies focused on the investigation and identification of the role of genes necessary for synaptic plasticity. These included genes already found to have a major role in associative learning (Davis 1996, Dubnau and Tully 1998, Waddell and Quinn 2001, Wu, Shih et al. 2013). However, even though a lot of the key molecules implicated in habituation were found to be common between different paradigms, it was observed that mutations in the same gene may result in opposite behavioural outputs in paradigms focusing on

habituation of different behaviours. These results stress the role of the different neuronal subsets involved in each type of habituation and point out that modulation of the same molecular pathways in different neuronal subsets affects differentially the behavioural response. For example, even though the cAMP pathway was shown to be crucial across the majority of habituation paradigms, adenylyl cyclase *rutabaga* mutations result both in increased and decreased habituation, depending on the paradigm studied and the neuronal circuits that underlie the specific response attenuation. Even though the sensory and motor circuits of *Drosophila* are very well described, only a few studies have focused on the identification of the neuronal subsets that underlie habituation, and further investigation of these circuits is deemed necessary.

2.4.2.1 Habituation of visually evoked behaviors

A. Giant Fiber Response Habituation

Drosophila shows a stereotypic sequence of leg extension and flight initiation triggered by sudden darkness (Wyman RJ 1984), or by the combination of visual stimulation and air flow produced by an approaching object (Hammond and O'Shea 2007). This response, called the jump-and-flight giant fiber response, is an “all or none” response that habituates after repeated stimulation. Habituation of the giant fiber response depends on the frequency of stimulation, recovers with time and dishabituates after introduction of a novel stimulus, such as an air puff. Interestingly, it was shown that repeated stimulation reduces the signal below threshold at least two neurons upstream of the jump and flight muscles. Electrical stimulation of the afferent neurons to the giant fiber in the brain is used to bypass the early sensory stages, permitting the study of the central synaptic input to the giant fiber neurons. The giant fiber response engages a widely studied neural circuit, and the circuitry postsynaptic to the giant interneuron is well characterized, with each cell and synapse in the circuit uniquely identified. However, the synaptic modification that underlies habituation of the escape response is located in neurons presynaptic to the giant fiber that have not been identified (Kelly 1983, Engel and Wu 1996, Hammond and O'Shea 2007).

Studies on the giant fiber response habituation revealed an important role for genes related to second messengers. More specifically, two of the major genes of the cAMP

pathway, adenylyl cyclase *rutabaga* (*rut)* and cAMP phosphodiesterase *dunce* (*dnc),* known for their roles in associative learning (Dudai, Jan et al. 1976, Aceves-Pina, Booker et al. 1983), present slower (*rut*) or faster habituation (*dnc*), when mutated. Surprisingly, *dnc-rut* double mutants habituate even faster than *dnc* mutants (Engel and Wu 1996), indicating that localization and kinetics of cAMP regulatory processes may be more significant to habituation than the overall cAMP levels. In addition, mutations affecting genes implicated in the cGMP second messenger pathway, like *foraging (for)* which encodes for the cGMP-dependent protein kinase (PKG) (Osborne, Robichon et al. 1997), resulted in decreased habituation rate in the giant fiber response (Engel, Xie et al. 2000).

Other important molecules in giant fiber response habituation are proteins implicated in synaptic neurotransmission and membrane excitability. CAKI, a presynaptic membrane-associated protein with domains homologous to the human CASK and the calcium/calmodulin-dependent kinase is involved in neurotransmitter release and was shown to be necessary for normal habituation. Null CAKI mutants present increased spontaneous neurotransmitter release of the indirect flight muscle NMJ when synaptic activity is modified, impaired response of the giant fiber pathway to continuous stimulation, altered electroretinographic responses both to single and continuous repetitive stimuli and abnormal optomotor behavior (Zordan, Massironi et al. 2005). Another family of proteins studied in giant fiber response habituation are the K+ channel subunits. Mutations in *ether-à-go-go* (*eag)* or *Shaker (Sh)*, which encode for α subunits of voltage-gated K+ channels, enhance habituation, whereas mutations in *Hyperkinetic (Hk)*, which encodes a β subunit of the Shaker channel or *slowpoke (slo)*, a Ca^{2+}-activated channel resulted in decreased habituation (Engel and Wu 1998). Finally, genetic analysis of habituation in the giant fiber pathway for the gene *period (per*), known for its role in circadian clock regulation, demonstrated that *per* mutants present faster habituation at unusually low stimulus frequencies, only when flies have been maintained under constant light (Megighian, Zordan et al. 2001).

B. Landing Response

The motor pattern of legs and wings during landing is a sterotyped response that shows habituation. To study habituation of the landing response, a single fly is tethered and

a dark bar is moving in its visual area, as if it is an object on which the fly can land. The leg extension response of the landing fly shows an “all or none” type of habituation, whereas the leg extension latency and the wing beat frequency are continuous variables, reduced upon the repetitive stimulus presentation. Landing response habituation shows spontaneous recovery and dishabituation after exposure to a vibrational stimulus and depends on stimulus intensity. Interestingly, generalization occurs only for visual stimuli and is specific to the direction of movement of the dark bar, indicating that habituation occurs before extensive convergence of visual pathways and after the convergence of neurons with adjacent receptive fields (Fischbach K-F 1988, Wittekind WC 1988).

Mutants in the cAMP pathway show deficits in landing response habituation. In contrast with the giant fiber response habituation, mutations in both *rutabaga* and *dunce* lead to increased landing response habituation (Wittekind WC 1988, Rees and Spatz 1989), a phenotype also presented in mutants with reduced protein phosphatase 1 isoenzyme (PP1) levels (Asztalos, von Wegerer et al. 1993).

2.4.2.2 Habituation of chemically evoked behaviors

A. Gustatory stimulus habituation

A hungry fly extents its proboscis when sugar water is applied to its front leg tarsae because of the taste receptors that are present there, a response called Proboscis Extension Reflex (PER) (Duerr and Quinn 1982, Le Bourg 1983). PER usually leads to eating, but when flies do not eat, the absence of reinforcement results in habituation to the stimulus sensed by the tarsae. Habituation of PER was shown after repeated (with 1 min interval) (Duerr and Quinn 1982, Le Bourg 1983) or continuous stimulation (lasting for 10 minutes) (Paranjpe, Rodrigues et al. 2012) and depends on the sugar concentration of the applied solution (Minois and Le Bourg 1997). During conditioning for PER habituation flies can taste but do not eat, thus eliminating the possibility of satiation or reinforcement. PER habituation presents generalization (Duerr and Quinn 1982, Bouhouche, Vaysse et al. 1993) and exposure to yeast odor results in recovery of the naïve response (dishabituation) (Le Bourg 1983, Fois, Medioni et al. 1991). Interestingly, habituation resides in neurons downstream of input convergence from both legs, since presentation of the stimulus to one leg leads to habituation of the other

as well (Duerr and Quinn 1982, Bouhouche, Vaysse et al. 1993). PER habituation is widely used due to its simplicity, and various genes were found necessary in the process.

As shown in giant fiber response habituation, the second messenger pathways are necessary in response attenuation. PER habituation is mediated by the *rutabaga*-dependent facilitation of the synapses between inhibitory GABAergic-glutamatergic neurons and cholinergic neurons in the subesophageal ganglion (SOG). Potentiation of both GABA and glutamate release from the inhibitory neurons was found necessary to decrease the response to the stimulus (Paranjpe, Rodrigues et al. 2012). Apart from *rutabaga*, other mutants such as *dunce* and *turnip* present decreased habituation (Duerr and Quinn 1982), whereas mutations in the cGMP-dependent protein kinase encoding *foraging* gene show enhanced habituation (Scheiner, Sokolowski et al. 2004). Another mutant with abnormal PER habituation is the anatomical mutant *nob* (*no-bridge*). Mutations in *nob* disrupt commissural tracts in the brain and show reduced habituation (Bouhouche, Vaysse et al. 1993). Interestingly, aged flies show decreased PER habituation, a result that suggests that the value of sugar as a stimulus increases with age in the fly (Fois, Medioni et al. 1991, Minois and Le Bourg 1997).

B. Olfactory stimulus habituation

The detailed characterization of the *Drosophila* olfactory system renders the fruitfly an exceptional animal model to study habituation of responses elicited by olfactory stimuli and identify the neuronal circuits and molecular mechanisms that underlie this process. Due to the advantages provided by the olfactory system, many different habituation paradigms based on responses to odors have been developed in the fly.

B.1. The olfactory jump response habituation

Drosophila responds with a jump and flight when subjected to a harmful odorant (McKenna, Monte et al. 1989), and brief repetitive pre-exposure to the odor with 1 minute interstimulus interval leads to attenuation of the response (Figure 11). The jump is an “all or none” response and habituation depends on the intensity and the frequency of the stimulus,

shows spontaneous recovery and dishabituation by mechanical stimulation (Mihalek, Jones et al. 1997, Asztalos, Arora et al. 2007, Asztalos, Baba et al. 2007, Joiner, Asztalos et al. 2007, Sharma, Keane et al. 2009).

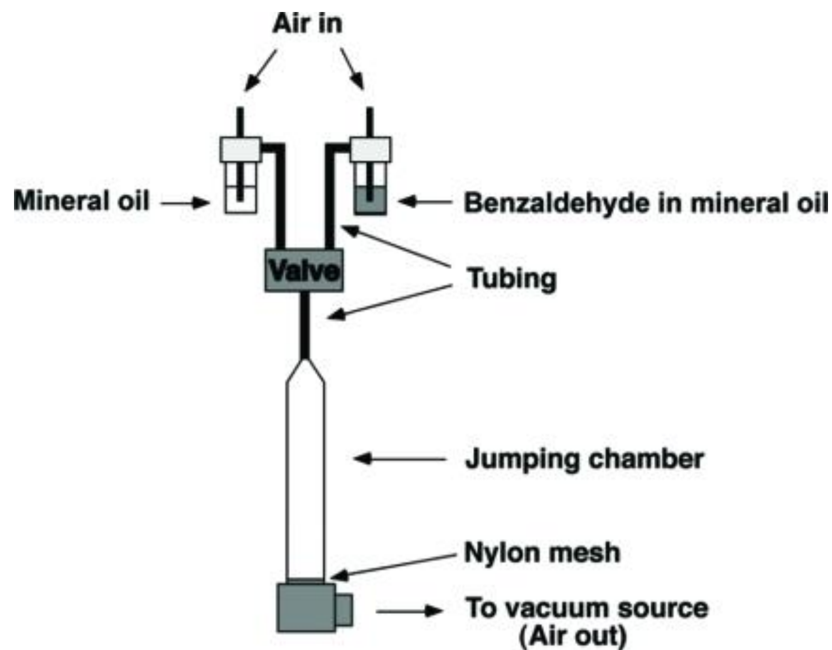


Figure 11. Apparatus for habituation of the olfactory jump response (Asztalos, Arora et al. 2007).

Olfactory jump habituation is affected by mutations in the second messenger pathways that were shown to be critical in other types of habituation as well. Mutations in *dnc* and *rut* mutants result in decreased habituation (Engel and Wu 1996, Asztalos, Arora et al. 2007). Another group of proteins with an important role in jump response habituation are the synaptic release-related proteins, such as Synapsin. Synapsin mutants present defective synaptic transmission and show faster olfactory jump habituation (Godenschwege, Reisch et al. 2004). Furthermore, mutations in potassium channel subunits show habituation deficits, with increased habituation in *ether a go-go* (eag) and *slowpoke* (*slo)* mutants and reduced habituation in *Hyperkinetic* (*Hk)* and *shaker* (*Sh)* mutants (Joiner, Asztalos et al. 2007). Another mutant showing increased jump habituation deficits is the *fickle* mutant, which carries a mutation in the homolog for Burton's tyrosine kinase (Asztalos, Baba et al. 2007).

To study olfactory jump habituation a high-throughput semi-automated system has been developed where the movement of individual flies can be tracked and their jump

response to repeated presentations of an odor is scored. Using this method, capable of screening 250 genotypes/month, 36 more genes implicated in the olfactory jump habituation have been identified (Sharma, Keane et al. 2009).

B.2. The olfactory startle response habituation

Flies that are exposed to a novel odorant exhibit an immediate and transient startle response, increasing their locomotion during the first 30 seconds of exposure (Cho, Heberlein et al. 2004). This response shows habituation after repeated exposures to the odor and has been mostly studied after repetitive exposure to ethanol vapour, with 6 minutes inter-stimulus intervals. Olfactory startle habituation is stimulus frequency dependent, shows spontaneous recovery, generalization and a mechanical shock results in dishabituation of the response (Cho, Heberlein et al. 2004, Wolf, Eddison et al. 2007). Since ethanol vapour exposure activates both the olfactory system and other non-olfactory mechanisms, animals without antennae were tested, showing that response attenuation was specific for the olfactory properties of ethanol. Surprisingly, in this paradigm the intensity of the applied stimulus is not correlated with the habituation rate (Cho, Heberlein et al. 2004). Furthermore, different odorants mediated by different primary olfactory neurons show generalization in this paradigm, suggesting that habituation occurs in higher order neuronal subsets and not the olfactory sensory neurons. Verifying this, ablation or functional silencing of the MBs reduces habituation without altering the initial response to the odorant (Cho, Heberlein et al. 2004).

In agreement with habituation studies in other paradigms, the cAMP pathway is crucial for normal olfactory startle response habituation, and mutations in *rutabaga* result in decreased locomotory startle response habituation. Another important protein for this type of habituation is encoded by *shaggy (sgg)*, a homolog of glycogen synthase kinase-3 (GSK-3). Decreased Shaggy levels result in reduced habituation, while increased levels promote the attenuation of the response. Interestingly, odor pre-exposure acutely regulates Shaggy by an inhibitory phosphorylation mechanism, suggesting that a signal transduction pathway that regulates Shaggy is engaged during habituation. Furthermore, Shaggy is required in neurons outside the MBs, since ubiquitous overexpression of the protein (wild type or a non-

phosphorylatable form) increases habituation, but local overexpression in the MBs does not (Wolf, Eddison et al. 2007).

To identify more genes implicated in olfactory startle response habituation a genetic screen was performed in 874 lines, resulting in 31 strains with habituation deficits. Many of the proteins that disrupt habituation in this paradigm localize at septate junctions, which are required for normal formation of the *Drosophila* perineural sheath. An interesting hit of that screen was *foraging* (*for*), which is known for its role in giant fiber response habituation and PER habituation. Mutations that disrupt the *for-T1* isoform, which is expressed in olfactory receptor neurons (ORNs) and the MBs result in increased habituation, whereas overexpression of *for-T1* in ORNs inhibits habituation, a phenotype similar to this of ORN inhibition. These findings suggest that *for-T1* may function in ORNs to decrease synaptic release upon repeated exposure to ethanol vapour (Eddison, Belay et al. 2012).

B.3. The unconditioned leg movement habituation

Odor exposure results in unconditioned leg movement, a response that habituates after repetitive presentation of the stimulus. A paradigm studying habituation of this response has been established, using various odorants with 5 minutes inter-stimulus interval to produce habituation, while feeding the flies with a sucrose solution resulted in recovery of the response (dishabituation). In addition, it was shown that generalization depends on the molecular features of the odors (e.g. carbon chain length and the presence of a target functional group) and presents a gradient across these molecular features (Chandra and Singh 2005).

B.4. Olfactory Avoidance Habituation

The innate response of the flies to avoid noxious odorants is modulated after pre-exposure to them. In the olfactory avoidance paradigm, habituation follows continuous exposure to an odor (CO_2 or ethyl butyrate) and is measured using a choice test in an upright Y-maze apparatus (Das, Sadanandappa et al. 2011). Depending on the duration of the stimulus exposure, habituation is categorized as short- and long-term. Short-term habituation

(STH) follows a 30 min exposure, is odorant-specific and shows spontaneous recovery within about 20 minutes. Four days of odor exposure, however, lead to long-term habituation (LTH), which shows odor selectivity as well, but lasts up to 6 days (Devaud, Keane et al. 2003, Das, Sadanandappa et al. 2011). The four days of exposure also result in decreased odor-evoked physiological responses in the respective odorant specific projection neurons and in volume increase specifically for the glomeruli activated by the odor (Sachse, Rueckert et al. 2007, Das, Sadanandappa et al. 2011).

B.4.1. Neuronal circuits implicated in olfactory avoidance habituation

The odor-specific attenuation of the response in olfactory avoidance habituation was shown to be localized in specific neuronal subsets of the antennal lobe. A series of experiments have demonstrated that olfactory habituation resides in the antennal lobe glomeruli, and more specifically in the projection neurons and local interneurons activated downstream of the olfactory sensory neurons (OSNs) in the signal transduction sequence. OSN activation in naïve flies for 30 min or 4 days with the transgenically expressed heat-activated cation channel TRPA1 (Pulver, Pashkovski et al. 2009), resulted in odor selective STH or LTH, respectively (Das, Sadanandappa et al. 2011). More importantly, substitution of the odor exposure with direct activation of specific projection neurons (PNs) with TRPA1 for 30 minutes led to odor-selective STH in naïve animals, whereas blocked synaptic transmission from PNs resulted in deficient habituation in conditioned flies. These results indicated that habituation occurs after the OSN level, and that the output of PNs is necessary and sufficient to induce habituation (Sudhakaran, Holohan et al. 2012). Apart from the projection neurons, neurotransmission from the multiglomerular, mainly GABAergic local interneurons (LNs) of the antennal lobe was found to be required for both STH and LTH. Local interneurons (LNs) receive input from OSNs and PNs and mediate feedforward and recurrent inhibition in the antennal lobe (Tanaka, Ito et al. 2009, Hu, Zhang et al. 2010, Huang, Zhang et al. 2010, Yaksi and Wilson 2010). Blocking the inhibitory LNs (iLNs) in conditioned flies disrupted habituation, whereas their activation was sufficient to induce response attenuation (Das, Sadanandappa et al. 2011). In addition, PN activation with TRPA1 and simultaneous reduction of vGlut or Rutabaga in the iLNs resulted in absence of habituation, stressing the role of LN neurotransmission in the process. Further experiments demonstrated that iLNs transmit

GABA and glutamate which bind on the ionotropic $GABA_A$ (Rdl) receptor and the NMDA receptor expressed postsynaptically in the PNs, thus repressing their activation (Sudhakaran, Holohan et al. 2012). Furthermore, the chemical synapses between the excitatory and inhibitory LNs were found to be important for 30-min habituation, since their disruption in a *shakB* mutant resulted in deficient habituation (Sudhakaran, Holohan et al. 2012). All these findings indicate that odor specific inhibition of distinct projection neurons that underlies habituation occurs through the activation of iLNs. OSN stimulation activates specific PNs, which in turn activate the LNs. These LNs, by transmitting GABA and glutamate, feedback onto the PNs and reduce their activation, leading to decreased response to the stimulus. This synapse-specific potentiation of recurrent inhibition onto active PNs enables the flies to reduce their response in an odor-specific manner (Sudhakaran, Holohan et al. 2012).

B.4.2. Essential genes for olfactory avoidance habituation

The Y maze choice test is one of the most extensively used olfactory habituation paradigm to access the neuronal circuits and genes implicated in the process. In agreement with the results obtained from other habituation paradigms, olfactory avoidance habituation is adenylate cyclase dependent and *rutabaga* mutants exhibit neither STH nor LTH. More specifically, *rutabaga* expression is required in the multiglomerular GABAergic local interneurons (LNs) of the antennal lobe for STH, LTH and for the glomeruli structural changes observed during LTH (Das, Sadanandappa et al. 2011). In addition to Rutabaga, normal STH requires two other proteins that function together in the LN1 subset of the LNs: Synapsin, a synaptic vesicle-clustering phosphoprotein, and Ca^{+2} calmodulin-dependent kinase II (CaMKII). Synapsin is phosphorylated and thus regulated by CaMKII, and its phosphorylation leads to synaptic vesicle mobilization and presynaptic facilitation of GABA release from the inhibitory LNs, which is necessary to inhibit the projection neuron activation (Sadanandappa, Blanco Redondo et al. 2013).

Long exposure to an odor that lasts for days elicits physiological, behavioural and structural changes and many genes were identified for their role in these processes. Exposure to an odor for four days results in decreased odorant-evoked calcium fluxes in PNs and increased volume of the glomeruli consisting of PNs specifically activated by the used odor.

This structural plasticity has been linked to the cAMP response element-binding protein (CREB), a protein necessary in long term associative memory (Silva, Kogan et al. 1998), and CREB-dependent protein synthesis is required in LNs for normal LTH, but not STH (Das, Sadanandappa et al. 2011). Furthermore, glutamatergic neurotransmission from the LNs onto the PNs is necessary for the increased glomerulus volume after 4 days of odor exposure and the behavioural attenuation of the response, since abrogation of vGlut specifically in the LNs and NMDA receptors specifically in the PNs disrupted both structural and behavioural plasticity (Das, Sadanandappa et al. 2011).

Apart from the cAMP pathway, another important pathway for long -term habituation is the microRNA (miRNA) pathway, known to be implicated in the local protein synthesis in synapses (Thomas, Pascual et al. 2014). Components of this pathway, such as the Ataxin-2 protein (Atx2), Me31B and Argonaute-1 are essential for the behavioral decrement of the response, the physiological changes in PN activation and the structural changes in glomeruli (McCann, Holohan et al. 2011, Sudhakaran, Hillebrand et al. 2014). These proteins are required presynaptically in LNs as well as postsynaptically in PNs (Sudhakaran, Hillebrand et al. 2014). Another protein associated with the miRNA pathway is the Drosophila Fragile X Mental Retardation Protein (dFMRP), an RNA-binding protein, which regulates local translation of target RNAs and is known for its role in learning and memory (Bolduc, Bell et al. 2008, Kanellopoulos, Semelidou et al. 2012). dFMRP functions together with Atx2, Me31B and Argonaute-1 both pre- and post-synaptically, potentiating the LN-PN synapse and regulating physiological and behavioral aspects of LTH (Sudhakaran, Hillebrand et al. 2014). dFMRP and Atx2 bind many target RNAs and regulate their translation, a process of great importance for the synapse-specific long-term plasticity required for LTH. One of their targets, CaMKII, is necessary for normal LTH and was shown to be locally translated both presynaptically in the LNs, and postsynaptically in the PNs (Hillebrand, Pan et al. 2010, Sadanandappa, Blanco Redondo et al. 2013, Sudhakaran, Hillebrand et al. 2014). These results, together with the CREB-depended protein synthesis necessary for normal LTH, demonstrate that long-term synaptic plasticity after long odor exposure lasting for days requires new protein synthesis and engages both de novo protein synthesis in the presynaptic local interneurons and activity-regulated local translation of synaptic mRNAs, both presynaptically and postsynaptically (Figure 12).

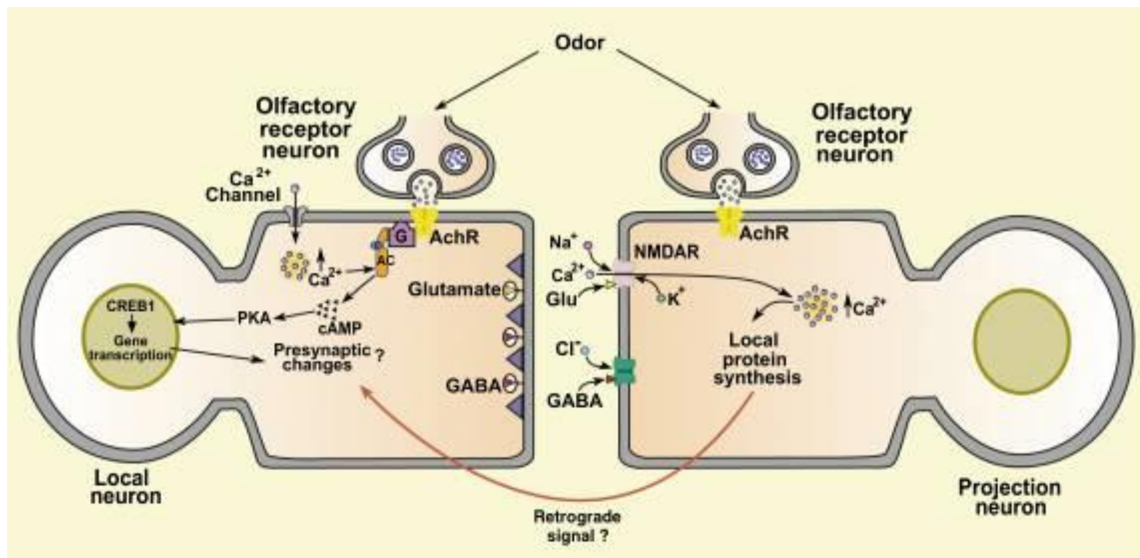


Figure 12. Cellular model of olfactory avoidance habituation in Drosophila (Glanzman 2011).

2.4.3 Habituation of other behaviors in intact flies

A. Shock avoidance habituation

The typical response of flies that experience an electric footshock is to avoid the stimulus, and the negative value of electric footshocks has been used to establish the most widely used associative learning paradigm in *Drosophila*, where electric footshocks are given as the unconditioned stimulus, simultaneously with an odor (conditioned stimulus) (Quinn, Harris et al. 1974, Tully and Quinn 1985). Repetitive exposure to low intensity electric footshocks leads to attenuation of the response. In the shock habituation paradigm, flies are pre-exposed to 15 electric footshocks with 4 sec inter-stimulus interval and are then subjected to a choice test in a T maze, where they select between a chamber presenting electric footshocks and one without. Pre-exposure to the stimulus results in habituation and decreased avoidance of the footshocks. Shock habituation depends on stimulus intensity, shows spontaneous recovery and exposure to an odour after conditioning results in dishabituation. In addition, multiple sessions of spaced training with 5-10 min intervals between the 15 electric shocks conditioning results in long term habituation, that lasts at least 90min. Interestingly, the MBs play a major role in shock habituation, with synaptic transmission from the α/β mushroom body neurons being required for protection from premature habituation when stimulus exposure is not long enough. Perturbation of the MBs

results in faster habituation, delayed spontaneous recovery of the initial response and inability for dishabituation when the animals are presented with another stimulus (Acevedo, Froudarakis et al. 2007). These results point to the MBs as the neuronal subset where non-associative and associative learning coincide and the behavioural output is modulated, leading to decreased or increased stimulus avoidance. Interestingly, habituation to the unconditional stimulus in the associative-learning paradigms is an observed phenomenon (Lloyd, Medina et al. 2014), and it is possible that the abnormal learning performance observed in *Drosophila* mutants, particularly in genes expressed in the MBs, is the consequence of fast devaluation of the unconditional stimulus that results in weaker associations. This hypothesis requires further experimental validation, which may elucidate the connection between habituation and cognition, and non-associative and associative learning in general.

B. Habituation of courtship conditioning (EDCM)

In *Drosophila melanogaster*, as in many other animals, courtship consists of a series of stereotypical behaviors carried out by a male in response to various sensory inputs. One aspect of courtship in male *Drosophila* is experience dependent and males that have courted fertilized females are virtually unresponsive to virgin females for 2-3 hr (Gailey, Jackson et al. 1984). Furthermore, males rapidly condition to immature males and the rejection from the young male eliminates the courtship behaviour, an effect that is called Experience-Dependent Courtship Modification (EDCM) (O'Dell 1994). EDCM is suggested to be habituation, although the only characteristics studied is response decrement and spontaneous recovery, that occurs after 1 hour of isolation. Mutations in molecules that participate in the cAMP pathway are shown to be important for EDCM, with *dnc* mutants exhibiting decreased EDCM (O'Dell 1994). However, additional studies are necessary to complete the characterization of EDCM and verify that the response attenuation conforms to the habituation parameters described by Thompson and Spenser.

2.4.4 Habituation studies in the thoracic nervous system

Habituation in the thoracic nervous system of *Drosophila* facilitates the study of synapses underlying the behaviour plasticity in the process. Fly decapitation offers a way to simplify the neuronal circuits that are activated during behaviour. Headless flies perform a number of behaviours that can be used to assay the general function of the thoracic nervous system, such as the maintenance of the upright position (righting reflex) or the bristle cleaning after tactile stimulation to the thoracic bristles (cleaning reflex) (Vandervorst and Ghysen 1980).

A. Habituation of the leg resistance reflex

The leg position in adult flies is controlled by a simple proprioceptive reflex, which requires a simple neuronal circuit consisting of a functional set of sensory neurons, the chordotonal neurons and their axonal projections. Upon repetitive stimulation, the strength of the reflex controlling leg position is attenuated, and this response decrement was shown to conform to the parametric features of habituation. Expression of mutant CaMKII transgenes in the sensory neurons that control the reflex eliminates habituation, showing increased response similar to sensitization. Expression of a mutant CaMKII incapable of achieving calcium independence decreased the initial responsiveness of the reflex but was then followed by strong facilitation of the response, eliminating habituation. The same phenotype was observed with the expression of a CaMKII inhibitory peptide in the sensory neurons, proposing a role for CaMKII in the presynaptic neurons in response modulation, level and dynamics (Jin, Griffith et al. 1998).

B. Habituation of the cleaning reflex

An air puff that moves a thoracic macrochaete (one of the large bristles) of a decapitated fly results in a stereotyped behaviour, where the fly sweeps a leg up as if to brush away an object. Repeated air puff stimulation with 1-5 sec interstimulus interval leads to a decrease in this response (Vandervorst and Ghysen 1980, Corfas and Dudai 1989). This response attenuation shows the parametric characteristics of habituation including

spontaneous recovery, potentiation of habituation, dependence on stimulus frequency and stimulus intensity, and dishabituation by a train of air puffs directed at a different bristle field (Corfas and Dudai 1989). Stimulus generalization was not observed in this paradigm, since habituation to stimulation of one bristle did not generalize to a second bristle that triggered a response in the same leg (Corfas and Dudai 1989). As shown in other habituation paradigms, the cAMP-pathway is necessary in this process, but its role was mainly focused on the recovery phase of the cleaning reflex, with mutations in *rutabaga* and *dunce* increasing the rate of recovery from habituation without altering habituation or affecting dishabituation (Corfas and Dudai 1989, Corfas and Dudai 1990).

The cleaning reflex provides an interesting model system to study habituation, especially due to the detailed characterization of the neuronal circuits involved in this behaviour. The fields of sensory bristles that trigger grooming by each leg are distinct and well-defined (Vandervorst and Ghysen 1980), their sensory neural projections have been characterized (Ghysen, 1980; Burg, Hanna, Kim, & Wu, 1993), and the motor neurons that mediate the reflex have been identified (Green 1981). The accessibility of the sensory neurons for electrophysiological recordings (Corfas and Dudai 1990) and the small-patch mosaic techniques that can be used to express a mutation in a single sensory neuron while its targets are wild-type (Burg and Wu 1989), make the cleaning reflex a great model for further studies focusing on the synaptic alterations underlying the behavioural plasticity in habituation.

3. Dishabituation

Dishabituation is the recovery of the naïve response to a habituated stimulus following the exposure to a novel stimulus (dishabituator) and is a process typically used as means to distinguish habituation from sensory adaptation and motor fatigue. Two basic hypotheses were formed to explain the mechanism of dishabituation, with experimental data supporting both of them. According to the dual-process theory, dishabituation occurs because an independent process like sensitization is superimposed on habituation (Groves and Thompson 1970), while the neuronal-model comparator theory for habituation proposed that dishabituation is an actual reversal of habituation (Sokolov 1960).

The dual-process theory of response plasticity to repeated stimulation, proposed by Groves and Thompson in 1970, suggested that repetitive exposure to a stimulus may decrease (habituation) or enhance (sensitization) an animal's response. Behavioral experiments using both the hindlimb flexion reflex in acute spinal cat and the acoustic startle response of intact rats showed that habituation and sensitization develop independently in the CNS in different neuronal subsets and interact to yield the final behavioral outcome (Groves and Thompson 1970). It was thus suggested, that dishabituation is not the reversal of habituation, but sensitization superimposed upon habituation. To examine this hypothesis various studies focused on the *Aplysia* gill- and siphon-withdrawal reflexes producing however, contradictory results. A study on the gill-withdrawal reflex demonstrated that both dishabituation and sensitization involve several mechanisms. These included facilitation at sensory neuron synapses, enhancement in the periphery, facilitation of excitatory interneurons and inhibition of the inhibitory ones, in different neurons that contribute preferentially depending on the time point after conditioning when the dishabituator is applied. Thus, the information processing is distributed both in space and in time and the recovery of the response after experiencing a dishabituator is a result of superimposed sensitization (Cohen, Kaplan et al. 1997). Another study on the gill withdrawal reflex combined behavioral and cellular neurophysiological data and showed that a common heterosynaptic facilitatory process underlies both sensitization and dishabituation, leading to increased effectiveness of the excitatory synaptic transmission between sensory and motor neurons (Carew, Castellucci et al. 1971). In addition, a siphon-withdrawal reflex analysis presented no qualitative differences between the major cellular mechanisms contributing to dishabituation and sensitization and verified the results previously obtained in the gill-withdrawal reflex. This would argue then that both processes involve multiple mechanisms, including heterosynaptic facilitation of sensory neuron-motor neuron postsynapric potentials (Antonov, Kandel et al. 1999). However, even though all previous studies verified the dual process theory explanation of dishabituation, another study in Aplysia did not confirm these results. Instead it suggested that dishabituation and sensitization can be behaviorally dissociated by their differential time of onset, sensitivity to stimulus intensity and emergence during development. More specifically, dishabituation showed a lower threshold and faster onset than sensitization, suggesting that the processes are different (Marcus, Nolen et al. 1988).

Additional studies on the molecular mechanisms of sensitization and dishabituation provided insight on these processes and further confirmed their differences. Sensitization of defensive gill and siphon withdrawal reflexes in Aplysia results, in part, from presynaptic facilitation of neurotransmitter release from mechanoreceptor sensory neurons that innervate the siphon skin and synapse with interneurons and motor neurons. Facilitation is associated with increased serotonin and enhances synaptic transmission due to prolonged presynaptic action potentials. These result from a decrease in a specific K^+ current and an enhancement of the Ca^{2+} transients elicited by depolarization. However, dishabituation is mediated only by serotonin and not by the prolonged duration of action potentials, which has little effect on neurotransmitter release when repeated activation of the sensory neurons has induced profound homosynaptic depression (habituation) (Hochner, Klein et al. 1986). In addition, whereas activation of PKA is sufficient to trigger facilitation of non-depressed synapses, activation of both PKA and PKC is required to facilitate depressed synapses, with the contribution of PKC becoming progressively more important as synaptic transmission becomes more depressed (Ghirardi, Braha et al. 1992). These results argue that even though some key players of the mechanisms underlying dishabituation and sensitization are the same, the processes are not.

An explanation of the contradictory results acquired on Aplysia dishabituation was given by a parametric study of dishabituation and sensitization on the gill withdrawal reflex, in a simplified preparation using two different test stimuli. Dishabituation and sensitization showed similar time courses and generally similar functions of shock intensity, but the two processes could be dissociated behaviorally, even though only under limited conditions. More specifically, dishabituation due to reversal of habituation occurred when the stimulus was applied shortly after habituation, while when a longer interval was introduced between the repeated stimulation and the dishabituator, the recovery of the response was a result of superimposed sensitization. These results suggested that both dishabituation and superimposed sensitization may underlie response recovery in Aplysia and they can both occur in the same preparation under different conditions (Hawkins, Cohen et al. 2006).

The mechanism of dishabituation was further studied in other animals. Results from the touch-elicited shortening reflex in the leech *Hirudo medicinalis* contributed to the hypothesis proposing that sensitization and habituation are different processes. More

specifically, even though both sensitization and dishabituation resulted in increased activity of an interneuron that connects the sensory and motor neurons, lesions of this neuron completely abrogated sensitization while they reduced but did not eliminate dishabituation (Sahley, Modney et al. 1994). Similarly, serotonin, which is involved in facilitation of the touch-elicited shortening reflex, mediates sensitization but not dishabituation in *Hirudo medicinalis*. Depletion of serotonin from the nervous systems using the toxin 5-hydroxytryptamine (5,7-DHT) attenuated early facilitation and sensitization but only reduced dishabituation of the touch-elicited shortening reflex (Ehrlich, Boulis et al. 1992). These results oppose the serotonin-mediated Aplysia dishabituation, suggesting that dishabituation may rely on different mechanisms depending on the reflex and the organism studied. Nevertheless, the disengagement of the processes which contribute to dishabituation and sensitization in the leech is consistent with the previous evidence that separates these processes in Aplysia and suggests that dishabituation might indeed be habituation reversal, as proposed by the stimulus-model comparator theory.

The stimulus-model comparator theory was proposed by Solokov in 1960, to explain how an animal responds to stimuli of its environment (Sokolov 1960). According to this theory, a response is elicited when an incoming stimulus does not match the already formed neuronal representation of the environment and significant stimuli result in increased responses compared to indifferent ones. In contrast to the dual-process theory, Sokolov suggested that dishabituation is a disturbance in the habituation process. The mechanism of dishabituation in the context of task-relevant "*significant*" stimuli and task-irrelevant "*indifferent*" stimuli was studied using the orienting reflex (OR) in human, the organism's immediate response to a change in the environment when that change is not sudden enough to elicit a startle reflex. As proposed by Sokolov, significant stimuli elicited stronger responses and showed enhanced dishabituation compared to the indifferent ones, while dishabituation was independent of sensitization both for significant and indifferent stimuli. In addition, sensitization was not affected by exposure to the dishabituating stimulus suggesting that it is an independent process (Steiner and Barry 2011, Steiner and Barry 2014). These findings argue against the dual-process theory's assertion that dishabituation is nothing more than a superimposed sensitization, independent of habituation. In contrast, they propose that dishabituation reflects the increased novelty associated with the recovery of the naïve

response to the initial stimulus and support Sokolov's assertion that dishabituation reflects a disruption to the habituation process.

The suggestion that dishabituation in mammals is a reversal of habituation was further confirmed by studies in rats. Olfactory habituation experiments in rats demonstrated that short-term habituation to odors requires metabotropic glutamate receptor mediated synaptic depression of cortical afferents and the antagonistic interaction between these receptors and the norepinephrine β-receptors underlies dishabituation. Introduction of an auditory stimulus after habituation to an odor resulted in elevated norepinephrine levels in the piriform cortex and dishabituation of the odor-evoked heart rate orienting bradycardia in awake rats, suggesting that dishabituation of odor-evoked reflexes is mediated by the reversal of synaptic mechanisms underlying habituation (Smith, Shionoya et al. 2009). Additional studies are necessary to further current understanding of the mechanisms of habituation at the molecular and systems level.

Purpose of this study.

The present PhD thesis aimed at the elucidation of the habituation and dishabituation mechanisms at a molecular and systems level, using the *Drosophila melanogaster* avoidance responses to aversive olfactory and mechanosensory stimuli.

Temporally specific engagement of distinct neuronal circuits regulating olfactory habituation in *Drosophila.*

Introduction

Habituation is a highly conserved behavioral modification whereby responses to repetitive or continuous stimuli not associated with concurrent salient stimuli or events are attenuated (Harris 1943). Habituation devalues the salience of a stimulus permitting animals to attend other, potentially more significant stimuli. Importantly, preventing premature habituation is essential to maintain information content long enough to allow association with other stimuli. This led to the notion that habituation is a 'building block for associative learning'.

Habituation paradigms have been used to assess cognitive abilities (Chard, Roulin et al. 2014) and recent studies indicate that genes involved in intellectual disability are linked to impaired habituation (Lugtenberg, Reijnders et al. 2016, Stessman, Willemsen et al. 2016). Habituation deficiencies have also been linked to disorders, such as schizophrenia (Akdag, Nestor et al. 2003, Ludewig, Geyer et al. 2003), migraines (Kalita, Bhoi et al. 2014, Kropp, Wallasch et al. 2015), attention-deficit/hyperactivity disorder (Jansiewicz, Newschaffer et al. 2004, Massa and O'Desky 2012) and autism-spectrum disorders (Bruno, Garrett et al. 2014, Lovelace, Wen et al. 2016, Tam, King et al. 2017). The implication of habituation in multiple cognitive disorders and its potential effects on associative learning highlight the significance of understanding the molecular mechanisms and neuronal circuitry that govern it.

Drosophila is a premier system for molecular approaches to understand habituation because of its advanced molecular and classical genetics. In fact, it is a well-established model for habituation of various sensory modalities such as taste (Cevik and Erden 2012), vision (Soibam, Shah et al. 2013) mechanosensory (Acevedo, Froudarakis et al. 2007) and escape responses (Engel and Wu 2009), reflecting that habituation is apparent in most, if not all, circuits and modalities of the nervous system. However, in most of these paradigms, the circuits engaged to process the stimulus and establish the experimentally measured attenuated behavioral response are unclear. Importantly, the advanced understanding of the *Drosophila* olfactory circuitry and stimulus processing facilitates exploration of the

mechanisms mediating decreased stimulus responsiveness and habituation to inconsequential odors. Such a recently described paradigm of olfactory habituation in *Drosophila*required 30 min of odor exposure and was mediated entirely by antennal lobe neurons (Das, Sadanandappa et al. 2011). In contrast, habituation to repetitive 30 s odor pulses required functional Mushroom Bodies (Cho, Heberlein et al. 2004), neurons on the central brain also implicated in associative learning and memory in flies (Cognigni, Felsenberg et al. 2018).

To resolve this paradox, we focused on the early behavioral dynamics of habituation upon continuous odor stimulation. To that end, we developed and characterized a novel habituation paradigm to rather brief continuous odors. The behavioral responses define two distinct phases, an initial phase we term habituation latency, when stimulus responsiveness is maintained, which is followed by a significant response decrement reflecting habituation. Analogous response dynamics have been reported for footshock habituation (Acevedo, Froudarakis et al. 2007). In addition, we investigated whether these phases engage and are mediated by distinct neuronal circuits. The results highlight the stimulus duration-dependent activation of specific neuronal subsets and their distinct roles in securing timely habituation latency and habituation induction.

Results

An experience-dependent odor-specific decrement in osmotaxis.

We used continuous exposure to odorants adjusted to elicit relatively mild aversive (3-octanol-OCT and benzaldehyde-BNZ) and attractive (ethyl acetate-ETA and 2,3-butanedione-BUT) osmotactic responses (Acevedo, Froudarakis et al. 2007) (Figure 1A–D). After 4 min of exposure to OCT, a highly significant ~60% avoidance attenuation was presented by both w^{1118} and w^{*} controls (Figure 1A). Similarly, a 50% decrease in BNZ avoidance was apparent after a 4-min exposure (Figure 1B). Moreover, attraction to ETA (Figure 1C) and BUT (Figure 1D) were similarly abated after a 4-min exposure to the respective odorants, suggesting an experience-dependent decrease in osmotaxis. Failure to avoid or move toward the test stimulus could not be attributed to odor-induced locomotor impairments, because most flies left the choice point in the absence of test odors and both naive and pre-exposed animals distributed equally in the arms of the maze (not shown).

Because the osmotactic attenuation was similar irrespective of odor valence, we used the milder aversive OCT for all subsequent experiments. Intriguingly, the initial 120 s of exposure define an osmotactic attenuation latency period with the odor apparently retaining its value and the flies responding as if naive (Figure 1). Latency to habituate with similar dynamics has also been described in the footshock habituation paradigm (Acevedo, Froudarakis et al. 2007) and appears operant in other *Drosophila* habituation paradigms that examined this early phase such as for the electrically induced giant fiber response (Engel and Wu 2009).

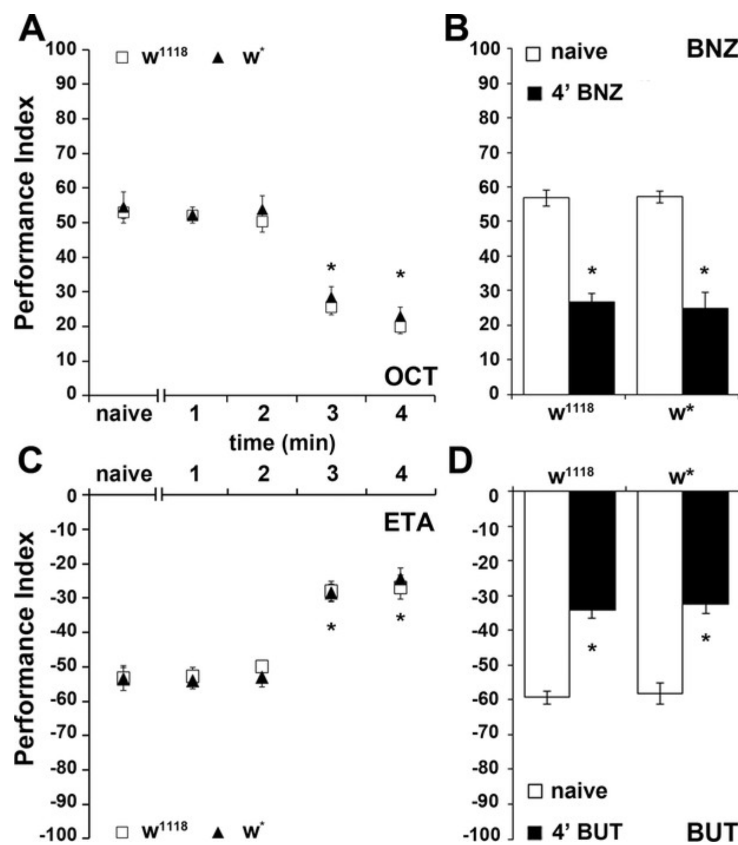


Figure 1. An experience-dependent odor-specific decrement in osmotaxis. Mean Performance Indices calculated as detailed in Material and methods are shown ±SEM. Positive values indicate aversion of the odorant and movement towards the air-bearing arm. Negative values indicate attraction to the odorants. Stars indicate significant differences. (**A**) Pre-exposure of the two control strains (w^{1118} and w*) to the aversive odorant 3-Octanol (1X OCT) for 3 or 4 min results in significant avoidance attenuation ($p<0.0001$, $n \geq 6$ for all groups), compared to flies that did not experience the odor except during testing (naive). (**B**) In contrast to naïve animals, pre-exposure to the aversive odor Benzaldehyde (1X BNZ) for 4 min resulted in significantly attenuated response in both strains ($p<0.0001$, $n \geq 6$ for all groups). (**C**) Pre-exposure of w^{1118} and w* flies to the attractant Ethyl Acetate (1X ETA) for 3 or 4 min precipitated a significant reduction in its attraction ($p<0.0001$, $n \geq 7$ for all

groups). (**D**) Exposure of both control strains to the attractive 2,3-Butanedione (1X BUT) for 4 min decreased significantly its attraction (p<0.0001, n ≥ 7 for all groups).

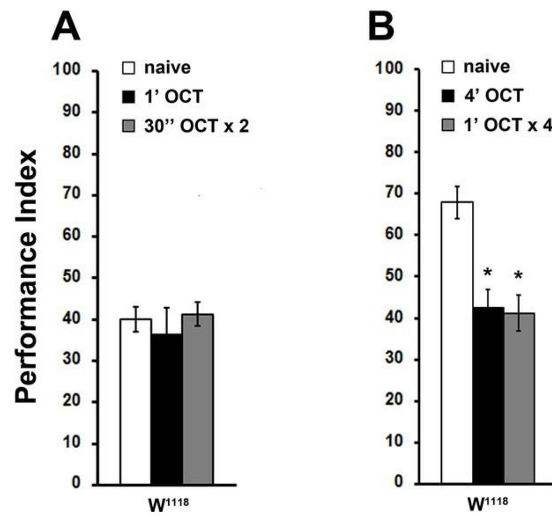


Figure 1—figure supplement 1. Continuous and pulsed OCT stimulation has the same effect on subsequent osmotaxis. Mean Performance Indices ± SEM are shown. Stars indicate significant differences from the naive response unless specified otherwise. (**A**) Repetitive 1X OCT stimulation (2 × 30 s) does not yield habituation, similar to the response after continuous odor exposure (ANOVA p=0.7125, n ≥ 14 for all groups). (**B**) Four 1 min 1X OCT pulses resulted in habituation (grey bar, p=0.0001), similar to the response after continuous odor exposure (black bar, p=0.0003). (n = 14 for all groups)

Is the osmotactic attenuation consequent of continuous exposure per se, or of the total stimulus exposure time irrespective of delivery method? To address this, the flies were subjected to discrete OCT pulses totaling the same exposure time as upon continuous exposure (see Materials and methods). The interstimulus interval (ITI) was kept around 25% of each pulse, because adaptation, which we aimed to avoid, has been reported proportional to odor stimulus duration (de Bruyne, Clyne et al. 1999). Significantly, OCT avoidance remained at naive levels after a single 1 min or two 30 s (with 8 s ITI) exposures (Figure 1—figure supplement 1). Moreover, avoidance was equally attenuated by one continuous 4 min exposure or 4, 1 min OCT pulses (15 s ITI) (Figure 1—figure supplement 1). These results indicate that attenuation of the avoidance response depends on total time of odor exposure, but not the mode of its delivery.

The decrement in osmotactic response conforms to habituation parameters.

Because it requires 4 min of total odor exposure to precipitate an osmotactic decrement, we wondered whether this behavioral response conforms to the classically defined habituation parameters of Thompson and Spencer (Thompson and Spencer 1966, Rankin, Abrams et al. 2009). Accordingly, animals that habituate to a repetitive or continuous stimulus should spontaneously recover if the stimulus is withheld. Indeed, a 3 or 6 min post-exposure rest resulted in spontaneous recovery of the osmotactic response to naive levels, both for aversive and attractive odors (Figure 2A,B).

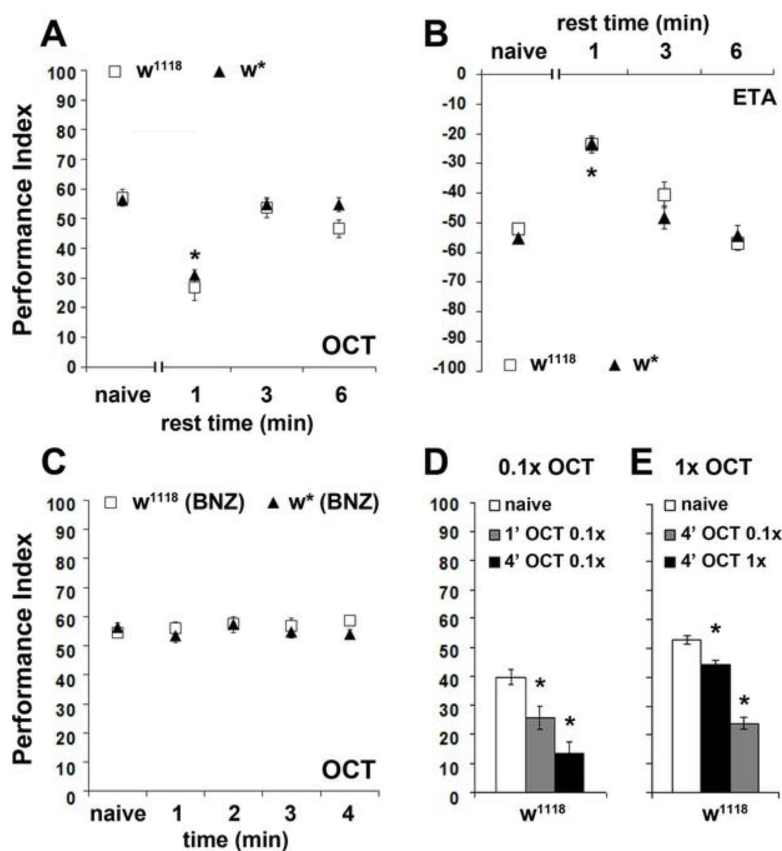


Figure 2. The osmotactic response attenuation conforms to habituation parameters. Mean Performance Indices ± SEM are shown in all figures. Positive values indicate aversion, while negative values indicate attraction. Stars indicate significant differences from the naïve response unless specified otherwise. (**A**) A 3 or 6 min rest after a 4-min exposure results in spontaneous recovery of 1X OCT avoidance. Spontaneous recovery was not observed after 1 min of rest ($p<0.0001$, $n \geq 9$ for all groups). (**B**) A 3 or 6 min rest following a 4-min exposure resulted in spontaneous recovery of attraction to 1X ETA, whereas 1 min of rest did not ($p<0.0001$, $n \geq 7$ for all groups). (**C**) Pre-exposure

to 1X BNZ for 1–4 min did not result in significant osmotactic decrement in subsequent 1X OCT avoidance (ANOVA p=0.7192, n ≥ 7 for all groups). (**D**) Pre-exposure and testing with 0.1X OCT results a significant decrease in response both after 4 min (black bar, p<0.0001), and after only 1 min of exposure (p=0.0086). (n ≥ 13 for all groups) (**E**) 4 min of exposure to 0.1X OCT followed by testing with 1X OCT (black bar) precipitated significant osmotactic attenuation (p=0.0096), but 4 min of pre-exposure to 1X OCT yielded deeper attenuation (p<0.0001) that was significant different to that of 0.1X OCT (p<0.0001). (n ≥ 8 for all groups)

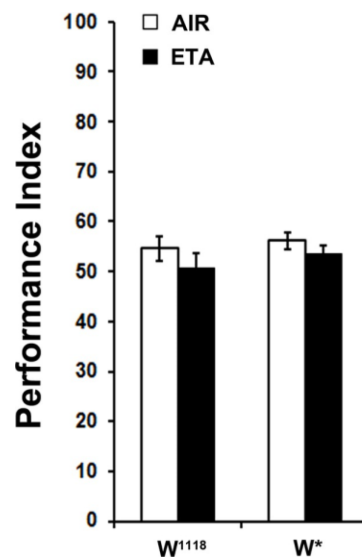


Figure 2—figure supplement 1. Exposure to the attractive odor ETA does not affect subsequent OCT avoidance. Mean Performance Indices ± SEM are shown in all figures. Stars indicate significant differences from the naive response unless specified otherwise. Pre-exposure to the attractive Ethyl Acetate (1X ETA) for 4 min did not result in diminished subsequent avoidance of OCT for both control strains (ANOVA p=0.1332 for w1118 and ANOVA p=0.2597 for w*, n = 8 for all groups).

The habituated response often exhibits generalization after exposure to similar stimuli (Thompson and Spencer 1966, Rankin, Abrams et al. 2009). Therefore, we investigated whether the osmotactic attenuation is specific to the pre-exposed odor, or flies generalize, presenting reduced responses to different odors, but of similar valence. After a habituation-inducing 4-min exposure to BNZ, flies avoided OCT normally (Figure 2C), suggesting no generalization of the response decrement to a different aversive odor. Moreover, pre-exposure to odors of opposite valence, such as the attractive ETA, also did not alter subsequent OCT avoidance (Figure 2—figure supplement 1), indicating that the osmotactic attenuation is odor-specific, a result also inconsistent with broad sensory fatigue. However,

we cannot rule out generalization to similar odors (for example 1-OCT and 3-OCT), which might activate overlapping neurons in circuits necessary for habituation, as previously shown for odorants with similar molecular features in the leg movement habituation paradigm (Chandra and Singh 2005). Given that we have used a limited set of odorants to test generalization, it is difficult to ascertain that osmotactic attenuation is not generalized.

Because weaker stimuli induce faster or more pronounced habituation (Rankin, Abrams et al. 2009), we investigated the effect of stimulus strength by exposing flies to dilute 0.1X OCT for 1 or 4 min and testing against 0.1X OCT. In agreement with Rankin et al., 2009, the diluted odor attenuated the response after only 1 min (Figure 2D), instead of the 4 min required for 1X OCT (Figure 1A), indicating that the weaker stimulus shortened the osmotactic attenuation latency as expected. Moreover, this response depended on the strength of the testing stimulus, because exposure to 0.1X OCT and testing against 1X OCT resulted in significant attenuation after 4 min, albeit decreased in magnitude compared to the decrement after exposure to 1X OCT (Figure 2E). Although the firing frequency of the OCT-responsive OSNs to different odorant concentrations is not known, imaging experiments on glomerulus activation indicated concentration dependent activation, with high concentration doubling the glomerular response (Yu, Ponomarev et al. 2004). Combined with the faster habituation upon exposure to lower OCT concentration, this suggests that the concentrations used are likely interpreted as different stimulus strengths.

Moreover, these results confirm that the response decrement is not a consequence of OSN adaptation, which occurs after odor pulses even as brief as less than 30 s and dynamically adjusts odor sensitivity (Cao, Jing et al. 2016). If the decrement depended on OSN adaptation, increased odor concentration would lead to faster adaptation and thus, faster response attenuation, but the opposite was observed. In addition, our data indicate that 1-min OCT exposure does not affect subsequent response to the same stimulus, strongly suggesting that it is not the OSNs, but neurons downstream in the olfactory pathway, that are implicated in the response decrement.

The experience-dependent osmotactic attenuation can be formally considered habituation if a relatively strong unrelated stimulus restores the naive response (dishabituation) (Thompson and Spencer 1966, Rankin, Abrams et al. 2009). We attempted

dishabituation using two distinct mechanical stimuli, electric footshock and vortexing. The strength and number of footshock stimuli required to reverse the osmotactic decrement with the weakest possible footshock were determined experimentally (see Materials and methods). A single 45-Volt footshock delivered after the 4-min OCT exposure restored subsequent avoidance to naive levels (Figure 3A) but did not affect OCT avoidance in naive flies (Figure 3B). The dishabituator should not be effective prior to odor exposure. Indeed, the footshock was effective only if delivered after OCT exposure, at the end of the apparent ~120 s latency period, but not prior to, or at the onset of odor presentation (Figure 3C). This suggests that the footshock likely interferes with processes occurring and potentially mediating habituation onset, as if effectively re-setting the latency period. Moreover, 3 s of vortexing immediately after the 4-min OCT exposure also resulted in recovery of the naïve response (Figure 3D), but did not affect responsiveness of vortexed but naive flies to OCT (Figure 3E).

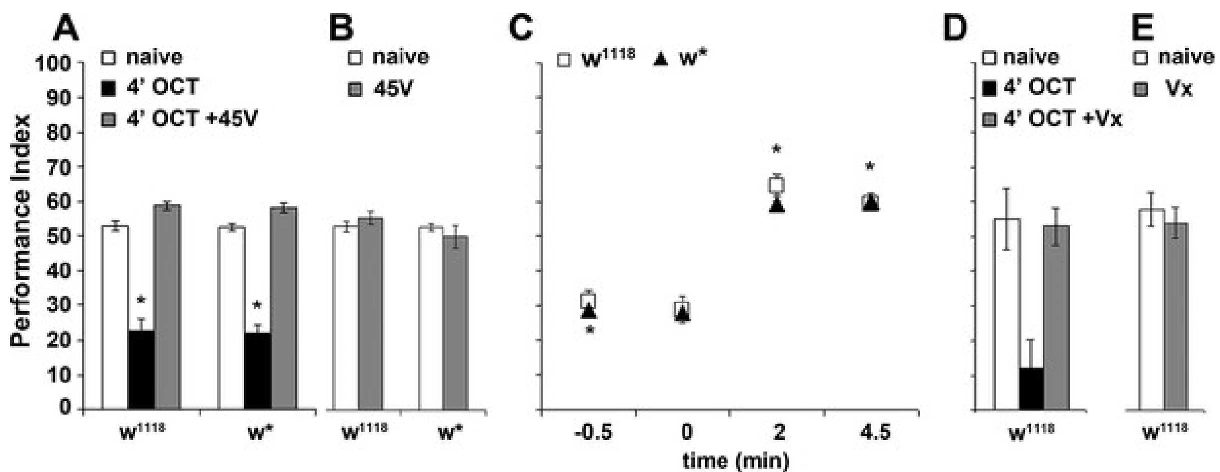


Figure 3. Dishabituation with mechanosensory stimuli results in recovery of the naive response. Mean Performance Indices ± SEM are shown in all figures. Stars indicate significant differences from the naive response unless specified otherwise. (**A**) Application of a 45V electric footshock after the 4 min odor exposure (grey bar) leads to reversal of the habituated response (black bar) in both w^{1118} and w* control strains (p=0.1412 and 0.0873, n ≥ 8 for all groups). (**B**) Application of a 45V electric footshock to naïve animals does not affect their response to the odorant (ANOVA p=0.3461, n ≥ 8 for all groups). (**C**) A 45V electric footshock was applied 30 s before (−0.5), concurrent with the onset of odor exposure (0), 2 min into the odor exposure (2), or 30 s post-exposure prior to testing. Dishabituation was evident only when the shock was delivered during or just after 1X OCT exposure (p<0.0001 for 2 and 4.5 min compared to −0.5, n ≥ 7 for all groups). (**D**) Application of a 3 s vortex at maximum speed (grey bar) after the habituating 4-min odor exposure (black bar, p=0.0010) led to

recovery (p=0.9729) of the naïve response (open bar). (n = 10 for all groups) (**E**) Application of a 3 s vortex to naive animals did not affect their response to the odorant (ANOVA p=0.5460, n = 18 for all groups).

Recovery of the response after footshock or vortexing and conformation to all other examined parameters demonstrates that the experience-dependent response attenuation is in fact olfactory habituation. Moreover, the initial ~120 s period represents habituation latency, potentially facilitating stimulus salience evaluation as previously suggested for habituation to repetitive electric footshocks (Acevedo, Froudarakis et al. 2007). If so, shortening or eliminating the latency period would result in early devaluation of the stimulus, effectively premature habituation.

Inhibitory local interneurons are necessary for habituation latency

Olfactory information is conveyed through the olfactory sensory neurons (OSNs) to the antennal lobe, consisting of local interneurons (LNs) and projection neurons (PNs) that transmit the information to higher order structures (Masse, Turner et al. 2009). Identification of neuronal subsets engaged in the latency period and habituation to the aversive OCT was facilitated by the well-defined olfactory circuitry in *Drosophila*.

We initially focused on the antennal lobe LNs, which are mostly (95%) GABAergic, activated both by sensory and projection neurons, and modulate PN output (Silbering, Okada et al. 2008, Tanaka, Endo et al. 2012). Potentiation of GABAergic inhibition from the LNs onto PNs has been suggested to mediate habituation after 30 min of odor exposure (Das, Sadanandappa et al. 2011). To determine whether LNs function similarly upon 4-min exposure, we conditionally blocked their synaptic output by transgenically expressing therein the temperature sensitive dynamin Shibire^{ts}(Shi^{ts}). At the restrictive temperature Shi^{ts} adopts an inactive conformation, blocking neurotransmitter reuptake, thus silencing neurons by depletion of the releasable neurotransmitter pool (Kitamoto 2001).

Shi^{ts} was expressed under the LN1Gal4 and GH298Gal4 drivers, which mark antennal lobe GABAergic inhibitory local interneurons (iLNs) (Okada R, Awasaki T et al. 2009, Acebes A, Martín-Peña A et al. 2011), arborizing in most glomeruli (Tanaka, Endo et al. 2012) and presenting extensive contacts with the PNs (Tanaka, Ito et al. 2009). We asked whether

silencing iLNs altered habituation latency by exposing the flies to OCT for only 1 min. Interestingly, 1 min exposure attenuated OCT avoidance, whereas controls retained their naive response (Figure 4A,B). The attenuated osmotaxis is in fact premature habituation demonstrated by its reversal to naive levels by a single post-pre-exposure dishabituating footshock (Figure 4A,B). It should be noted that for simplicity all driver heterozygotes are not presented in Figure 4, but their performance (Suppl File 2), was similar to the *shibire*^{ts}/+controls. Therefore, antennal lobe GABAergic iLNs appear to have a dual role. Upon brief odor exposure, they modulate antennal lobe activity to preserve stimulus value, contributing to habituation latency, while upon prolonged, 30-min exposure they were reported to facilitate habituation (Das, Sadanandappa et al. 2011).

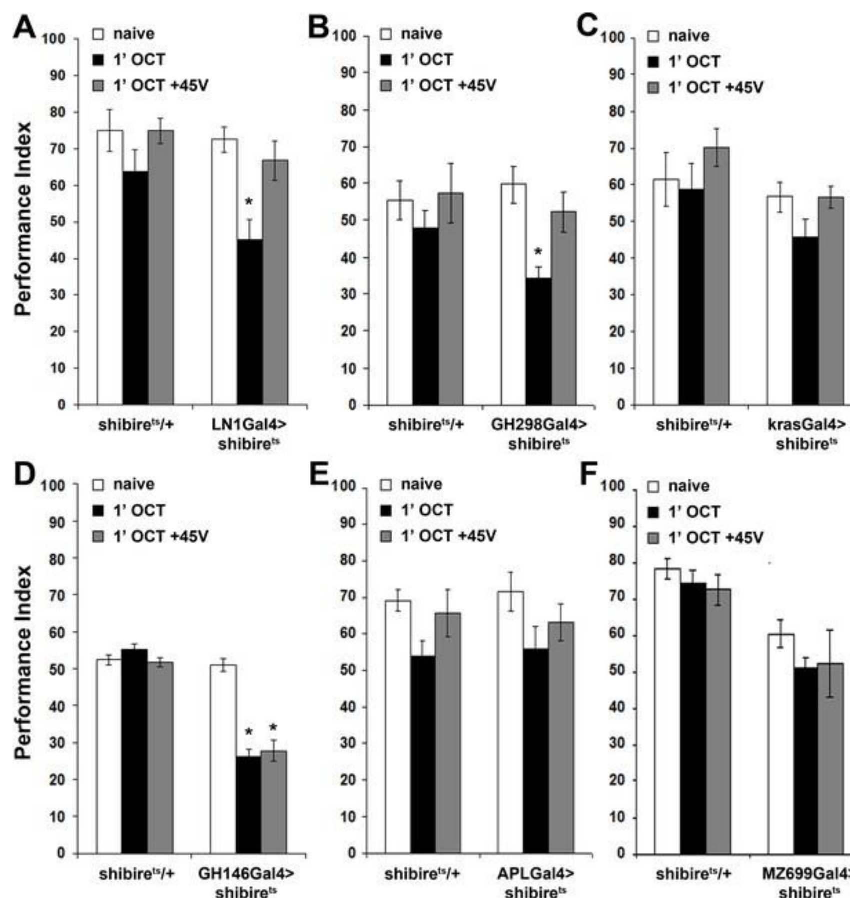


Figure 4. Inhibitory local interneurons and excitatory projection neurons are necessary for habituation latency. Mean Performance Indices ± SEM are shown in all figures. Stars indicate significant differences from the naive response unless specified otherwise. (**A**) Functional silencing of iLNs with UAS-shibire^{ts} under LN1GAL4 resulted in premature/facilitated habituation after only 1 min of 1X OCT exposure (black bar, p=0.0012), which recovers to naive levels after a single 45V electric footshock (grey bar, p=0.6268). In contrast, UAS-shibire^{ts}/+controls did not present significant

differences among treatment groups. (n ≥ 9 for all groups) (**B**) Functional silencing of the GH298GAL4-marked iLNs with UAS-shibire^{ts} facilitates habituation, apparent after 1 min of odor exposure (black bar, p<0.0001), which recovered to naïve levels by a 45V electric footshock (grey bar, p=0.4332). In contrast, UAS-shibire^{ts}/+controls did not present significant differences among treatment groups. (n ≥ 10 for all groups) (**C**) Functional silencing of the krasGAL4-marked eLNs driving UAS-shibire^{ts} did not affect responsiveness to 1X OCT after 1 min of exposure. Controls were similarly unaffected (black bar, ANOVA p=0.1171, n ≥ 10 for all groups). (**D**) Functional silencing of the GH146Gal4-marked excitatory projection neurons with UAS-shibire^{ts} resulted in facilitated habituation after 1 min of exposure (black bar, p<0.0001) and blocked footshock dishabituation (grey bar, p<0.0001). Control UAS-shibire^{ts}/+flies did not present significant response differences irrespective of treatment. (n ≥ 8 for all groups) (**E**) Blocked neurotransmission from APL neurons did not affect the response to 1X OCT following 1 min of exposure, similar to the responses of control groups (black bar, ANOVA p=0.1573, n ≥ 11 for all groups). (**F**) Expression of UAS-shibire^{ts} in MZ699GAL4 neurons to functionally silence the iPNs did not precipitate differences from the naive response, or from control flies (black bar, ANOVA p=0.4033, n ≥ 10 for all groups).

In contrast, silencing the mostly (60–66%) cholinergic, excitatory LNs (eLNs) under krasavietzGal4 (Acebes A, Martín-Peña A et al. 2011, Shang Y 2007) did not yield significant effects (Figure 4C). However, eLNs are also connected to ePNs and to iLNs via electrical synapses. These electrical synapses modulate PN output through direct excitation upon exposure to weak stimuli and indirect inhibition after strong stimulus exposure (Huang, Zhang et al. 2010, Yaksi and Wilson 2010). Although the chemical component of these eLN synapses is not implicated and as Shi^{ts} does not affect gap junction activity, we cannot rule out the possibility that electrical coupling of these synapses contributes to maintenance of the avoidance response, by maintaining excitation.

Collectively then, modulation of PN output by inhibitory GABAergic neurotransmission from the iLNs, and possibly via eLN electrical synapses, is crucial for stimulus value preservation and inhibition of premature habituation. LNs are known to modulate the PN output by broadening or narrowing their response dynamics (Silbering, Okada et al. 2008). LN activation also mediates periodic PN inhibition, thought to synchronize the responses of the latter and impact activation of Mushroom Body neurons (MacLeod and Laurent 1996).

Furthermore, iLN activation decreases PN firing rates at high odor concentrations (Wilson and Laurent 2005). Hence, attenuating iLN output may functionally mimic exposure to low odor concentration, which results in faster habituation (Figure 2D). Alternatively, signals un-modulated by the iLNs could be interpreted as 'noise', decreasing stimulus salience and facilitating habituation. In contrast, eLNs function in gain and redistribution of odor-evoked activity over a larger ensemble of PNs at low odor concentrations (Shang Y 2007). Since we use a relatively strong odor, we cannot exclude a role for the eLNs in habituation to dilute odorants. In addition, since strong stimulus exposure could lead to iLN activation via eLN-iLN gap junctions (Yaksi and Wilson 2010), it is possible that these chemical synapses are also necessary for habituation latency.

Excitatory projection neurons are essential for habituation latency.

Because iLNs modulate PN activity, we examined the role of the latter in habituation latency. PNs form three antennocerebral tracks (inner-iACT, middle-mACT and outer-oACT) (Stocker 2001), connecting the antennal lobe with higher order structures - the Mushroom Bodies (MBs) and the Lateral Horn (LH) (Wong, Wang et al. 2002, Tanaka, Awasaki et al. 2004). Because a driver clearly marking oACT neurons is not currently available to the best of our knowledge, we used the GH146Gal4 and MZ699Gal4 drivers to silence the iACT and mACT, respectively. GH146Gal4 marks 60% of the PNs, which account for approximately 90 mainly excitatory neurons (Liang, Li et al. 2013) that project axons through the iACT and innervate both MBs and LH (Wong, Wang et al. 2002).

Surprisingly, silencing GH146 neurons did not eliminate OCT avoidance (Figure 4D). This suggests that residual PNs not marked by GH146Gal4 or electrical synapses not affected by Shi^{ts}-mediated silencing suffice to convey odor information leading to odor avoidance. However, silencing GH146 neurons decreased habituation latency, resulting in premature habituation after only 1 min of OCT exposure and interestingly, eliminated dishabituation (Figure 4D). This suggests that neurotransmission from these neurons prevents premature habituation and promotes dishabituation. Independent validation of the reduced habituation latency was obtained by constitutive silencing of GH146 neurons with Tetanus Toxin Light Chain (Figure 4—figure supplement 1).

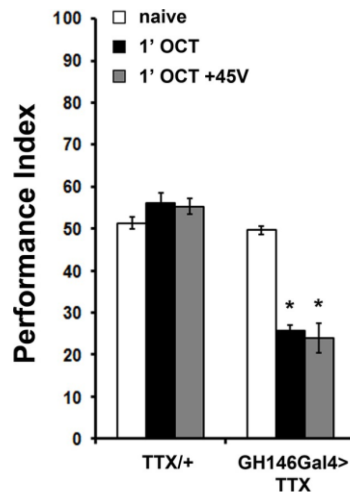


Figure 4—figure supplement 1. Expression of Tetanus Toxin Light Chain (TTX) under GH146Gal4 results in premature habituation. Mean Performance Indices ± SEM are shown in all figures. Stars indicate significant differences from the naive response unless specified otherwise. Expression of Tetanus Toxin Light Chain (TTX) in the projection neurons under GH146Gal4 blocks neurotransmission and results in premature habituation after only 1 min of 1X OCT exposure (black bar, $p<0.0001$) and inability to dishabituate (grey bar, $p<0.0001$). ($n \geq 6$ for all groups).

Importantly, besides excitatory neurons giving rise to the iACT, GH146Gal4 also marks the GABAergic and octopaminergic (Liu and Davis 2009, Wu, Shih et al. 2013) anterior paired lateral (APL) neurons, also known to be activated by odors (Silbering, Okada et al. 2008). APL neurons innervate the MBs and contribute to associative learning and memory (Liu and Davis 2009)l(Wu, Shih et al. 2013). They were silenced under APLGal4 (Wu, Shih et al. 2013), to determine whether they contribute to the decreased habituation latency upon synaptic blocking under the GH146Gal4 driver. Abrogating APL neurotransmission did not facilitate habituation (Figure 4E) and reducing GABA production in GH146Gal4-marked neurons via a GAD-RNAi, also did not shorten habituation latency (Figure 4—figure supplement 2). Because the latter is expected to affect both the inhibitory PNs and the GABAergic APL neurons marked by the GH146Gal4 driver, these results confirm that only the excitatory iACT PNs are essential for maintenance of habituation latency.

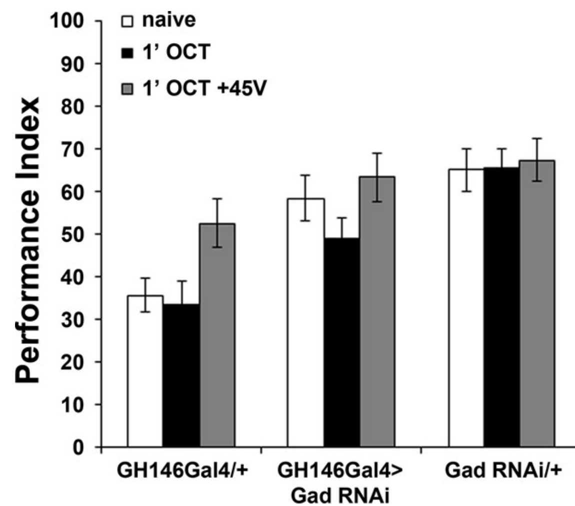


Figure 4—figure supplement 2. GABA attenuation in GH146-marked neurons does not affect habituation latency. Mean Performance Indices ± SEM are shown in all figures. Stars indicate significant differences from the naive response unless specified otherwise. Attenuation of GABA production by RNAi-mediated abrogation of Glutamic Acid Decarboxylase (Gad) within GH146Gal4 neurons did not precipitate premature habituation to OCT (black bar, ANOVA p=0.1832, n ≥ 9 for all groups).

Furthermore, silencing the 38 mainly GABAergic mACT projection neurons innervating the lateral horn (Liang, Li et al. 2013) under MZ699Gal4 (Tanaka, Ito et al. 2009), did not facilitate habituation after 1 min OCT exposure (Figure 4F). This strongly indicates that the mACT is dispensable for habituation latency. However, since Shi^{ts} expression affects only the chemical synapses between the iPNs and the LH, and not gap junctions between iPNs and ePNs (Shimizu and Stopfer 2017), it is possible that iPN-ePN electrical coupling drives maintenance of the response. Electrical coupling of iPNs and ePNs was been shown to amplify the antennal lobe response to certain odorants and may in fact enhance the response to the training odor, thus inhibiting habituation. Together with the GH146Gal4 silencing experiments, these results indicate that innervation of the MBs and LH by excitatory iACT neurons, but not the mACT PNs, is essential to sustain habituation latency and prevent premature habituation to brief continuous odor exposure.

The MBs are essential for habituation latency and dishabituation

The role of the iACT in response maintenance suggested that the MBs may also be engaged in the latency phase of odor habituation as they are for habituation to ethanol pulses

(Cho, Heberlein et al. 2004) and electric footshock (Acevedo, Froudarakis et al. 2007). If necessary for habituation latency, then eliminating the MBs should lead to premature habituation. To address this hypothesis, we used two complementary approaches. Chemical ablation with hydroxyurea (HU) (Acevedo, Froudarakis et al. 2007) and the *mbm¹* mutant presenting structurally aberrant, greatly reduced MBs (Raabe, Clemens-Richter et al. 2004). The Berlin strain was used as the appropriate cognate genetic control for *mbm¹* (Raabe, Clemens-Richter et al. 2004). Critically, both MB-ablated and *mbm¹* flies avoid OCT and other odorants normally (Raabe, Clemens-Richter et al. 2004, Acevedo, Froudarakis et al. 2007), verifying in essence that the LH without MB inputs is fully capable of mediating innate, unmodulated avoidance (Figure 5A,B-naïve) and attraction (Figure 5—figure supplement 1).

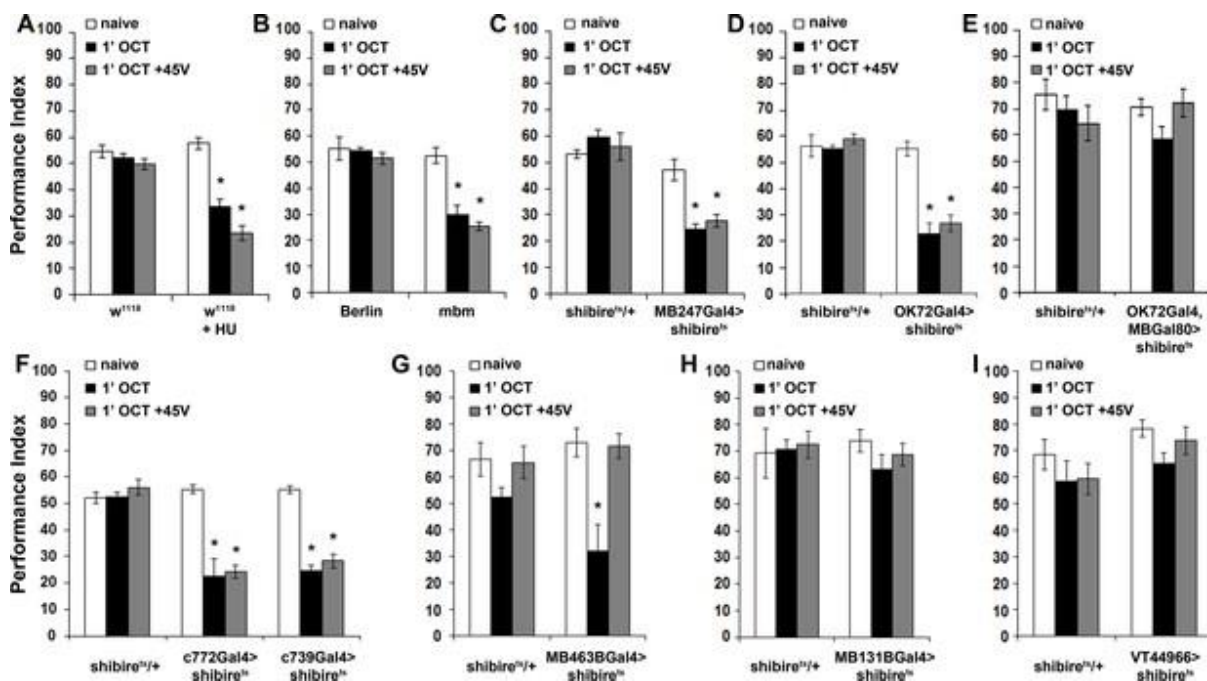


Figure 5. The mushroom bodies are essential for habituation latency and normal dishabituation. Mean Performance Indices ± SEM are shown in all figures. Stars indicate significant differences from the naïve response unless specified otherwise. (**A**) Flies with HU-ablated mushroom bodies (MBs) exhibit premature habituation after a 1-min exposure to 1X OCT (black bar, $p<0.0001$) and inability to dishabituate (grey bar, $p<0.0001$), while the response of the control groups was not affected. ($n \geq 8$ for all groups) (**B**) *mbm¹* mutants also presented facilitated habituation after a 1-min exposure to this odorant (black bar, $p<0.0001$) and inability to dishabituate (grey bar, $p<0.0001$). ($n \geq 6$ for all groups) (**C**) Silenced neurotransmission from the MBs with MB247Gal4 driving UAS-shibire^{ts} resulted in premature habituation following a 1 min 1X OCT exposure (black bar, $p<0.0001$) and inability to dishabituate (grey bar, $p<0.0001$). ($n \geq 8$ for all groups) (**D**) Blocked neurotransmission in OK72Gal4-

expressing neurons also facilitated habituation after a 1-min odor exposure (black bar, p<0.0001), and inability to dishabituate (grey bar, p<0.0001). (n ≥ 6 for all groups) (**E**) Functional silencing of all OK72Gal4-marked neurons apart from the MBs under OK72Gal4;MBGal80 does not affect odor habituation after 1 min of exposure (black bar, ANOVA p=0.0752, n ≥ 10 for all groups). (**F**) Functional silencing of the αβ MB neurons under c772Gal4 and c739Gal4 facilitated habituation after only 1 min of 1X OCT exposure (black bars, p=0.0002 for c772Gal4 and p<0.0001 for c739Gal4) and resulted in inability to dishabituate (grey bars, p=0.0005 for c772Gal4 and p<0.0001 for c739Gal4) in contrast to controls (UAS-shibire^{ts}/+) that did not alter their response. (n ≥ 6 for all groups) (**G**) Functional silencing of the α’β’ MB neurons under MB463BGal4 facilitated habituation after 1 min of 1X OCT exposure (black bar, p=0.0011) in contrast to controls that did not alter their response, and showed normal dishabituation (grey bar, p=0.9842). (n ≥ 8 for all groups) (**H-I**) Blocked neurotransmission from the γ MB neurons results in normal OCT responsiveness after 1 min of exposure under both MB131BGal4 (black bar, ANOVA p=0.3026, n ≥ 8 for all groups) and VT44966 (black bar, ANOVA p=0.0651, n ≥ 8 for all groups).

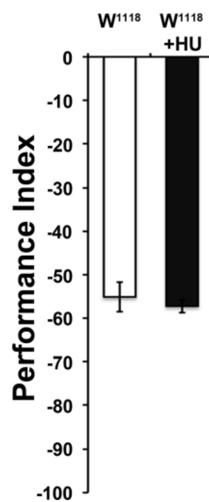


Figure 5—figure supplement 1. Attraction to 1X ETA was not altered by Hydroxyurea (HU)-dependent ablation of the MBs. Mean Performance Indices ± SEM are shown in all figures. Stars indicate significant differences from the naive response unless specified otherwise (ANOVA p=0.5251, n > 7 for all groups).

MB ablation (Figure 5A) or reduction (*mbm*^{1}), led to premature habituation after 1 min of OCT exposure (Figure 5B). This drastically reduced habituation latency indicates that normally the MBs inhibit olfactory habituation, possibly by modulating the LH-mediated innate OCT avoidance. To verify this and determine whether the premature habituation of MB-ablated and *mbm*^{1} flies was developmental in origin, we silenced the MBs using

MB247Gal4 (Zars, Fischer et al. 2000). Although driver heterozygotes are not included in Figure 5, no significant differences from *shibire*^{ts}/+were uncovered (Supplementary File 1).

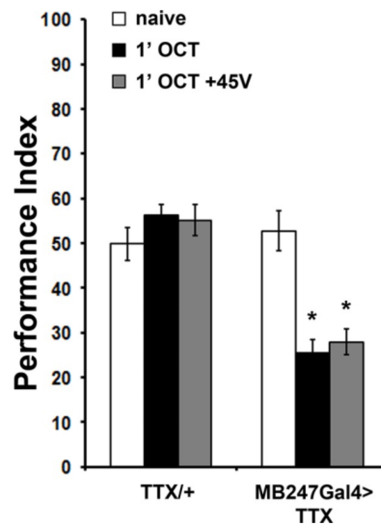


Figure 5—figure supplement 2. Expression of Tetanus Toxin Light Chain (TTX) in the MBs results in premature habituation. Mean Performance Indices ± SEM are shown in all figures. Stars indicate significant differences from the naive response unless specified otherwise. Expression of Tetanus Toxin Light Chain (TTX) in the MBs under MB247Gal4 blocks neurotransmission and results in premature habituation to 1X OCT (black bar, $p<0.0001$) and inability to dishabituate (grey bar, $p<0.0001$). ($n \geq 8$ for all groups)

Remarkably, 1 min of OCT exposure was sufficient to elicit habituation upon functionally silencing the MBs (Figure 5C), just as for MB-ablated and *mbm*^{1}animals (Figure 5A,B). This was independently validated by expression of Tetanus Light Chain under MB247Gal4 (Figure 5—figure supplement 2). Further confirmation for the role of the MBs in habituation latency was obtained with OK72Gal4 (de Haro, Al-Ramahi et al. 2010) (Figure 5D). Because in addition to the MBs OK72Gal4 marks antennal lobe neurons (Devaud, Keane et al. 2003), we used OK72Gal4;MBGal80 to silence only the latter but spare the MBs, a manipulation which did not yield facilitated habituation, verifying that the MB component of OK72Gal4 is necessary for habituation latency (Figure 5E). Therefore, the MBs are essential for response maintenance during the first minute(s) of odor exposure. In addition, this demonstrates that the facilitated habituation of HU-ablated and *mbm*^{1} flies is unlikely consequent of altered development or brain anatomy re-arrangement. Furthermore, structurally and functionally intact MBs are also required for dishabituation, as their complete

ablation, partial abrogation in *mbm¹* mutants, or functional silencing, eliminated dishabituation after 1-min OCT exposure (Figure 5A–D).

The three broad types of MB intrinsic neurons, the αβ, α′ β′and γ (Crittenden, Skoulakis et al. 1998) are differentially implicated in olfactory learning and memory (Yu, Akalal et al. 2006, Krashes, Keene et al. 2007, Blum, Li et al. 2009, Zhang and Roman 2013). Because neurotransmission from αβ neurons prevents premature habituation to footshocks (Acevedo, Froudarakis et al. 2007), we investigated whether these neurons function similarly for olfactory habituation. The role of the αβ neurons was examined using c772Gal4 and c739Gal4, which label preferentially αβ and γ neurons and almost exclusively αβ neurons, respectively (Aso, Grubel et al. 2009). Silencing the MBs with either driver resulted in premature habituation after 1 min of OCT exposure and inability to dishabituate (Figure 5F), confirming the results obtained with MB247Gal4 (Figure 5C). Therefore, αβ neurons are indispensable for habituation latency. Interestingly, silencing the α′β′ neurons with the highly selective split-Gal4 line MB463B (Aso, Hattori et al. 2014) also facilitated habituation, but did not affect dishabituation (Figure 5G). Finally, silencing the γ neurons with the MB131B split-Gal4 (Aso, Hattori et al. 2014) and the independent γ driver VT44966, did not affect habituation (Figure 5H,I).

Therefore, neurotransmission from αβ and α′β′, but not γ MB neurons and their activation ostensibly by the PNs upon odor exposure, is essential for stimulus value maintenance, which underlies normal habituation latency. Because only the αβ neurons are required for normal dishabituation with footshock, it appears that the dishabituating stimulus engages them differentially and distinguishes them functionally from their α′β′counterparts.

Distinct neuronal subsets are required to establish olfactory habituation.

Collectively, the results indicate that habituation latency is an active process requiring synaptic activity of distinct neuronal subsets engaged in odor information processing. However, of equal importance is timely habituation. To identify neurons mediating habituation we silenced the neuronal assemblies involved in the olfactory pathway and assessed avoidance after 4 min of OCT exposure, adequate for control flies to habituate (Figure 1A).

Blocking iLN or eLN antennal lobe interneurons was permissive to habituation after 4 min of OCT stimulation (Figure 6A,B). Perhaps, this is expected for the iLNs since their activity is required for response maintenance (Figure 4A,B), but not for the eLNs, which do not function in habituation latency (Figure 4C). We next investigated the role of the excitatory and inhibitory PNs marked by GH146Gal4 and MZ699Gal4, respectively. Surprisingly, given their role for habituation latency, silencing GH146 neurons blocked habituation after 4 min of OCT (Figure 6C). However, reducing GABAergic neurotransmission from the inhibitory GH146Gal4-marked neurons including the APL had no effect (Figure 6—figure supplement 1), indicating that the cholinergic neurons necessary for response maintenance also participate in establishing habituation. Significantly, blocking MZ699 neurons also eliminated habituation (Figure 6D), indicating that these neurons function specifically in the habituation and not during the preceding latency phase (Figure 4F). Therefore, both excitatory iACT and inhibitory mACT PN neurons are necessary to establish odor habituation. The performance of all remaining controls is presented in Supplementary File 2.

To verify the role of PNs in habituation and determine whether their activation is sufficient to mediate the process, we depolarized them artificially by activation of the heat-activated TRPA1 channel (Pulver, Pashkovski et al. 2009) for 1 or 4 min without odor stimulation and then tested the flies for OCT avoidance. Activation of either PN subset for 1 min did not alter OCT avoidance (Figure 6E,F), as it did not when the GH146-marked PNs were depolarized for 4 min (Figure 6E). However, activation of the inhibitory MZ699-marked mACT PNs for 4 min attenuated OCT avoidance without prior exposure to the odor (Figure 6F). These results suggest that prolonged activation of iPNs innervating the LH is necessary and sufficient for habituation, while the ePNs are also necessary, because silencing them blocks habituation (Figure 6C), but not sufficient to drive OCT habituation. It is likely that mACT PNs are functionally 'downstream' of those of the iACT in promoting habituation, possibly by inhibiting the LH-mediated innate odor avoidance.

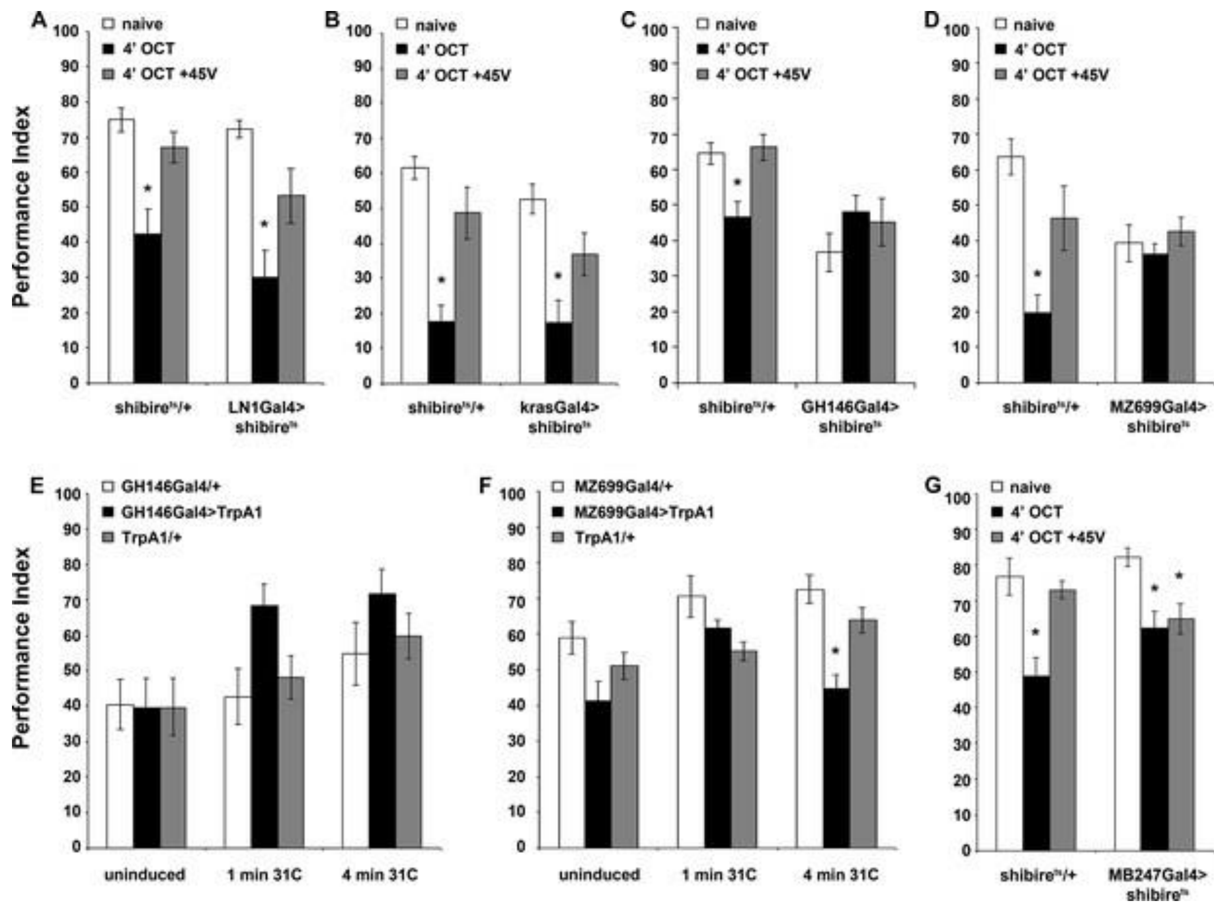


Figure 6. Distinct neuronal subsets are required for olfactory habituation. Mean Performance Indices ± SEM are shown in all figures. Stars indicate significant differences from the naive response unless specified otherwise. (**A**) Blocked neurotransmission from iLNs results in normal habituation after 4 min of OCT exposure (black bar, p<0.0001) and normal dishabituation (grey bar, p=0.0867), similar to controls. (n ≥ 10 for all groups) (**B**) Blocked neurotransmission from the eLNs yielded normal habituation to 4 min 1X OCT exposure (black bar, p<0.0001) and normal dishabituation (grey bar, p=0.0977), similar to controls. (n ≥ 12 for all groups) (**C**) Blocked neurotransmission from the GH146Gal4-marked ePNs resulted in abrogated habituation, with the response to OCT remaining at naïve levels even after 4 min of exposure (black bar, ANOVA p=0.3640), while controls habituated (black bar, p=0.0026) and dishabituated normally (grey bar, p=0.9159). (n ≥ 11 for all groups) (**D**) Blocked neurotransmission from iPNs under MZ699Gal4 resulted in abrogated habituation, with the response to 1X OCT remaining at naïve levels after 4 min of exposure (black bar, ANOVA p=0.6121), while controls habituated (black bar, p=0.0002) and dishabituated normally (grey bar, p=0.1491). (n ≥ 10 for all groups) (**E**) Activation of the GH146Gal4-marked PNs with UAS-TRPA1 but without odor exposure did not alter significantly the response to 1X OCT after 1 min (black bar, ANOVA p=0.03) or 4 min of activation (black bar, ANOVA p=0.2707). (n ≥ 10 for all groups) (**F**) Activation of the iPNs under MZ699Gal4 to drive UAS-TRPA1 did not alter significantly the response to 1X OCT after 1 min (black

bar, ANOVA p=0.0190), but 4 min of activation sufficed to produce significant habituation (black bar, p<0.0001 when compared to MZ699Gal4/+). (n ≥ 13 for all groups) (**G**) Blocked neurotransmission from the MBs under MB247Gal4 resulted in normal habituation to 4 min of 1X OCT exposure (black bar, p=0.0033) and inability to dishabituate (grey bar, p=0.0075) while control flies habituate (black bar, p=0.0003) and dishabituate normally (grey bar, p=0.8229). (n ≥ 10 for all groups)

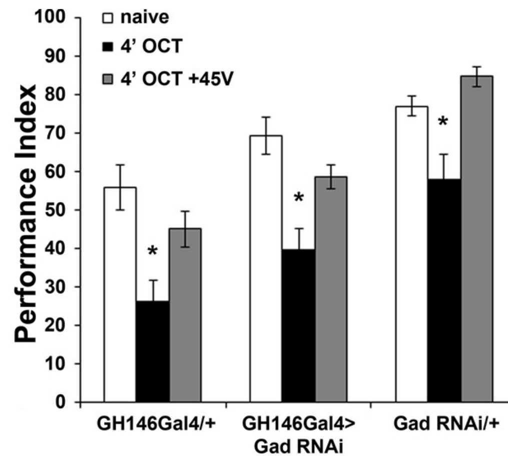


Figure 6—figure supplement 1. GABA attenuation in GH146-marked neurons does not affect habituation. Mean Performance Indices ± SEM are shown in all figures. Stars indicate significant differences from the naive response unless specified otherwise. Attenuation of GABA production by RNAi-mediated Gad abrogation within GH146Gal4 neurons did not alter habituation to 1X OCT after 4 min of exposure (black bar, p<0.0001) or dishabituation (grey bar, p=0.2233). (n ≥ 11 for all groups).

Finally, we investigated the role of the MBs in habituation. Surprisingly, collective silencing of the MBs under MB247Gal4 resulted in normal habituation (Figure 6G). Therefore, the MBs are dispensable for establishing or expression of odor habituation, in contrast to their function during habituation latency.

The rutabaga adenylyl cyclase is essential for normal olfactory habituation.

The cAMP signaling pathway has been implicated in habituation of various modalities and circuits in Drosophila (Engel and Wu 2009). Rutabaga, the major Adenylyl Cyclase in the adult *Drosophila* brain (Han, Levin et al. 1992), has been shown necessary for odor habituation after repetitive (Cho, Heberlein et al. 2004), or continuous odor exposure (Das, Sadanandappa et al. 2011). More specifically, Rut is required in LN1 neurons to mediate habituation after 30 min odor exposure (Das, Sadanandappa et al. 2011). Given the

differences, we uncovered in the neuronal subsets implicated in habituation to 4 min and 30 min odor exposure, we investigated the role of Rut in the latency and habituation phases to OCT stimulation.

The *rut*^{2080} (Levin, Han et al. 1992) mutant presented premature habituation after only 1 min of OCT exposure (Figure 7A), suggesting a role for this protein in habituation latency. To elucidate whether Rut is indeed required for habituation latency within the LNs, PNs, or the MBs, where it is preferentially expressed (Han, Levin et al. 1992), we expressed a UAS-*rut* transgene in these neurons of *rut*^{2080} mutants. Rut re-expression in LNs was sufficient to restore habituation latency after 1 min of OCT exposure (Figure 7B). This was independently validated by RNA interference (RNAi)-mediated Rut abrogation in adult LNs under LN1Gal4;Gal80^{ts}, which phenocopied the premature habituation of *rut*^{2080} mutants (Figure 7C), confirming the role of Rut within the iLNs for habituation latency. In contrast, Rut re-expression in PNs (Figure 7D), or the MBs (Figure 7E), did not rescue the premature habituation phenotype of *rut*^{2080} mutants, indicating that the protein is not required therein for response maintenance.

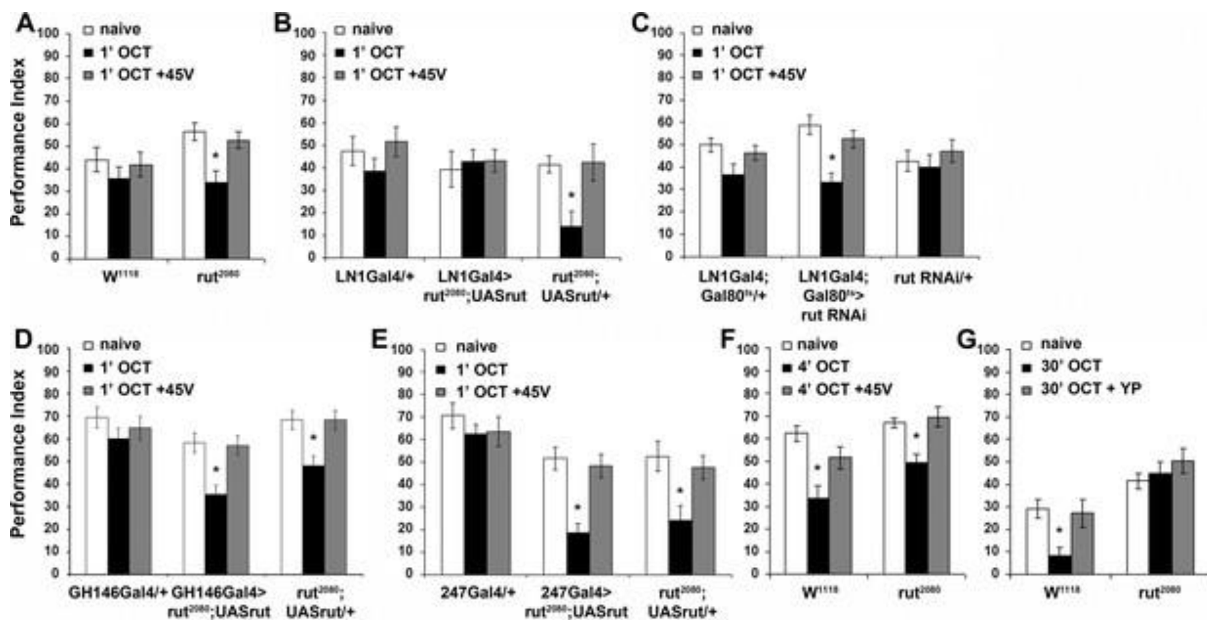


Figure 7. The Rutabaga Adenylyl Cyclase is required for olfactory habituation. Mean Performance Indices ± SEM are shown in all figures. Stars indicate significant differences from the naive response unless specified otherwise. (**A**) Rut^{2080} mutants presented facilitated habituation to 1X OCT after only 1 min of exposure (black bar, $p=0.0011$) and normal dishabituation (grey bar, $p=0.7873$). Controls responded indistinguishably from naive animals to 1 min of 1X OCT (black bar, ANOVA $p=0.5346$).

(n ≥ 14 for all groups) (**B**) Targeted Rut re-expression in *rut*^{2080} mutants specifically in iLNs rescued their premature habituation (black bar, ANOVA p=0.8976), while *rut*^{2080}; UASrut/+animals habituated prematurely (black bar, p=0.0079). (n ≥ 10 for all groups) (**C**) Rut abrogation via RNAi-mediating transgene expression in iLNs mimics the facilitated habituation of *rut*^{2080} (black bar, p<0.0001), while both controls did not (LN1Gal4;Gal80^{ts} ANOVA p=0.0606; rutabaga RNAi/+ANOVA p=0.5997). (n ≥ 18 for all groups) (**D**) Targeted Rut re-expression in *rut*^{2080} mutants specifically in ePNs under GH146Gal4 did not rescue their premature habituation (black bar, p=0.0024), which is similar to that presented by *rut*^{2080} mutants (black bar, p=0.0041). However, dishabituation was normal in both cases. (n ≥ 10 for all groups) (**E**) Targeted Rut re-expression in *rut*^{2080} MBs under MB247Gal4 did not rescue the premature habituation (black bar, p<0.0001) in accord to the performance of *rut*^{2080} mutants (black bar, p=0.0060). Dishabituation was normal in both cases. (n ≥ 10 for all groups) (**F**) *rut*^{2080}habituated normally to 1X OCT after 4 min of exposure (black bar, p=0.0039) and dishabituated normally (grey bar, p=0.8037). (n ≥ 15 for all groups) (**G**) Rutabaga mutants did not habituate after 30 min 1X OCT exposure (black bar, ANOVA p=0.4287), in contrast to control flies that habituated normally (black bar, p=0.0067) and dishabituated with a yeast puff (grey bar, p=0.9279). (n ≥ 12 for all groups)

Habituation of *rut*^{2080} mutants was normal after 4 min of OCT exposure (Figure 7F), indicating that Rut is not required for habituation. This is not unexpected, since Rut is required within the LNs, which are specifically required for habituation latency (Figure 4A), but are dispensable for habituation (Figure 6A). However, Rut has been reported essential for habituation to 30 min odor stimulation (Das, Sadanandappa et al. 2011). This difference is consistent with the notion that habituation to 4 min odor stimulation is distinct from habituation to 30 min of odor exposure, or it might be consequent of the different odor stimuli or experimental setups used in the two paradigms. To differentiate between these alternatives, we established habituation to 30-min OCT stimulation in our experimental setup. We demonstrate that as for 4-min habituation, continuous or pulsed OCT stimulation for 30-min results in response attenuation in control flies (Figure 7—figure supplement 1A). This long exposure habituated response recovered spontaneously after 6 or 30 min of rest (Figure 7—figure supplement 1B,C) in agreement with a prior report (Das, Sadanandappa et al. 2011). However, we were unable to dishabituate the long odor exposure habituation with footshock or vortexing (Figure 7—figure supplement 1D,E), as for habituation to 4 min of OCT. Interestingly, habituation after 30 min of OCT exposure was dishabituated with a short puff of yeast paste odor (Figure 7—figure supplement 1F, Figure 7G).

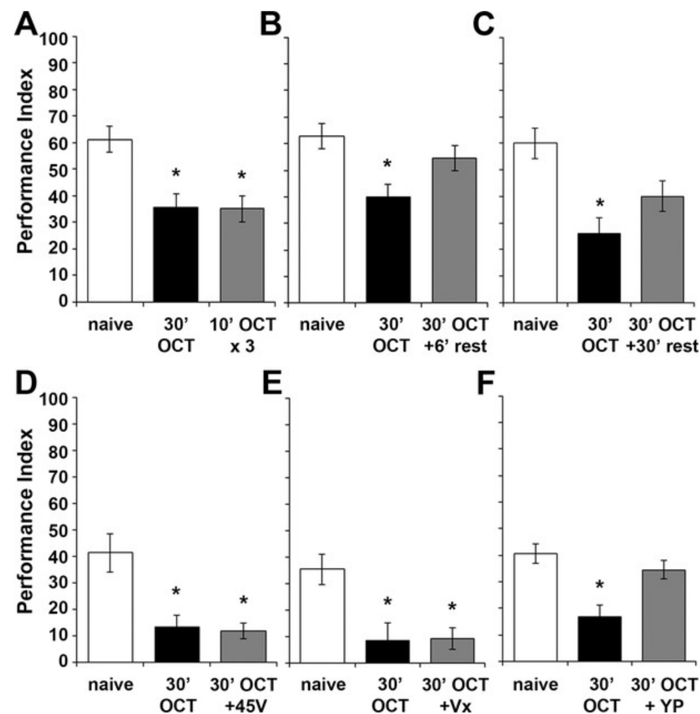


Figure 7—figure supplement 1. Habituation parameters after continuous exposure to OCT for 30 min. Mean Performance Indices ± SEM are shown in all figures. Stars indicate significant differences from the naïve response unless specified otherwise. Three 10-min 1X OCT pulses elicited habituation to this odor (grey bar, p=0.0009), mimicking the effect of continuous 30-min exposure to the odor (black bar, p=0.0010). (n ≥ 15 for all groups) (**B**) A 6-min rest interval following 30 min of 1X OCT was adequate for spontaneous recovery of the naive response (grey bar, p=0.3282, n = 10 for all groups). (**C**) A 30 min rest after 30 min 1X OCT exposure also resulted in spontaneous recovery of the naive response (grey bar, p=0.0122, n = 10 for all groups). (**D**) Exposure to OCT for 30 min elicited habituation (black bar, p=0.0012) and inability to dishabituate by a 45V electric footshock (grey bar, p=0.0007). (n = 11 for all groups) (**E**) Exposure to OCT for 30 min resulted in habituation (black bar, p=0.0039) and inability to dishabituate by a 3 s vortex (grey bar, p=0.0061). (n ≥ 9 for all groups) (**F**) 30 min of 1X OCT exposure resulted in habituation to that odor (black bar, p=0.0003), but a 3 s yeast puff sufficed to dishabituate the response (grey bar, p=0.4588). (n ≥ 11 for all groups).

These results suggest that the response attenuation after prolonged odor stimulation is distinct from habituation to 4 min of exposure. Importantly, we verified that Rut is essential for habituation to 30-min OCT stimulation (Figure 7G), as has been shown by Das *et al* using different odors and experimental apparatus (Das, Sadanandappa et al. 2011). This indicates that Rut and by extension cAMP signaling, appear essential within inhibitory LNs, for habituation latency and prolonged odor habituation. This highlights the differential role of

Rut in the dynamic engagement of circuits and molecular mechanisms therein, to ensure and regulate responses to continuous brief or prolonged inconsequential stimuli.

Discussion

A novel olfactory habituation paradigm.

We describe a novel olfactory habituation paradigm to brief odor stimuli and operationally define two distinct phases in the response dynamics. The initial period of ~120 s we term habituation latency is characterized by maintenance of responsiveness to the odor. This is followed by manifestation of the habituated response, characterized behaviorally by attenuated osmotaxis. Focusing on the behavioral dynamics early in the process complements previous work olfactory habituation to continuous odor stimulation in *Drosophila* (Das, Sadanandappa et al. 2011, Sadanandappa, Blanco Redondo et al. 2013). A number of criteria differentiate these two paradigms from other types of habituation to olfactory stimuli as discussed below.

We show that *Drosophila* habituate equally well to continuous or pulsed olfactory stimuli (Figure 1, Figure 1—figure supplement 1, Figure 7—figure supplement 1A). This likely reflects the nature of olfactory stimuli, which typically are continuous rather than pulsed. On the other hand, habituation of the startle response to ethanol vapor (Cho, Heberlein et al. 2004) may specifically require short (30 s) pulses due to its sedative properties and this may also be reflected by the rather long 6 min ITIs compared to the 15 s to 2.5-min intervals used herein for OCT. Short odor pulses are also required for the odor-mediated jump and flight response habituation (Asztalos, Arora et al. 2007), suggesting that pulsing may be necessary to evoke the startle response per se.

An important property shared with all habituation paradigms in Drosophila and other systems is spontaneous recovery of the response (Thompson and Spencer 1966, Rankin, Abrams et al. 2009). This is another differentiating parameter among habituation paradigms in Drosophila. For the olfactory habituation paradigms, whereas 6 min suffice for spontaneous recovery after 4 and 30 min continuous odor exposure (Figure 2A,B and Figure 7—figure supplement 1B,C), 15–30 (Cho, Heberlein et al. 2004) to surprisingly 60 min (Asztalos, Arora et al. 2007) are required for recovery in the olfactory startle paradigms. Habituation to

mechanosensory stimuli typically also requires shorter spontaneous recovery times, with habituation of the giant fiber-mediated jump-and-flight response requiring a mere 2 min (Engel and Wu 1996) and electric footshock habituation 6 min (Acevedo, Froudarakis et al. 2007). Interestingly, other non-mechanosensory habituation paradigms require long spontaneous recovery periods with 30 min for habituation of the proboscis extension reflex (PER), (Paranjpe, Rodrigues et al. 2012) and surprisingly, 2 hr for habituation of odor-induced leg response (Chandra and Singh 2005). We posit that these differences reflect the engagement of distinct neuronal circuits mediating habituation to these diverse stimuli and the properties and connections of the neuronal types that comprise them.

Olfactory habituation phases are mediated by distinct neuronal subsets.

Overall, our data suggest that latency and habituation to brief odor exposure involve modulation of LH output, a neuropil innately encoding response valence to odor stimuli (Fisek and Wilson 2014, Schultzhaus, Saleem et al. 2017). We propose that habituation latency involves processes that are not permissive to, or actively prevent stimulus devaluation. Latency duration depends on stimulus strength (Figure 2D), as suggested (Thompson and Spencer 1966, Rankin, Abrams et al. 2009) and is consistent with the notion that it is adaptive not to devalue strong, hence potentially important stimuli, expediently. In fact, we posit that habituation latency serves to facilitate associations with concurrent stimuli, a requirement for associative learning. Shortened latency leading to premature habituation is predicted to compromise associative learning.

Importantly, maintaining responsiveness early upon odorant exposure requires activity of GABAergic inhibitory neurons (Figure 4A,B), which are essential for lateral inhibition of antennal lobe glomeruli (Figure 8A). LN activation appears to prevent saturation by strong continuous odors and hence reduce PN activity (MacLeod and Laurent 1996, Olsen, Bhandawat et al. 2007). Therefore, shortening habituation latency by blocking GABAergic neurotransmission in the antennal lobe may effectively reduce stimulus intensity, expediting habituation as suggested by the dilute odor experiments (Figure 2D). This interpretation is further supported by the decreased habituation latency upon silencing the iACT PNs (Figure 4D) conveying olfactory signals to the MBs and the LH (Stocker RF 1997, Tanaka, Awasaki et al. 2004), but not by the mACT neurons (Figure 4F) innervating only the LH (Liang, Li et al.

2013). Since iACT PNs are mainly excitatory, it appears that response maintenance requires excitatory signaling to the LH and the MBs (Figure 8A).

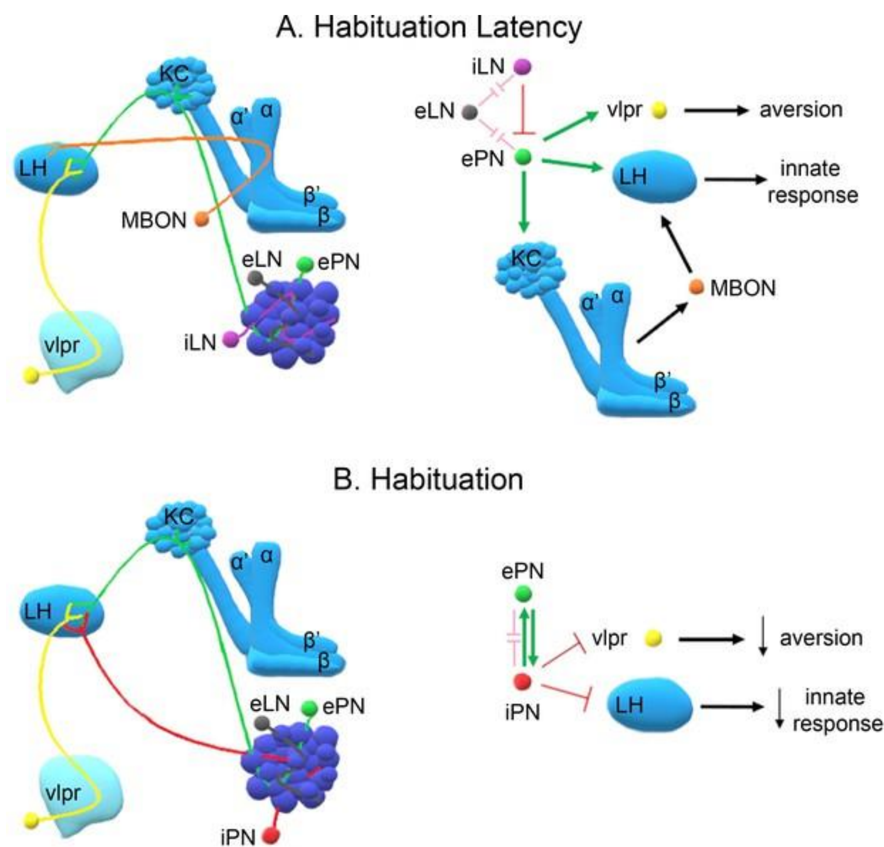


Figure 8. A model of the neuronal subsets underlying (A) Habituation Latency and (B) Habituation, after exposure to aversive stimuli. The antennal lobe, the mushroom bodies, the lateral horn (LH) and the ventrolateral protocerebrum (vlpr) are depicted with shades of blue, lighter blue showing higher order neurons. Distinct neuronal subsets are marked with different colors; iACT excitatory projection neurons (ePN-green), inhibitory local interneurons (iLN-purple), excitatory local interneurons (eLNs-grey), Mushroom Body Output Neurons (MBONs-orange), ventrolateral protocerebrum neurons (vlpr-yellow), mACT inhibitory projection neurons (iPNs-red). Green arrows indicate activation while red blunt arrows indicate inhibition. Pink blunt arrows indicate electrical synapses.

All MB neuronal types except the γ, are essential for habituation latency (Figure 5). This suggests that at least part of the excitatory signal conveyed by the iACT PNs impinges upon the αβ and α′ β′ MB neurons (Stocker RF 1997, Tanaka, Awasaki et al. 2004), which is consistent with their role in associative learning (Busto, Cervantes-Sandoval et al. 2010) and the proposal that habituation latency facilitates it. Neurotransmission from the MBs to LH neurons mediating aversive responses likely engages MB output neurons (MBONs), to

maintain the valence and intensity of the odor and sustain aversion (Figure 8A). Distinct MBONs are known to drive both attraction and aversion to odors (Aso, Sitaraman et al. 2014) and their potentially differential involvement in habituation is currently under investigation.

Dishabituation results in stimulus value recovery and apparently resets habituation latency (Figure 3C). Clearly it requires neurotransmission via the GH146-marked neurons and MBs because silencing these neurons disables dishabituation (Figure 4D, Figure 5C,D,F, Figure 6G), consistently with their role in response maintenance. These results lead us to hypothesize that dishabituating stimuli might converge on the MBs and/or iACT, possibly stimulating excitatory neurotransmission to the LH, to reinstate stimulus aversion. This hypothesis is currently under investigation as well.

In contrast, habituation requires prolonged or repeated exposure to the odorant and functional iACT and mACT PNs (Figure 8B) converging on the LH (Tanaka, Awasaki et al. 2004, Liang, Li et al. 2013). Interestingly, the mainly GABAergic mACT PNs (Okada R, Awasaki T et al. 2009) receive input both from the olfactory sensory neurons and the excitatory iACT PNs (Wang, Gong et al. 2014). Their depolarization also activates the excitatory iACT neurons via direct chemical synapses (Shimizu and Stopfer 2017). This apparent feedback loop may be required for mACT activation after prolonged exposure to aversive odors, since these neurons were reported to respond mainly to attractive stimuli (Strutz, Soelter et al. 2014). We propose that prolonged aversive odor exposure enhances iACT activation, which in turn leads to habituation (Figure 6F, Figure 8B), while shorter exposure does not activate the iACT neurons, reflected by their dispensability for habituation latency (Figure 4F). Importantly, the mACT innervates the LH downstream of the iACT PNs, providing feedforward inhibition (Wang, Gong et al. 2014). These characteristics likely underlie the necessary and sufficient role of mACT PNs in habituation upon 4-min odor stimulation. Collectively, our results are consistent with the proposal that mACT activation inhibits the innate LH-mediated avoidance response to the aversive odorant, establishing habituation (Figure 8B). However, full mACT activation appears to also require iACT neurotransmission, which if abrogated eliminates habituation (Figure 6C) but is insufficient to establish it on its own (Figure 6E).

Because the MZ699 Gal4 driver also marks ventrolateral protocerebrum (vlpr) neurons it is possible that they also play a role in habituation. In fact, vlpr neurons function in

aversive odor responses, are activated by excitatory iACT PNs, inhibited by the inhibitory PNs, and are afferent to the LH (Liang, Li et al. 2013). Thus, they could act in parallel or synergistically to mACT PNs to establish the habituated response. As we are not aware of a specific vlpr driver, it is impossible at the moment to address this possibility directly. Briefly then, our collective results strongly suggest a novel role for the inhibitory PNs innervating the LH, and possibly vlpr neurons, in inhibition of the innate response and habituation (Figure 8B). The kinetics of inhibitory projection neuron activation and their output on downstream neurons could serve as a measure of the duration of odor exposure. Upon prolonged exposure, these neurons mediate inhibition of odor avoidance, thus devaluing the stimulus.

Analysis of the neuronal subsets underlying habituation has focused on aversive odors. However, considering the neuronal clusters involved in the process, it would be relatively safe to assume that our results extend to attractive odor habituation as well. It is possible that the neuronal circuitry comprised of PNs, the LH and MBs may be mediating habituation independently of odor valence. However, specific neuronal clusters may differ in odor valance-dependent activation or inhibition of other circuit components with opposing effects on the behavioral readout. For example, inhibitory PNs (iPNs) mediate attraction by releasing GABA in the LH to inhibit avoidance (Strutz, Soelter et al. 2014). If inhibited themselves, the resultant attenuated attraction will likely drive a behavioral output of habituation to an attractive odor.

In accord with this notion, attractive and aversive odors are represented in different AL glomerular clusters (Seki, Dweck et al. 2017) and this valence-dependent organization is preserved into higher brain centers. In fact, the posterior-dorsal LH responds to attractive and its ventral complement to aversive odors (Seki, Dweck et al. 2017), while third order neurons convey information from ventral LH to the vlpr (Liang, Li et al. 2013, Strutz, Soelter et al. 2014) and from the dorsal LH to the superior medial protocerebrum (Fisek and Wilson 2014). This organization potentially reflects differential recruitment of these neuronal clusters in habituation to aversive and attractive odors. The circuits involved in habituation to attractive odors and their specific contribution to the process will be the focus of future work.

Habituation after prolonged odor stimulation.

Although behaviorally there is significant osmotactic attenuation after both 4 and 30 min aversive odor exposure, our experiments suggest that these represent distinct types of olfactory habituation. Habituation after 4 min of odor exposure does not require the MBs, but rather the projection neurons innervating the LH (Figure 6C,D, Figure 6F). Habituation after 30 min of exposure is also independent of MB function (Das, Sadanandappa et al. 2011), but appears to be entirely mediated by iLNs and reside within the AL (Das, Sadanandappa et al. 2011). This clear difference suggests that the specific potentiation of inhibitory synapses shown to underlie habituation after 30 min of exposure is not necessary for habituation to the brief 4-min exposure. Additionally, while Rut is required within the iLNs during the latency period upon brief odor exposure (Figure 4A,B), it is surprisingly required within the same neurons for habituation to long odor exposure (Das, Sadanandappa et al. 2011). Therefore, Rut-driven activity within the iLNs yields opposing time-dependent behavioral outputs in accord with the abovementioned notion that the same circuit components may drive opposing outputs.

Furthermore, the fact that mechanosensory stimuli are not effective dishabituators after 30 min of odor exposure as they are after 4 min (Figure 7—figure supplement 1D,E), augments the conclusion these are different types of olfactory habituation and suggests that distinct dishabituators likely recruit different neuronal subsets to modulate the habituated response. Such neuronal circuits and the effect of different dishabituators in response recovery are currently under investigation.

Altogether, the results indicate different mechanisms for 4 min and 30 min habituation to aversive odors with the former mediated by the interaction between iPNs, ePNs and their targets in the LH, while the latter is based on the inhibition of ePNs by iLNs at the AL level. However, it is possible that the potentiated PN inhibition would decrease their output to the LH to drive reduced avoidance. This argues that the LH could be involved in the behavioral output indicating habituation after 30 min of OCT exposure as well. An AL-mediated reduction in the perceived intensity or valence of a chronically present odor probably serves an adaptive evolutionary role distinct from short exposure to the same stimulus. In fact, filtering away the chronic odor at the antenna, the first olfactory synaptic

station, might facilitate evaluation of additional odors at higher order neurons such as the MBs or the LH.

Significantly, this interpretation is congruent with timescale habituation in mice, where short-timescale odor habituation is mGluR-dependent and mediated by the anterior piriform cortex while long-timescale habituation requires NMDAR and is mediated by the olfactory bulb (Chaudhury, Manella et al. 2010). In addition, studies in mice, rats and primates have shown that habituation of the higher order neurons is faster and more prominent than in olfactory bulb neurons (Zhao, Holahan et al. 2015, Zhao, Wang et al. 2016). Therefore, temporal and spatial principles for olfactory habituation appear broadly conserved between insects and mammals, despite their evolutionary distance.

Materials and methods

Drosophila strains

Drosophila were cultured in standard wheat-flour-sugar food supplemented with soy flour and $CaCl_2$ at 22–25C, unless specified otherwise. Animals expressing Gal80^{ts} (TARGET system) were raised at 18C until hatching and then placed at 30C for 3 days prior to testing. Animals expressing the tetanus toxin light chain transgene (UAS-TTX) (Sweeney, Broadie et al. 1995) were raised at 18C until hatching, then placed at 21–22C for 2 days prior to testing. TT_{LC} cleaves synaptobrevin, a protein required for synaptic vesicle docking, thus silencing presynaptic neurons constitutively (Humeau, Doussau et al. 2000).

The control strain Berlin and the *mushroom body miniature* (*mbm^{1}*) mutants have been described previously (Heisenberg, Borst et al. 1985). Control flies carrying the *w^{1118}*mutation had been backcrossed to the Canton-S for at least 10 generations (*w^{1118}*strain). As a second control, *w**, an independent mutation in the *white* gene was used. All other strains had been backcrossed to the Cantonised-*w^{1118}* for four to six generations prior to use in behavioral experiments. The *rutabaga* mutant *rut^{2080}*was described previously (Levin, Han et al. 1992). Transgenes used to block neurotransmission were the UAS-TTX (Keller, Sweeney et al. 2002), encoding the tetanus toxin light chain and the UAS-shi^{ts}, which bears a temperature-sensitive mutation in dynamin, encoded by the gene *shibire* (Kitamoto 2001). To achieve neuronal

hyperpolarization, the transgene UAS-TRPA1 was used for overexpression of the TRPA1 channel (Rosenzweig, Brennan et al. 2005).

GH146, MB247, c772 and c739 Gal4 drivers have been described before (Acevedo, Froudarakis et al. 2007, Pavlopoulos, Anezaki et al. 2008) and similarly for OK72 (Acebes and Ferrus 2001). MB463B and MB131B targeting the α’β’ and γ lobe, respectively, were kindly provided by Y. Aso (Janelia Research Campus, Howard Hughes Medical Institute, Ashburn, VA) (Aso, Hattori et al. 2014). The VT44966 γ lobe driver was obtained from the Vienna Drosophila Resource Center (VDRC, #203571), as was the UAS-Gad RNAi (VDRC, #32344). The APLGal4 driver was described previously (Wu, Shih et al. 2013). The UAS-rutabaga was obtained from Bloomington (#9405) and has been described before (Zars, Fischer et al. 2000). MBGal80 was obtained from Ron Davis (Scripps Florida), while LN1Gal4, LN1Gal4;Gal80^{ts} (Sudhakaran, Holohan et al. 2012) and UAS-rutabaga RNAi (VDRC#5569) (Das, Sadanandappa et al. 2011) were provided by M. Ramaswami (Trinity College, University of Dublin, Dublin, Ireland). GH298Gal4 (Stocker RF 1997) was obtained from A. Ferrus (Instituto Cajal, C.S.I.C., Madrid, Spain) and krasavietzGal4 (Shang Y 2007) was provided by A. Fiala (Georg-August-Universität Göttingen, Göttingen, Germany). MZ699Gal4 (Tanaka, Endo et al. 2012) was kindly provided by Liqun Luo (Department of Biological Sciences Stanford University, Stanford, CA).

Hydroxyurea treatment

Hydroxyurea ablation of the MBs was achieved using 75 mg/ml hydroxyurea (HU) as described previously and each batch of HU-treated adults was monitored histologically for the extent of mushroom body ablation before using flies from the particular brood for testing (Acevedo, Froudarakis et al. 2007).

Behavioral analyses

To obtain flies for behavioral analyses, Gal4 driver homozygotes were crossed *en masse* to UAS-shi^{ts}, UAS-TTX and UAS-TRPA1. Similarly, UAS-shi^{ts}, UAS-TTX, UAS-TRPA1 and Gal4 driver homozygotes were crossed *en masse* to *w^{1118}*, to obtain heterozygous controls. For the rescue experiments virgins *rut^{2080}*;UAS-rutabaga were crossed to Gal4 driver homozygotes or *w^{1118}*. Since the *rutabaga* gene is on the X chromosome, behavior was tested only in male

mutant and control flies. For the rutabaga RNAi experiment LN1Gal4;Gal80^{ts} homozygotes were crossed to UAS-rutabaga RNAi homozygotes or w^{1118}. UAS-rutabaga RNAi homozygotes were also crossed to w^{1118} to obtain the heterozygous control flies. For the Gad RNAi experiments, GH146Gal4 homozygotes were crossed to UAS-Gad RNAi and w^{1118}, while w^{1118} flies were crossed to UAS-Gad RNAi to obtain heterozygous controls. Flies for all experiments were raised at 25C, except for the neuronal activation experiments with UAS-TRPA1 and the rutabaga RNAi experiment, where flies were raised at 18C. All flies used in behavioral experiments were tested 3–5 days after emergence. Behavioral experiments were performed under red light at 23–24C and 60–70% humidity.

Osmotaxis

Odor avoidance and attraction were quantified by exposing approximately 50 flies at the choice point of a standard T-maze (Acevedo, Froudarakis et al. 2007) to an air-stream (500 ml/min) carrying the odor in one arm and fresh air in the other. The odorants utilized for these experiments were 1000 µl of 3-octanol (OCT) (Acros), 100 µl of benzaldehyde (BNZ) (Sigma), 10 µl of a 0.1% dilution in water of ethyl acetate (ETA) (Sigma) and 10 µl of a 0.5% dilution of 2,3-butanedione (Sigma). Flies were given 90 s to choose between aversive odors and air. In contrast, control experiments (not shown) determined that 180 s for the choice between attractive odors and air gave the most consistent and reliable indices. At the end of the choice period, flies in each arm were trapped and counted. The performance index (PI) was calculated as the percentage of the fraction of flies that avoid the odor and congregate in the unscented (air) arm minus the fraction of flies that prefer the odor-bearing arm.

Olfactory habituation

Olfactory habituation experiments were performed under the conditions described above. For the ‘training phase’, approximately 50 flies were exposed in the upper arm of a standard T-maze to either attractive (ETA, BUT) or aversive odors (OCT, BNZ) for the indicated times. After a 30 s rest period, the flies were lowered to the center of the maze for testing their osmotactic response by a choice of air vs. either the previously experienced, or a novel odor. At the end of the choice period (90 for aversive and 180 s for attractive odors), the flies in each arm were trapped and counted and the performance index was calculated as described

above. UAS-shi^{ts} harboring strains were placed in a 32C incubator for 30 min prior to the start of the 'training phase' to inactivate the transgenic temperature-sensitive Shibire protein, which recovers its full activity within 15 min after removal from 32C (Kitamoto 2001, McGuire, Le et al. 2001). Flies overexpressing the wild-type rutabaga gene in the *rut*^{2080} mutant background, and flies overexpressing the UAS-Gad RNAi, were kept at 30C overnight, to maximize trangene expression. To examine spontaneous recovery, flies were given rest periods of variable lengths as indicated, 6 min being the experimentally derived standard recovery time, within the upper arm of the maze after 4 min odor pre-exposure. For the spontaneous recovery after 30 min of pre-exposure, flies were returned to food vials for the 6 or 30 min rest period. Subsequently, they were tested against the odor they were pre-exposed to, versus air.

For the pulsed odor stimulation, we divided the 1 min of continuous odor exposure into two 30 s odor pulses with an 8 s interstimulus interval (ITI), the 4-min odor exposure into four 1 min odor pulses with the proportional 15 s ITI and finally the 30-min exposure was divided into three 10 min pulses with 2.5 min ITI. ITI length was kept at a quarter of the odor exposure duration, since adaptation has been correlated with stimulus duration (de Bruyne, Clyne et al. 1999).

To determine the conditions for dishabituation with electric shock, control experiments were performed first to determine the stimulus strength and number of shocks required. Dishabituation was attempted at different shock stimulus strengths with the following results: OCT PI for naive: 59.2 ± 2.3. Habituated OCT PI: 20.6 ± 1.7. Dishabituation with 30 V, OCT PI: 54.8 ± 2.1; with 45 V, OCT PI: 58.7 ± 2.6; with 90V OCT PI: 58.3 ± 2.2. Moreover, the number of shocks did not have a significant effect on dishabituation (1 × 90V shock OCT PI: 58.7 ± 2.3; 2 × 90V shock OCT PI: 59.4 ± 2.8). Since the 90V and 45V dishabituating shocks had equal effects, the weaker of the two was selected.

Vortexing was used as another mechanical stimulus to produce dishabituation. Flies were subjected to 3 s of vortexing at maximal speed immediately after odor exposure. Finally, for the 30 min odor pre-exposure habituation experiments flies were exposed to air drawn at 500 ml/min over a 30% (w/v) aqueous solution of Brewers yeast (Acros Organics) for 3 s after the

odor pre-exposure to dishabituate. The performance index (PI) for habituation and dishabituation was calculated as described above.

For neuronal activation experiments, expression of UAS-TRPA1 was driven to the neurons of interest. Flies used in activation experiments were raised at 18C and expression of the transgene was induced at 31C (using a heat block), for 1 min or 4 min prior to testing.

Statistical analysis

Untransformed (raw) data were analyzed parametrically with JMP3.1 statistical software package (SAS Institute Inc., Cary, NC). If significant, initial ANOVA tests were followed by Dunnett's and Least Square Means Contrast analyses and the experimentwise error rate was adjusted as suggested by Sokal and Rohlf (Appendix B) (Rohlf 1995).

Supplementary Data

Genotype	**Naïve**	**1 min OCT**	**1 min OCT +45V**	**ANOVA**
LN1Gal4/+	74.56 ± 4.30	54.82 ± 4.57	64.27 ± 5.81	$F_{(2,30)}$=4.08, **p=0.0278**
GH298Gal4/+	71.62 ± 3.51	47.85 ± 7.09	62.66 ± 4.06	$F_{(2,29)}$=5.47, **p=0.0101**
krasGal4/+	52.83 ± 4.45	27.54 ± 3.38	53.54 ± 6.01	$F_{(2,34)}$=9.20, **p=0.0007** [1]
GH146Gal4/+	82.71 ± 2.92	75.22 ± 3.83	74.88 ± 6.52	$F_{(2,34)}$=0.89, **p=0.4209**
APLGal4/+	79.98 ± 2.99	62.87 ± 5.29	78.20 ± 4.36	$F_{(2,31)}$=4.64, **p=0.0178**
MZ699Gal4/+	92.73 ± 1.70	78.49 ± 2.50	86.25 ± 2.75	$F_{(2,35)}$=9.14, **p=0.0007** [2]
MB247Gal4/+	73.18 ± 4.75	62.22 ± 5.07	77.33 ± 3.55	$F_{(2,44)}$=3.13, **p=0.0540**
OK72Gal4/+	81.31 ± 3.35	67.92 ± 5.44	71.27 ± 4.13	$F_{(2,31)}$=2.40, **p=0.1086**
OK72Gal4/+;MBGal80/+	77.52 ± 4.00	65.89 ± 4.84	76.60 ± 4.06	$F_{(2,39)}$=2.27, **p=0.1170**

C739Gal4/+	82.13 ± 4.46	69.30 ± 4.09	70.62 ± 2.90	$F_{(2,29)}$=3.32, **p=0.0514**
C772Gal4/+	83.68 ± 3.62	84.12 ± 2.02	80.97 ± 3.55	$F_{(2,29)}$=0.29, **p=0.7478**
MB463Gal4/+	76.11 ± 5.42	59.58 ± 5.68	69.98 ± 6.30	$F_{(2,31)}$=2.32, **p=0.1167**
MB131BGal4/+	79.90 ± 4.33	64.00 ± 6.08	70.57 ± 4.79	$F_{(2,29)}$=2.38, **p=0.1121**
VT44966/+	59.46 ± 4.70	43.91 ± 3.67	55.52 ± 3,90	$F_{(2,39)}$=3.79, **p=0.0318**

Supplementary File 1. Responses of control animals, heterozygous for the Gal4 drivers when naive, after 1 min pre-exposure and after 1 min pre-exposure followed by one 45V electric footshock application. Data are represented as mean ± SEM. Subsequent Dunnett's test: p=0.0016 for 1 min OCT and p=0.9918 for 1 min OCT +45V two subsequent Dunnett's test: p=0.0003 for 1 min OCT and p=0.1084 for 1 min OCT +45V

Genotype	**Naïve**	**4 min OCT**	**4 min OCT +45V**	**ANOVA**
LN1Gal4/+	57.73 ± 5.94	14.88 ± 4.84	55.06 ± 4.49	$F_{(2,35)}$=21.91, **p<0.0001** [1]
krasGal4/+	54.55 ± 4.32	15.73 ± 4.98	43.64 ± 2.55	$F_{(2,36)}$=23.22, **p<0.0001** [2]
GH146Gal4/+	77.14 ± 4.01	48.13 ± 5.31	72.62 ± 3.63	$F_{(2,44)}$=12.74, **p<0.0001** [3]
MZ699Gal4/+	68.60 ± 4.69	37.74 ± 6.76	63.01 ± 4.54	$F_{(2,36)}$=8.95, **p=0.0008** [4]
MB247Gal4/+	77.48 ± 4.69	39.75 ± 3.72	65.68 ± 3.99	$F_{(2,45)}$=23.31, **p<0.0001** [5]

Supplementary File 2. Responses of control animals, heterozygous for the Gal4 drivers when naïve, after 4 min pre-exposure and after 4 min pre-exposure followed by one 45V electric footshock application. Data are represented as mean ± SEM Subsequent Dunnett's test: p<0.0001 for 4-min OCT and p=0.9061 for 4-min OCT +45V two subsequent Dunnett's test: p<0.0001 for 4 min OCT and p=0.1594 for 4-min OCT +45V three subsequent Dunnett's test: p<0.0001 for 4-min OCT and p=0.6941 for 4-min OCT +45V four subsequent Dunnett's test: p=0.0007 for 4-min OCT and p=0.7259 for 4-min OCT +45V five subsequent Dunnett's test: p<0.0001 for 4-min OCT and p=0.1046 for 4-min OCT +45V

The neuronal circuits and mechanisms of dishabituation in Drosophila.

Introduction

Dishabituation is the instant recovery of the naïve response to a habituated stimulus following exposure to a novel stimulus and it is described as one of the ten characteristics of habituation (Rankin, Abrams et al. 2009). Dishabituation has been widely used in habituation studies as means to demonstrate that the observed response attenuation is not a consequence of fatigue or sensory adaptation. Studies on the mechanism of habituation in *Aplysia* have demonstrated that recovery of the response following the experience of a novel stimulus can be the result of both sensitization superimposed on habituation and reversal of habituation (Carew, Castellucci et al. 1971, Hochner, Klein et al. 1986, Marcus, Nolen et al. 1988, Ghirardi, Braha et al. 1992, Cohen, Kaplan et al. 1997, Antonov, Kandel et al. 1999, Hawkins, Cohen et al. 2006). However, studies in leeches, rats and humans propose that dishabituation is independent of sensitization and the novel stimulus actually reverses habituation (Ehrlich, Boulis et al. 1992, Smith, Shionoya et al. 2009, Steiner and Barry 2011, Steiner and Barry 2014).

Dishabituation is crucial for animal survival and associative learning, since it provides instant response recovery to an often ongoing repetitive stimulus and allows animals to reevaluate environmental cues. However, this process is not well studied; the neuronal circuits that underlie dishabituation have not been identified, and most of the results regarding the molecular components of this process were observations in studies focusing on habituation. In *C. elegans*, dishabituation of the response to mechanical stimuli requires functional glutamate vesicular transporters (Rankin and Wicks 2000) while in rats dishabituation of odor-evoked heart rate orienting bradycardia depends on norepinephrine β-receptors and their antagonistic interaction with the metabotropic glutamate receptors mediating synaptic depression and lead to habituation (Smith, Shionoya et al. 2009).

The Drosophila olfactory circuitry provides an excellent system to study habituation and dishabituation. We have previously established an olfactory habituation paradigm where continuous exposure to an odor for four minutes results in attenuation of the response and can be dishabituated with mechanical stimuli or a yeast puff. Using this paradigm, we have

demonstrated that modulation of the lateral horn (LH) output by the olfactory projection neurons drives habituation to odors (Semelidou, Acevedo et al. 2018). Interestingly, the mushroom bodies (MBs), the bilateral neuropils known for their role in mediating habituation latency (Acevedo, Froudarakis et al. 2007, Semelidou, Acevedo et al. 2018) and learning and memory (Crittenden, Skoulakis et al. 1998, Heisenberg 2003), are necessary for dishabituation after electric shock stimulation (Semelidou, Acevedo et al. 2018). However, the remaining neuronal subsets that comprise the circuit which directs the recovery of a habituated response have not been defined. Using the advanced genetic tools available in Drosophila we identified the neuronal subsets afferent to the MBs that are necessary for response recovery after mechanical stimulation and proposed a mechanism for dishabituation to olfactory stimuli.

Results

The dopaminergic neurons are necessary for dishabituation to mechanical stimuli.

Continuous exposure to an aversive odor for 4 minutes results in attenuation of olfactory avoidance, which recovers after mechanical stimulation (dishabituation). Dishabituation occurs instantly and depends on the activation of the mushroom bodies (Semelidou, Acevedo et al. 2018), the center of learning and memory in Drosophila (Crittenden, Skoulakis et al. 1998, Heisenberg 2003). Aiming to identify the afferent neurons that convey the dishabituation signal to the MBs, we initially focused on the dopaminergic neurons that innervate the MBs and are known to mediate electric shock reinforcement in associative learning (Schwaerzel, Monastirioti et al. 2003, Schroll, Riemensperger et al. 2006). To determine whether activation of the dopaminergic neurons is necessary for dishabituation, we conditionally silenced these neurons by transgenically expressing therein the temperature sensitive dynamin Shibire^{ts}, which blocks synaptic transmission. At the restrictive temperature, Shibire^{ts} adopts an inactive conformation, blocking neurotransmitter reuptake, thus silencing neurons by depletion of the releasable neurotransmitter pool (Kitamoto 2001).

Using the TH-Gal4 driver (Friggi-Grelin, Coulom et al. 2003) we expressed Shibire^{ts} in ~280 dopaminergic neurons and attempted dishabituation with two mechanical stimuli: electric footshock of 45V and 90V, and vortexing. After a 4-min odor-exposure, which is

sufficient to cause habituation (Semelidou, Acevedo et al. 2018), animals experienced a single electric footshock of 45 or 90 Volts or were vortexed for 3 seconds. Silencing the TH-Gal4 dopaminergic neurons did not affect habituation, but disrupted dishabituation irrespective of the dishabituating stimulus type used (Figure 1A-C). In contrast, control animals recovered their initial response to the odor after exposure to electric shock or vortex. Importantly, blocking dopaminergic neurotransmission did not compromise the naive response to electric shocks (Suppl. Figure 1A). These results indicate that dopaminergic neurons are activated (directly or indirectly) by the mechanical stimuli that act as dishabituators and neurotransmission from these neurons is indispensable for recovery of the habituated response.

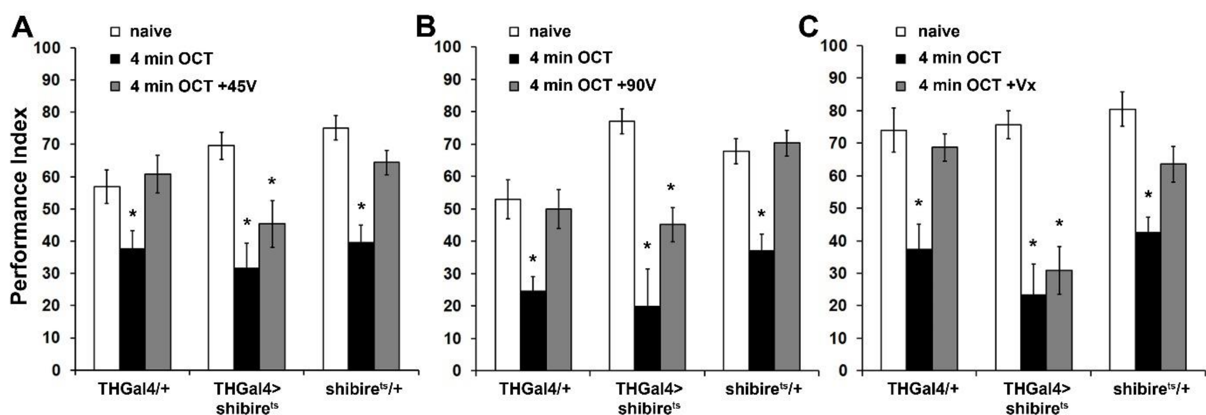


Figure 1. Dopaminergic neurotransmission is necessary for dishabituation after stimulation with olfactory stimuli. Mean Performance Indices ± SEM are shown in all figures. Stars indicate significant differences from the naive response unless specified otherwise. **(A)** Functional silencing of the dopaminergic neurons with UAS-shibire^{ts} under TH-Gal4 resulted in normal habituation after 4 min OCT exposure (black bar, p=0.0003) but no dishabituation of the olfactory response after application of a 45V electric shock (grey bar, p=0,0097). Both control lines showed normal habituation and dishabituation. (n ≥ 9 for all groups) **(B)** Functional silencing of the dopaminergic neurons with UAS-shibire^{ts} under TH-Gal4 resulted in normal habituation after 4 min OCT exposure (black bar, p<0.0001) but no dishabituation of the olfactory response after application of a 90V electric shock (grey bar, p=0,0058). Both control lines showed normal habituation and dishabituation. (n ≥ 7 for all groups) **(C)** Functional silencing of the dopaminergic neurons with UAS-shibire^{ts} under TH-Gal4 resulted in normal habituation after 4 min OCT exposure (black bar, p<0.0001) but no dishabituation of the olfactory response after application of a 45V electric shock (grey bar, p=0,0005). Both control lines showed normal habituation and dishabituation. (n ≥ 8 for all groups)

Activation of the PPL1 cluster of dopaminergic neurons is necessary and sufficient for dishabituation to mechanical stimuli.

Drosophila dopaminergic TH-Gal4 neurons are subdivided into eight clusters, three of which innervate the MBs (Mao and Davis 2009). The PPL1 neurons, previously shown to project to the vertical lobes, junction area, heel and distal peduncle of the MBs (Mao and Davis 2009) are important for aversive memory formation and their activation is sufficient to act as a reinforcer, as their artificial activation substitutes the electric shock in associative memory conditioning (Claridge-Chang, Roorda et al. 2009, Aso, Siwanowicz et al. 2010). To examine the role of these neurons in dishabituation, we blocked their synaptic output expressing Shibire^{ts} under two drivers, MB504B-Gal4 (Vogt, Schnaitmann et al. 2014) and C061;MBGal80 (Krashes, DasGupta et al. 2009). MB504B-Gal4 marks four dopaminergic neurons, PPL1γ1pedc>αβ, PPL1γ2α'1, PPL1α'2α2 (also known as MB-MP1, MB-MV1, MB-V1), and the neuron that projects to the tip of the α lobe (Vogt, Schnaitmann et al. 2014). Blocking neurotransmission from these neurons disrupted dishabituation after electric shock stimulation, while habituation remained normal (Figure 2A). Similarly, silencing the PPL1γ1pedc>αβ dopaminergic neuron by Shibire^{ts} expression under the C061;MBGal80 driver, results in failure to recover the response after a 45V electric shock, without affecting olfactory habituation (Figure 2B). In contrast, control animals habituate and dishabituate normally (Figure 2A-B). Collectively, these results validate the role of the dopaminergic neurons in dishabituation, narrowing down the important neurons to the small PPL1 cluster.

Since blocking neurotransmission from a subset of the PPL1 neurons disturbed dishabituation, we next activated these neurons to examine if dopamine release from such a specific neuronal subset is sufficient to induce response recovery. To activate these neurons we expressed the heat-activated TRPA1 channel, to depolarize neurons in temperature-dependent manner (Pulver, Pashkovski et al. 2009). However, the temperature increase to 32C for 10 s itself was sufficient to act as a dishabituator in control animals that did not express TRPA1 (Suppl. Figure 1B), making impossible to activate the PPL1 neurons with this transgene. Thus, to activate the PPL1 neurons we expressed a channelrhodopsin-2 (ChR2) mutant, ChR2-XXL, a powerful optogenetic tool developed to control neuronal activity. Expression of ChR2-XXL under the C061;MBGal80 driver and subsequent optogenetic activation of these neurons with a continuous light pulse at 460nm for 3 s after the 4-min odor exposure was adequate to induce dishabituation (Figure 2C). Importantly, continuous

light stimulation for 3 s did not induce recovery of the response in control animals that do not express the channel (Figure 2D), validating that the light pulse itself does not act as a dishabituator.

Collectively, these results indicate that electric shock activates the dopaminergic PPL1 neurons and this activation is necessary and sufficient to induce dishabituation in animals habituated to an odor.

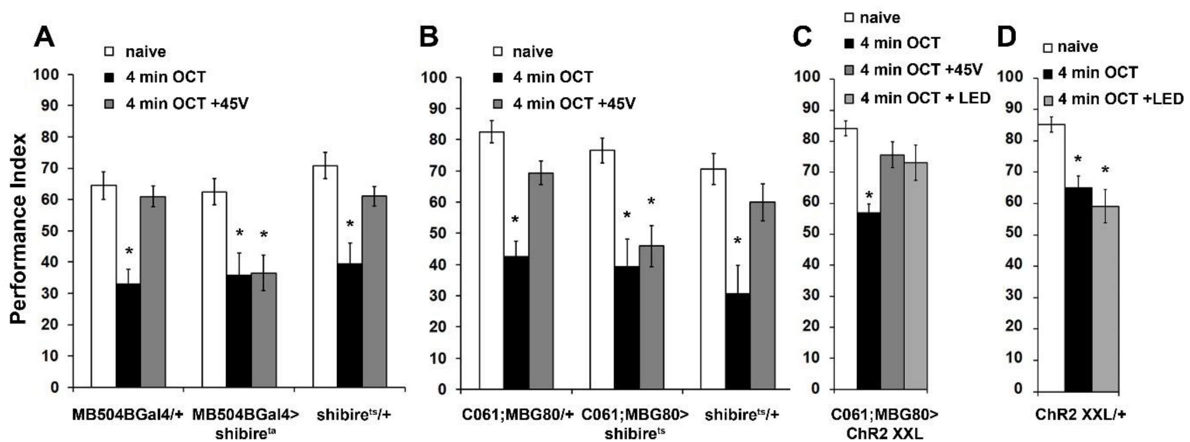


Figure 2. Dopaminergic PPL1 neurons are necessary and sufficient for dishabituation of the olfactory response. Mean Performance Indices ± SEM are shown in all figures. Stars indicate significant differences from the naive response unless specified otherwise. **(A)** Functional silencing of the PPL1 dopaminergic neurons with UAS-shibire^{ts} under MB504B-Gal4 resulted in normal habituation after 4 min OCT exposure (black bar, p=0.0037) but no dishabituation of the olfactory response after application of a 45V electric shock (grey bar, p=0,0049). Both control lines showed normal habituation and dishabituation. (n ≥ 10 for all groups) **(B)** Functional silencing of the PPL1 dopaminergic neurons with UAS-shibire^{ts} under C061;MB80 resulted in normal habituation after 4 min OCT exposure (black bar, p<0.0008) but no dishabituation of the olfactory response after stimulation with a 45V electric shock (grey bar, p=0,0075). Both control lines showed normal habituation and dishabituation. (n ≥ 13 for all groups) **(C)** Activation of the PPL1 dopaminergic neurons with a 460mn light pulse for 3 s in animals that express UAS-ChR2XXL under C061;MB80 induced dishabituation without application of a stimulus after the odor exposure (light grey bar, p=1369). Habituation (black bar, p<0.0001) and dishabituation after application of a 45V electric shock (grey bar, p=0.3129) were not affected. (n = 11 for all groups) **(D)** Control animals that do no express Channelrhodopsin ChR2XXL do not present recovery of the response after exposure to 460mn light pulse for 3 s (grey bar, p=0.0003). (n ≥ 11 for all groups)

Neurotransmission from the Anterior Paired Lateral (APL) neurons is essential for dishabituation.

Having identified the role of dopaminergic neurons in dishabituation, we next examined if silencing other neurons that innervate the mushroom bodies has the same effect on olfactory avoidance recovery. The Anterior Paired Lateral (APL) neurons are single neurons per brain hemisphere that innervate broadly the MBs and are activated both by electric shock and odor stimuli (Liu and Davis 2009). To block synaptic transmission from these neurons we expressed Shibire^{ts} under a specific APL-Gal4 driver (Wu, Shih et al. 2013). Even though silencing the APL neurons did not affect habituation to the odor, animals were unable to dishabituate after experiencing a 45V electric shock (Figure 3A). Interestingly, when a stronger shock of 90V was delivered, we observed recovery of the attenuated negative osmotaxis (Figure 3B). These results indicate that the APL neurons are necessary for dishabituation after mild electric stimulation, but a stronger dishabituator can bypass the contribution of these neurons, leading to recovery of the naive response.

The APL neurons are both GABAergic and octopaminergic. They are implicated in olfactory learning through GABA neurotransmission onto the αβ MB lobes (Liu and Davis 2009) and in anesthesia resistant memory through octopaminergic transmission onto the α'β' lobes (Wu, Shih et al. 2013). To examine if octopaminergic or GABAergic transmission from the APL neurons is necessary for dishabituation, we used RNA interference to downregulate the expression of the rate-limiting enzymes in octopamine and GABA synthesis in these neurons. Remarkably, adult APL-specific abrogation of tyramine beta-hydroxylase (TBH), which catalyzes the last step in *octopamine* biosynthesis resulted in normal habituation but failed dishabituation upon 45V electric shock stimulation (Figure 3C), as previously shown after functional silencing these neurons (Figure 3A). In contrast, when the glutamic acid decarboxylase (Gad) RNAi was expressed under the APLGal80^{ts} driver, animals exhibited habituation and dishabituation (Figure 3D) indistinguishable from that of control lines. To verify that GABAergic neurotransmission is indeed dispensable in dishabituation, we silenced the GABAergic neurons by overexpression of DORK, an outward rectifier K^+ channel, under the Gad-Gal4 driver (Nitabach, Blau et al. 2002). In agreement with the previous results, animals that lack GABAergic neurotransmission showed normal dishabituation after stimulation with a 45V shock (Figure 3E). Collectively then, octopaminergic and not GABAergic

neurotransmission from the APL neurons is necessary for the recovery of the habituated response after mechanical stimulation.

Another set of neurons that innervates the mushroom bodies and is involved in long-term memory storage and retrieval consists of the dorsal-anterior-lateral (DAL) neurons (Chen, Wu et al. 2012). DAL neurons innervate the mushroom body calyx, and specifically the region where the dendrites of the αβ lobes reside. To study the role of these neurons in dishabituation, we expressed Shibire^{ts} using the specific DAL-Gal4 driver (Chen, Wu et al. 2012). In contrast to the dopaminergic and the APL octopaminergic neurons, silencing the DAL neurons did not affect dishabituation after 45V stimulation (Figure 3F), suggesting that these neurons are dispensable in the process.

The role of mushroom body neurons in dishabituation.

Neurotransmission from the mushroom bodies is necessary for dishabituation of a habituated odor response after stimulation with a 45V electric footshock (Semelidou, Acevedo et al. 2018). The mushroom body neurons can be further divided in the αβ, α'β', and γ lobes (Crittenden, Skoulakis et al. 1998) that have differential roles in olfactory learning and memory (Yu, Akalal et al. 2006, Krashes, Keene et al. 2007, Blum, Li et al. 2009, Zhang and Roman 2013). To further examine the role of these neuronal subsets in dishabituation, we first silenced the αβ neurons, expressing Shibire^{ts} under the c739-Gal4 driver. In contrast with the results obtained with the pan-mushroom body MB247-Gal4 driver (Semelidou, Acevedo et al. 2018), blocking neurotransmission only from the αβ lobes did not disrupt dishabituation (Figure 4A). These results, together with the normal dishabituation of animals lacking neurotransmission from the DAL neurons that mainly innervate the αβ lobes suggest that these neurons are dispensable for dishabituation.

Similarly, Shibire^{ts} expression specifically in the γ lobe neurons with the MB131B-Gal4 driver resulted in normal habituation and dishabituation after stimulation with a 45V electric shock (Figure 4B). The role of the α'β' neurons remains to be examined to further understand how the mushroom bodies are implicated in dishabituation. Our results on the role of octopamine in dishabituation (Figure 3D), together with results in anesthesia resistant memory that link the APL octopaminergic neurotransmission with octβ2R activation in the α'β' lobes (Wu, Shih et al. 2013) suggest a possible role for the α'β' MB lobe neurons in dishabituation.

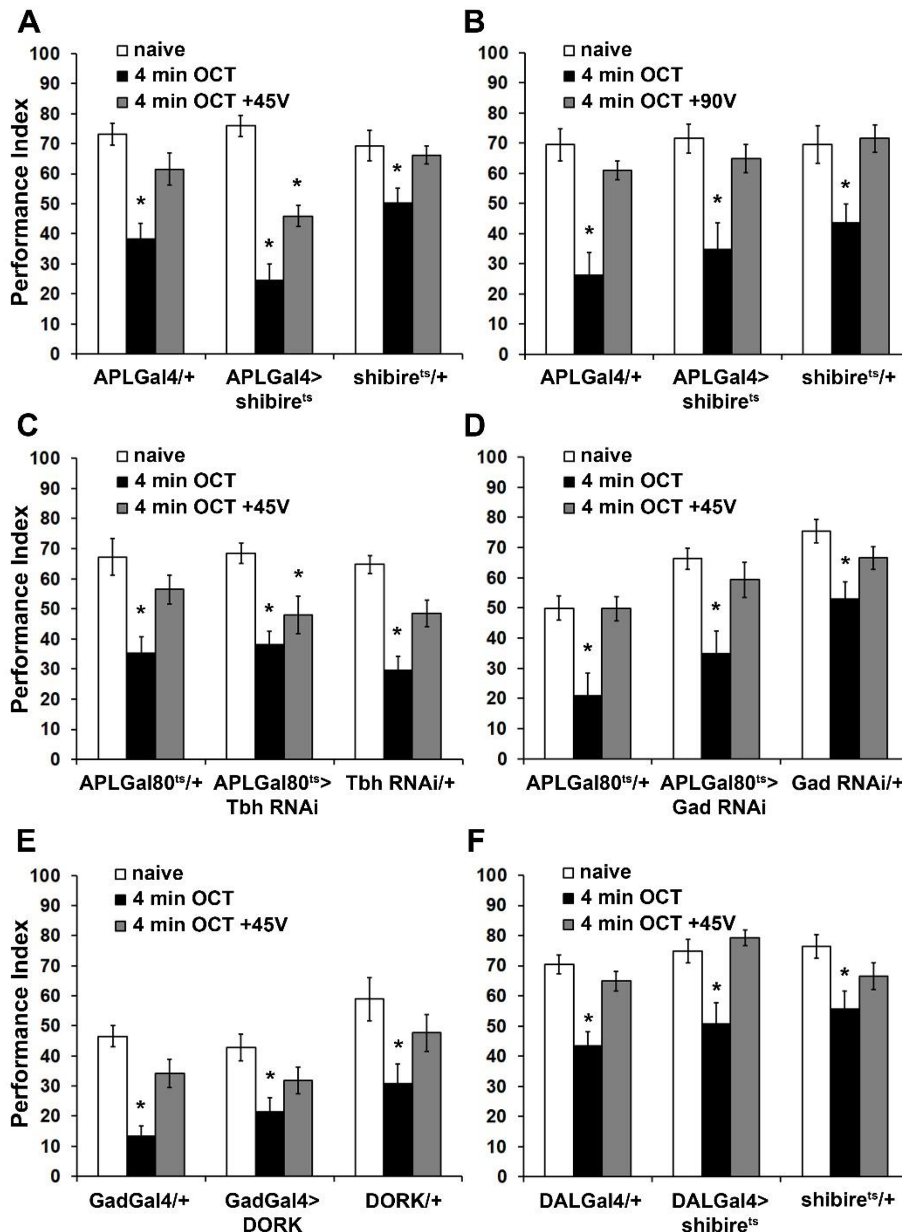


Figure 3. Octopaminergic neurotransmission from the APL neurons is necessary for dishabituation after stimulation with a 45V electric shock. Mean Performance Indices ± SEM are shown in all figures. Stars indicate significant differences from the naive response unless specified otherwise. **(A)** Functional silencing of the APL neurons with UAS-shibire^{ts} under APL-Gal4 resulted in normal habituation after 4 min OCT exposure (black bar, $p<0.0001$) but no dishabituation of the olfactory response after application of a 45V electric shock (grey bar, $p<0.0001$). Both control lines showed normal habituation and dishabituation. ($n \geq 12$ for all groups) **(B)** Functional silencing of the APL neurons with UAS-shibire^{ts} under APL-Gal4 resulted in normal habituation after 4 min OCT exposure (black bar, $p=0.0006$) and normal dishabituation of the olfactory response after application of a 90V

electric shock (grey bar, p=0.7219). Both control lines showed normal habituation and dishabituation. (n ≥ 9 for all groups) **(C)** Tbh abrogation via RNAi-mediating transgene expression in the APL neurons of adult flies resulted in normal habituation after 4 min OCT exposure (black bar, p=0.0002) but no dishabituation of the olfactory response after application of a 45V electric shock (grey bar, p=0.0074). Both control lines showed normal habituation and dishabituation. (n ≥ 12 for all groups) **(D)** Gad1 abrogation via RNAi-mediating transgene expression in the APL neurons of adult flies resulted in normal habituation after 4 min OCT exposure (black bar, p=0.0006) and dishabituation of the olfactory response after application of a 45V electric shock (grey bar, p=0.6313). Both control lines showed normal habituation and dishabituation. (n ≥ 15 for all groups) **(E)** Functional silencing of the GABAergic neurons with UAS-DORK under Gad-Gal4 resulted in normal habituation after 4 min OCT exposure (black bar, p=0.0024) and normal dishabituation of the olfactory response after application of a 45V electric shock (grey bar, p=0.1716). Both control lines showed normal habituation and dishabituation. (n ≥ 7 for all groups) **(F)** Functional silencing of the DAL neurons with UAS-shibire^{ts} under DAL-Gal4 resulted in normal habituation after 4 min OCT exposure (black bar, p=0.0019) and normal dishabituation of the olfactory response after application of a 45V electric shock (grey bar, p=0.7627). Both control lines showed normal habituation and dishabituation. (n ≥ 14 for all groups)

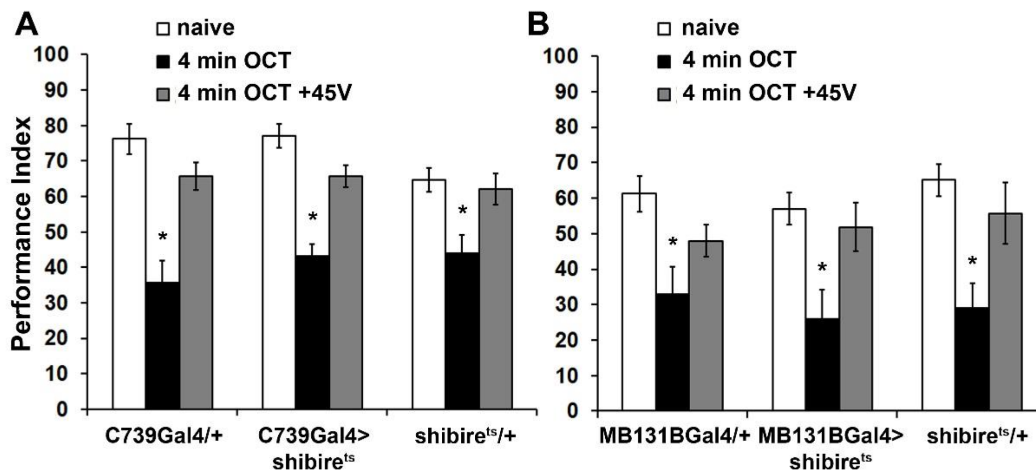


Figure 4. The αβ and γ lobes of the MBs are dispensable for dishabituation. Mean Performance Indices ± SEM are shown in all figures. Stars indicate significant differences from the naive response unless specified otherwise. **(A)** Functional silencing of the αβ lobe neurons with UAS-shibire^{ts} under C739-Gal4 resulted in normal habituation after 4 min OCT exposure (black bar, p<0.0001) and recovery of the olfactory response after application of a 45V electric shock (grey bar, p=0.0326). Both control lines showed normal habituation and dishabituation. (n ≥ 10 for all groups) **(B)** Functional silencing of the γ lobe neurons with UAS-shibire^{ts} under MB131B-Gal4 resulted in normal habituation after 4 min

OCT exposure (black bar, p=0.0036) and recovery of the olfactory response after application of a 45V electric shock (grey bar, p=0.8189). Both control lines showed normal habituation and dishabituation. (n ≥ 13 for all groups)

GABAergic inhibition of the αβ MB lobes is not necessary for dishabituation.

Our results on the role of the αβ and γ MB neurons in dishabituation indicate that they are dispensable in the process (Figure 4A-B). However, the PPL1γ1pedc>αβ dopaminergic neurons that are necessary and sufficient in dishabituation (Figure 2B-C), innervate a specific MB compartment of the γ lobe and form synapses with MB output neurons (MBONs) of this compartment, which send their axons to the αβ MB lobes (Tanaka, Tanimoto et al. 2008, Mao and Davis 2009, Aso, Sitaraman et al. 2014). Considering that the MBON-PPL1γ1pedc>αβ are GABAergic (Aso, Sitaraman et al. 2014), we hypothesized that the PPL1 neurons may activate the GABAergic MBON-PPL1γ1pedc>αβ neurons which, in turn, inhibit the αβ MB neurons and facilitate dishabituation. To determine whether inhibition of the αβ lobes is necessary for dishabituation, we abrogated the expression of known GABA receptors specifically in the αβ lobes of adult flies using two drivers, c739Gal80^{ts} and c772Gal80^{ts}. However, expression of Rdl RNAi, GABA-B-R1 RNAi, GABA-B-R2 RNAi, or GABA-B-R3 RNAi in the αβ MB lobes did not affect dishabituation after a 45V electric shock (Figure 5A-I). These results demonstrate that the αβ MB neurons are dispensable for dishabituation and neither their inhibition nor their activation is necessary for response recovery after electrical stimulation. Furthermore, they indicate that other neurons, post-synaptic to the PPL1γ1pedc>αβ, are crucial for normal dishabituation.

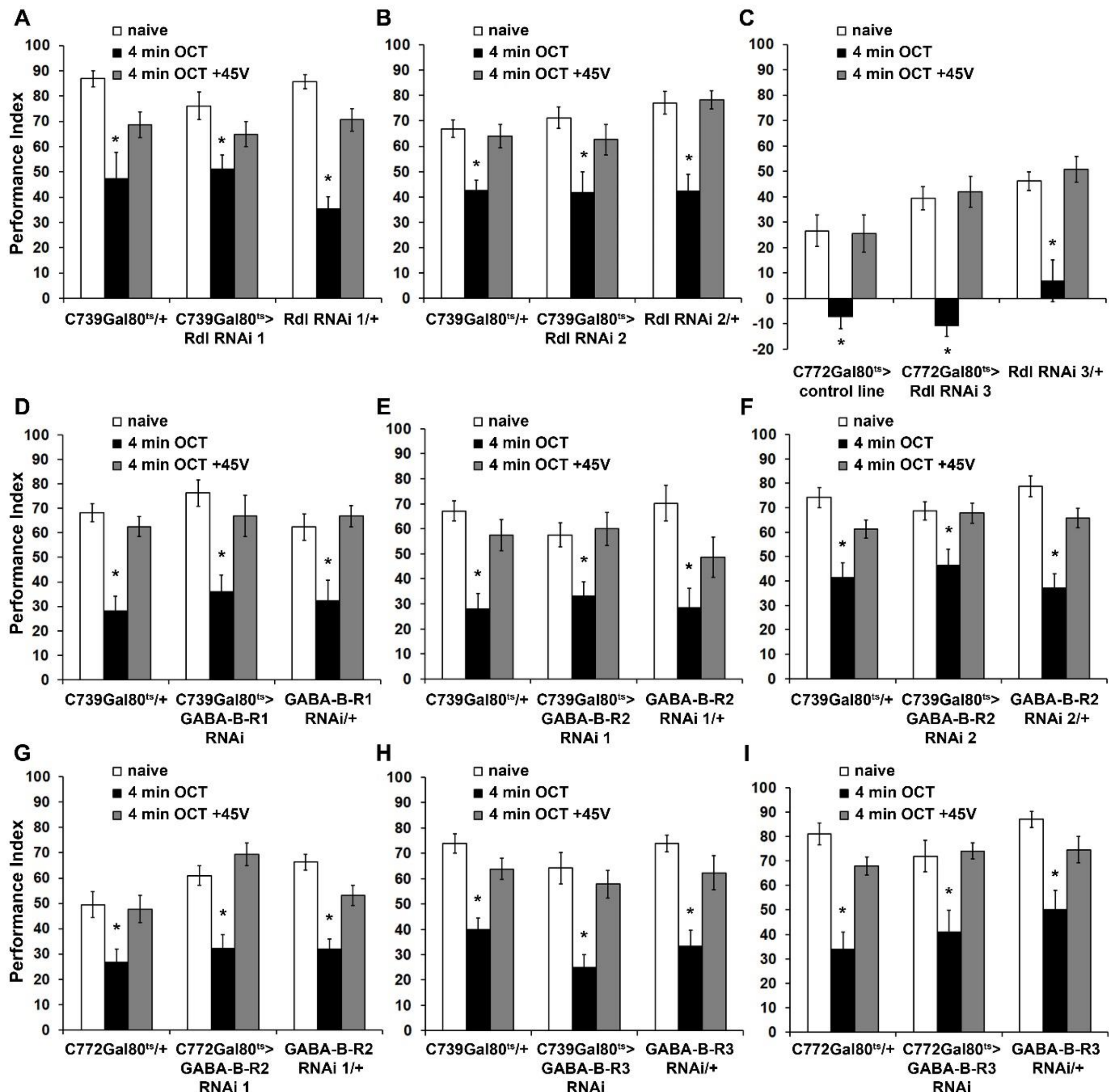


Figure 5. Inhibition of the αβ MB lobes through GABAergic neurotransmission is not necessary in dishabituation. Mean Performance Indices ± SEM are shown in all figures. Stars indicate significant differences from the naive response. **(A-B)** Rdl abrogation via RNAi-mediating transgene expression in the αβ neurons of adult flies under C739Gal80^{ts} resulted in normal habituation after 4 min OCT exposure (black bars, $p=0.0076$ for RNAi 1 and $p=0.0049$ for RNAi 2) and dishabituation of the olfactory response after application of a 45V electric shock (grey bars, $p=0.2433$ for RNAi 1 and $p=0.5006$ for RNAi 2). Control lines presented normal habituation and dishabituation. ($n \geq 8$ for all groups) **(C)** Rdl abrogation via RNAi-mediating transgene expression in the αβ neurons of adult flies under C772Gal80^{ts} resulted in normal habituation after 4 min OCT exposure (black bars, $p<0.0001$) and dishabituation of the olfactory response after application of a 45V electric shock (grey bars, $p=0.9089$). Control lines presented normal habituation and dishabituation. ($n \geq 10$ for all groups) **(D)** GABA-B-R1 abrogation via RNAi-mediating transgene expression in the αβ neurons of adult flies under

C739Gal80^{ts} resulted in normal habituation after 4 min OCT exposure (black bars, p=0.0007) and dishabituation of the olfactory response after application of a 45V electric shock (grey bars, p=0.5387). Control lines presented normal habituation and dishabituation. (n ≥ 9 for all groups) **(E-F)** GABA-B-R2 abrogation via RNAi-mediating transgene expression in the αβ neurons of adult flies under C739Gal80^{ts} resulted in normal habituation after 4 min OCT exposure (black bars, p=0.0076 for RNAi 1 and p=0.0056 for RNAi 2) and dishabituation of the olfactory response after application of a 45V electric shock (grey bars, p=0.9394 for RNAi 1 and p=0.9886 for RNAi 2). Control lines presented normal habituation and dishabituation. (n ≥ 10 for all groups) **(G)** GABA-B-R2 abrogation via RNAi-mediating transgene expression in the αβ neurons of adult flies under C772Gal80^{ts} resulted in normal habituation after 4 min OCT exposure (black bars, p=0.0003) and dishabituation of the olfactory response after application of a 45V electric shock (grey bars, p=0.3864). Control lines presented normal habituation and dishabituation. (n ≥ 12 for all groups) **(H-I)** GABA-B-R3 abrogation via RNAi-mediating transgene expression in the αβ neurons of adult flies under C739Gal80^{ts} and C772Gal80^{ts} resulted in normal habituation after 4 min OCT exposure (black bars, p<0.0001 for C739Gal80^{ts}>RNAi and p=0.0050 for C772Gal80^{ts}>RNAi) and dishabituation of the olfactory response after application of a 45V electric shock (grey bars, p=0.6449 for C739Gal80^{ts}>RNAi and p=9616 for C772Gal80^{ts}>RNAi). Control lines presented normal habituation and dishabituation. (n ≥ 8 for all groups)

Discussion

Studies in Aplysia have introduced two hypotheses for dishabituation: superimposed sensitization and reversal of habituation. The first, based on the dual process theory for habituation and sensitization (Groves and Thompson 1970), suggested that dishabituation is sensitization superimposed upon habituation. Even though early studies on the Aplysia gill-reflex and withdrawal-reflex dishabituation with mechanical stimuli confirmed this model, additional studies showed controversial results, both verifying and contradicting superimposed sensitization as a dishabituation mechanism. Interestingly, differences between sensitization and dishabituation depended on the stimulation time point and the intensity of the dishabituating stimulus (Marcus, Nolen et al. 1988, Hawkins, Cohen et al. 2006). Further studies in invertebrates, mammals and in human demonstrated that dishabituation is a reversal in habituation, as proposed by the stimulus-model comparator theory (Sokolov 1960). In the present study dishabituation was induced by a single pulse of mechanical stimulation immediately after habituation, and the stimulus with the lowest

intensity (45V electric shock) was used in the majority of the experiments, since studies in Aplysia demonstrated that immediate application of the stimulus and stimulus intensity are the important factors to dissect dishabituation and sensitization (Marcus, Nolen et al. 1988, Hawkins, Cohen et al. 2006). Furthermore, as previously shown, stimulation of naïve flies with a 45V electric shock or 3 s vortex did not produce an increased response to the odor (Semelidou, Acevedo et al. 2018), results that indicate that sensitization to the mechanical stimulus does not drive dishabituation in our paradigm. In contrast, our results indicate that dishabituation requires activation of neuronal subsets dispensable during habituation, that feedback onto the habituated neurons and modulate their response.

In the present study, we used a Drosophila olfactory habituation paradigm to investigate the neuronal subsets that underlie dishabituation after mechanical stimulation. We have previously demonstrated that the MBs are necessary for the recovery of the odor response after stimulation with electric shock (Semelidou, Acevedo et al. 2018). Focusing on neurons afferent to the MBs, we show that the dopaminergic and the APL neurons play an important role in dishabituation. Both these neuronal subsets innervate the MBs, are activated by electric shocks and are necessary in associative learning and memory (Liu and Davis 2009, Pitman, Huetteroth et al. 2011, Wu, Ren et al. 2012, Wu, Shih et al. 2013, Kaun and Rothenfluh 2017). Functional silencing of all dopaminergic neurons perturbed dishabituation of the olfactory response irrespectively of the mechanical stimulation used as a dishabituator (Figure 1), while dopamine transmission from the PPL1 cluster of dopaminergic neurons and octopamine transmission from the APL neurons are indispensable in the recovery of the response after a 45V electric shock (Figure 2, Figure 3). Interestingly, even though the MB neurons are necessary for normal dishabituation (Semelidou, Acevedo et al. 2018), silencing specifically the αβ or γ lobes did not disrupt the recovery of the response after stimulation with a 45V electric shock (Figure 4). These results propose that the α’β’ MB lobes receive input from the APL and PPL1 neurons and are crucial for dishabituation, and we aim to examine this hypothesis directly.

Both Gal4 lines used in this study to drive expression in the PPL1 neurons mark the PPL1γ1pedc>αβ which innervate the MB γ lobe. However, even though PPL1γ1pedc>αβ activation is necessary and sufficient to induce dishabituation, silencing the γ lobe did not disrupt the recovery of the response after a 45V electric shock (Figure 4). Previous studies have shown that the mushroom body output neurons (MBONs) confined on the heel of the

MBs where PPL1γ1pedc>αβ synapse (MBONγ1pedc>αβ) are GABAergic and send their axons to the αβ lobes (Aso, Sitaraman et al. 2014). However, our results indicate that the activation of the MBONγ1pedc>αβ by PPL1 neurons and further inhibition of the αβ lobes by the MBONs does not contribute in dishabituation of the odor response (Figure 5).

These results suggest that other neurons activated by the PPL1γ1pedc>αβ are important in dishabituation. Indeed, PPL1γ1pedc>αβ send their axons to the superior medial and superior lateral protecerebrum (SLP) (Jefferis, Potter et al. 2007, Fisek and Wilson 2014) as do some of the MBONs that innervate the β' lobe (MBONβ2β'2α) and induce avoidance (Koseki, Matsui et al. 2016) and most of the α' MBONs that send their axons to the lateral horn (LH) (Aso, Sitaraman et al. 2014), a neuropil that encodes response valence to odor stimuli and is inhibited during habituation (Semelidou, Acevedo et al. 2018). Interestingly, a LH type II neuron projects into the SLP (Jefferis, Potter et al. 2007, Fisek and Wilson 2014), connecting this brain region with the LH. It is possible that SLP neurons modify the LH activation and subsequently modulate the recovery of the olfactory response after stimulation with an electric shock (Figure 8). However, the PPL1γ1pedc>αβ neurons are not the only neurons activated by electric shock. Other neurons of the PPL1 cluster, such as the PPL1-γ2α'1 and PPL1-α'2α2, innervate the vertical MB lobes and activate MBONs that project to the LH (Aso, Sitaraman et al. 2014), and can thus modulate the response of neurons implicated in habituation (Figure 8).

Apart from the cholinergic MBONs that arborize from the vertical α and α' lobes and innervate the lateral horn (LH) (Aso, Sitaraman et al. 2014), previous studies have shown that activation of the glutamatergic MBONs that innervate the horizontal β, β' and γ lobes induces avoidance to odor (Aso, Sitaraman et al. 2014, Owald, Felsenberg et al. 2015). Our results demonstrate that octopaminergic transmission from the APL neurons that broadly innervate the MBs, is necessary for normal dishabituation (Figure 3). Importantly, previously studies on anesthesia resistant memory (ARM) have shown that APL neurons activate the α'β' MB lobes through activation of the octβ2R receptor (Wu, Shih et al. 2013). Our hypothesis is that APL neurons are (directly or indirectly) activated by electric shock and activate the α'β' MB lobes through the octβ2R. Consequently, the α'β' MBONs that innervate LH activate the habituated neurons and restore the response to the odor. Further experiments will be conducted to examine the role of the α'β' neurons and octβ2R in dishabituation (Figure 8). Interestingly, octopamine is a monohydroxylic analog of norepinephrine (Farooqui 2007), which is

important in olfactory dishabituation in rats. Olfactory habituation in rats depends on the metabotropic glutamate receptor mediated synaptic depression of cortical afferents, and dishabituation with an auditory stimulus elevates norepinephrine levels that activates the noradrenergic ß-receptors of the anterior piriform cortex. These receptors act antagonistically to the mGluR group III receptor necessary for habituation, and thus reverse the synaptic mechanisms underlying response attenuation (Smith, Shionoya et al. 2009).

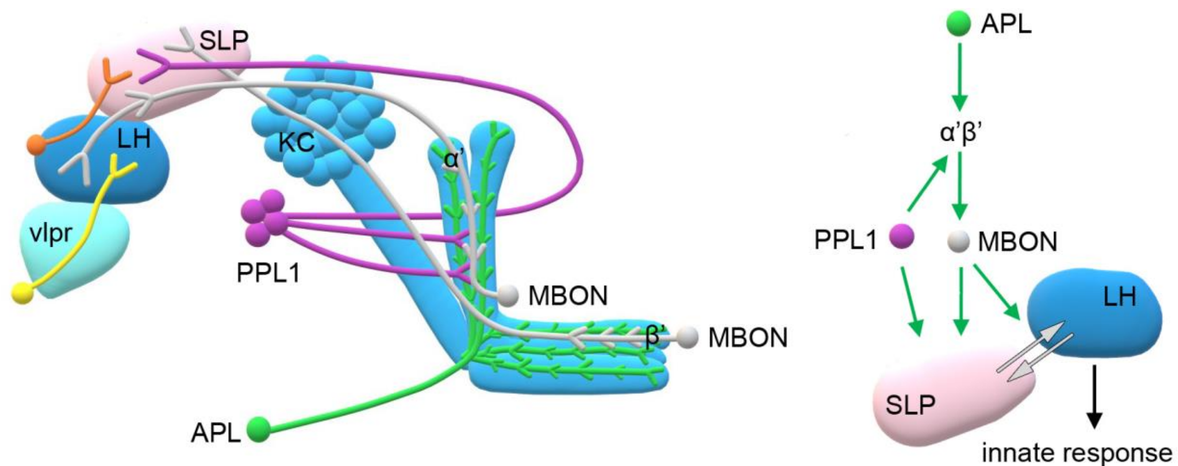


Figure 6. A proposed model of the neuronal subsets underlying dishabituation. Distinct neuronal subsets are marked with different colors; Anterior Paired Lateral neurons (APL-green), dopaminergic PPL1 neurons (PPL1-purple), mushroom body output neurons (MBON-light grey), Lateral Horn type II neurons (orange), ventrolateral protocerebrum neurons (vlpr-yellow). Green arrows indicate activation. Light grey arrows indicate possible interaction.

Collectively, our results suggest that dishabituation requires activation of dopaminergic and octopaminergic neurons that possibly feedback onto the LH via the MBs and the SLP neurons and modulate the response to the odor. To further investigate this hypothesis, we will perform imagining experiments using trans-Tango, a technique for anterograde transsynaptic circuit tracing and manipulation (Talay, Richman et al. 2017), to identify the pre- and post-synaptic terminals of the PPL1 and APL neurons that are necessary in dishabituation.

This is the first study, to our knowledge, to investigate the neuronal circuits involved in dishabituation in *Drosophila melanogaster.* Dishabituation allows immediate recovery of a response and behavioral plasticity, enabling the animals to re-evaluate stimuli when the

context or contingency has changed. It is a process as important as habituation, endowing highly adaptive responses in dynamic environments. Abnormal dishabituation would be detrimental to brain plasticity, leading in permanent stimulus devaluation, thereby affecting conditional learning as well. More studies on dishabituation are necessary, to elucidate the molecular and cellular components of this process.

Materials and Methods

Drosophila strains

Drosophila were cultured in standard wheat-flour-sugar food supplemented with soy flour and $CaCl_2$ at 22–25C, unless specified otherwise. Animals expressing $Gal80^{ts}$ (TARGET system) were raised at 18C until hatching and then placed at 30C for 2 days prior to testing.

Control flies carrying the w^{1118} mutation had been backcrossed to the Canton-S for at least 10 generations (w^{1118} strain). All other strains had been backcrossed to the Cantonised-w^{1118} for four to six generations prior to use in behavioral experiments. Transgenes used to block neurotransmission were the UAS-$shibire^{ts}$, which bears a temperature-sensitive mutation in dynamin, encoded by the gene *shibire* (Kitamoto 2001) and UAS-DORK, which encodes for an outward rectifier K^+ channel that exhibits no voltage or time dependence of the open state and behaves as a K^+-selective hole in the cell membrane, similar to the neuronal “leak” conductance (Nitabach, Blau et al. 2002) . To achieve neuronal hyperpolarization, the transgene UAS-TRPA1 was used for overexpression of the thermosensitive TRPA1 channel (Rosenzweig, Brennan et al. 2005) and the transgene UAS-chr2-XXL which encodes for the D156C mutant of Channelrhodopsin-2 that shows extra high expression and long open state, was used for the optogenetic activation experiments (Dawydow, Gueta et al. 2014).

TH-Gal4 was previously described (Friggi-Grelin, Coulom et al. 2003), as was c061;MBGal80 (Krashes, DasGupta et al. 2009) which was obtained from Scott Waddell (Oxford University, Oxford, UK), and MB504B-Gal4 (Vogt, Schnaitmann et al. 2014) which was obtained from Ron Tanimoto (Tohoku University, Sendai, Japan). The APL-Gal4 driver was described previously (Wu, Shih et al. 2013). c772 and c739-Gal4 targeting expression in the

αβ MB lobes have been described before (Acevedo, Froudarakis et al. 2007, Pavlopoulos, Anezaki et al. 2008), as was MB131B-Gal4, which marks the γ lobe MB neurons and was kindly provided by Yoshinori Aso (Janelia Research Campus, Howard Hughes Medical Institute, Ashburn, VA) (Aso, Sitaraman et al. 2014). Gad-Gal4 was described previously (Jackson, Newby et al. 1990, Buchner 1991), as was DAL-Gal4 (Chen, Wu et al. 2012). The UAS-Gad RNAi was obtained from the Vienna Drosophila Recource Center (VDRC, #32344). The UAS-Tbh RNAi (VDRC, #51667) was kindly provided by Scott Waddell (Oxford University, Oxford, UK). Rdl RNAi 1 (VDRC, #41101) was provided by Scott Waddell (Oxford University, Oxford, UK), Rdl RNAi 2 (P{UAS-Rdl.RNAi.8-10}J, FBti0153036) (Liu, Krause et al. 2007) was provided by Ron Davis (Scripps Florida), while Rdl RNAi 3 was obtained from Bloomington (#52903). GABA-B-R1 RNAi was obtained from Bloomington (#28353). GABA-B-R2 RNAi 1 and 2 (VDRC #1785 and VDRC #110268, respectively) were provided by Scott Waddell (Oxford University, Oxford, UK). GABA-B-R3 RNAi (VDRC, #108036) was provided by Scott Waddell (Oxford University, Oxford, UK). For the Rdl RNAi 3 which was in a y^1v^1 background, the control line for TRiP RNAi carrying P{CaryP}attP40 was obtained from Bloomington (#36304) and was crossed to the Gal4 line to obtain heterozygote controls with the right genetic background.

Behavioral analyses

To obtain flies for behavioral analyses, Gal4 driver homozygotes were crossed *en masse* to UAS-shibire^{ts}, UAS-DORK, UAS-TRPA1 and UAS-Chr2-XXL. Similarly, UAS-shibire^{ts}, UAS-DORK, UAS-TRPA1, UAS-Chr2-XXL and Gal4 driver homozygotes were crossed *en masse* to *w*^{1118}, to obtain heterozygous controls. For the RNAi experiments APL;Gal80^{ts}, C739Gal80^{ts} and C772Gal80^{ts} homozygotes we crossed to the RNAi lines and to *w*^{1118} to obtain heterozygous controls. Similarly, homozygotes for the RNAi transgenes were crossed to *w*^{1118} to obtain heterozygous controls. In the case of the TRiP RNAi Rdl line, C772Gal80^{ts} homozygotes was crossed to the control line for the TRiP RNAi which carries the same genetic background as the transgenic line. Flies for all experiments were raised at 25C, except for the RNAi experiments, where flies were raised at 18C. All flies used in behavioral experiments were tested 3–5 days after emergence. Behavioral experiments were performed under red light at 23–24C and 60–70% humidity.

Olfactory habituation and dishabituation

Olfactory habituation experiments were performed under the conditions described above and the experimental setup was previously described (Semelidou, Acevedo et al. 2018). For the 'training phase', approximately 50 flies were exposed in the upper arm of a standard T-maze to Octanol (OCT) for 4 minutes. Immediately after the odor exposure flies were stimulated with the dishabituator which was a 45V electric shock, a 90V electric shock or were vortexed for 3 sec. After a 30 sec rest period, the animals were lowered to the center of the maze for testing their osmotactic response, choosing between air and OCT. The choice period lasted for 90 sec and then the flies in each arm were trapped and counted. The performance index (PI) was calculated as the percentage of the fraction of flies that avoid the odor and congregate in the unscented (air) arm minus the fraction of flies that prefer the odor-bearing arm. UAS-shibire^{ts} harboring strains were placed in a 32C incubator for 30 min prior to the start of the 'training phase' to inactivate the transgenic temperature-sensitive Shibire protein, which recovers its full activity within 15 min after removal from 32C (Kitamoto 2001, McGuire, Le et al. 2001). Flies overexpressing the RNAi transgenes were kept at 30C for two days to induce transgene expression.

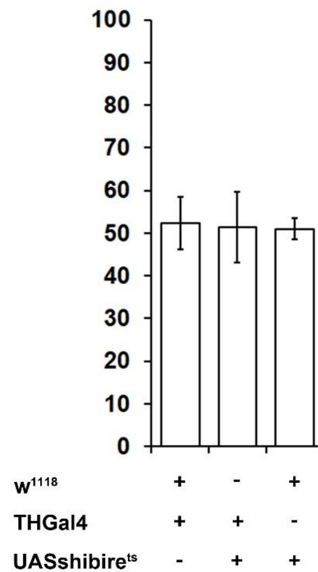
The conditions for dishabituation with electric shock were described previously (Semelidou, Acevedo et al. 2018) and similar dishabituation performances were observed with electric shocks of 30V, 45V, and 90V. Moreover, the number of shocks did not have a significant effect on dishabituation. Vortexing was used as another mechanical stimulus to produce dishabituation. Flies were subjected to 3 s of vortexing at maximal speed immediately after odor exposure.

For neuronal activation experiments with the UAS-TRPA1 overexpression, flies were raised at 18C and expression of the transgene was induced at 31C (using a heat block), for 10 sec after odor exposure. For the optogenetic activation, expression of UAS-Chr2-XXL was driven to the neurons of interest. Flies were transferred in a glass Erlenmeyer flask immediately after odor exposure and were stimulated with a continuous light pulse at 460nm for 3 sec. Then, they were transferred back to the training arm where they rested for 30 sec before the choice period.

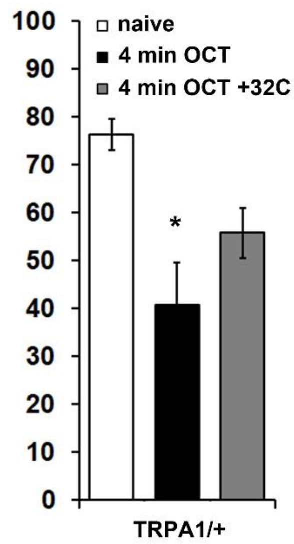
Statistical analysis

Untransformed (raw) data were analyzed parametrically with JMP3.1 statistical software package (SAS Institute Inc., Cary, NC). If significant, initial ANOVA tests were followed by Dunnett's and Least Square Means Contrast analyses and the experimentwise error rate was adjusted as suggested by Sokal and Rohlf (Appendix B) (James Rohlf and Sokal, 2012).

Supplemental Material



Suppl Figure 1. Blocked neurotransmission from the dopaminergic neurons does not affect shock avoidance. Mean Performance Indices ± SEM are shown in all figures. Functional silencing of the dopaminergic neurons with UAS-shibire^{ts} under THGAL4 did not affect avoidance to 45V electric shocks (ANOVA p=0.9864, n = 6 for all groups).



Suppl Figure 2. Elevated temperature is adequate to recover the olfactory response. Mean Performance Indices ± SEM are shown in all figures. Stars indicate significant differences from the naive response. Elevated temperature at 32C for 10 s after odor exposure was adequate to recover the habituated olfactory response (grey bar, p=0.0377, n = 10 for all groups).

The role of sertonergic receptors in electric shock habituation.

Introduction

Serotonin is a neurotransmitter widely distributed in the central and peripheral nervous systems of both mammals and insects. Serotoninergic transmission is implicated in various behaviors, such as circadian activity (Glass, Grossman et al. 2003)(Glass, Grossman et al. 2003)(Glass, Grossman et al. 2003)(Glass, Grossman et al. 2003)[84](Glass, Grossman et al. 2003)(Glass, Grossman, Farnbauch, & DiNardo, 2003)[323][32384](Glass, Grossman et al. 2003)(Glass, Grossman et al. 2003)(323)[323](Smart and Biello 2001, Glass, Grossman et al. 2003, Yuan, Lin et al. 2005, Yuen, Jiang et al. 2005), sleep (Boutrel, Franc et al. 1999, Yuan, Joiner et al. 2006), aggression (Johnson, Becnel et al. 2009, Alekseyenko, Chan et al. 2014) and learning and memory (Sarnyai, Sibille et al. 2000, Johnson, Becnel et al. 2011, Lee, Lin et al. 2011). Importantly, abnormalities in serotonergic receptor activation have been linked to various disorders, such as anxiety (Heisler, Chu et al. 1998, Ramboz, Oosting et al. 1998, Bailey and Toth 2004, Gordon and Hen 2004, Li, Holmes et al. 2004), depression (Lemonde, Turecki et al. 2003, Alexandre, Popa et al. 2006, Mohammad, Aryal et al. 2016), and schizophrenia (Bleich, Brown et al. 1988, Akhondzadeh 2001, Chaudhry, Soni et al. 2002).

In *Drosophila melanogaster*, serotonergic neurons project to most brain regions (Lundell and Hirsh 1994, Monastirioti 1999) and five serotonin receptors have been identified in the Drosophila genome: 5-HT1A, 5-HT1B, 5-HT2A, 5-HT2B, and 5-HT7. Drosophila serotonergic receptors are G protein-coupled receptors and share considerable sequence similarity with their mammalian homologs (Saudou, Boschert et al. 1992, Colas, Launay et al. 1995, Portas, Bjorvatn et al. 2000, Blenau, Daniel et al. 2017). Drosophila 5HT-1 receptor stimulates adenylate cyclase, while the 5HT-dro2A and the 5HT-dro2B inhibit adenylate cyclase and activate phospholipase C (Saudou, Boschert et al. 1992).

Serotonin has an important role in olfactory learning and memory. In mice, downregulation of 5HT1A impairs hippocampal-dependent learning and leads to functional abnormalities in the hippocampus (Sarnyai, Sibille et al. 2000). In Drosophila, blocking the 5HT1A attenuates short-term memory (also referred to as learning) while 5HT1A, 5HT2, and 5HT7 are important for memory consolidation and retrieval, and pharmacological approaches

have shown that functional interactions between the serotonergic receptors mediate short-term and long-term memory (Johnson, Becnel et al. 2011). Interestingly, the expression of these receptors is distributed in more than one neuronal subset that play a role in learning and memory. 5HT1A and 5HT1B are expressed postsynaptically in the Mushroom bodies (MBs), the center of learning and memory in Drosophila (Heisenberg 2003). Activation of these receptors results in reduced cAMP levels while activation of the 5-HT2 receptors that are located postsynaptically on neurons of the protocerebrum are coupled to Gαq and their activation increases cAMP levels. It was proposed that 5HT2 receptors expressed in these neurons facilitate the integration of sensory information into the MBs and functionally interact with the 5-HT1A (Johnson, Becnel et al. 2011). Serotonin levels also affect anesthesia resistant memory (ARM), which was enhanced in animals with elevated serotonin levels and inhibited in those with reduced serotonin. ARM formation requires serotonin release from two dorsal paired medial (DPM) neurons onto the mushroom bodies (MBs) (Lee, Lin et al. 2011).

Extensive studies have focused on the role of serotonin in anxiety, and decreased serotonin neurotransmission has been linked to increased anxiety levels (Heisler, Chu et al. 1998, Ramboz, Oosting et al. 1998, Bailey and Toth 2004, Gordon and Hen 2004, Li, Holmes et al. 2004, Mohammad, Aryal et al. 2016). Interestingly, it was proposed that anxiety helps the animals anticipate and assess potential danger in ambiguous situations, since it results in increased response to stimuli (Blanchard, Griebel et al. 2001, McNaughton and Corr 2004, Belzung and Philippot 2007, Mohammad, Aryal et al. 2016).

Habituation, the attenuation of the response after repetitive presentation of a stimulus is essential for the animals to evaluate the stimuli of the environment and filter them depending on their significance. Abnormal habituation has been linked to various disorders, including schizophrenia (Akdag et al., 2003; Ludewig et al., 2003), migraines (Kalita et al., 2014; Kropp et al., 2015), attention-deficit/hyperactivity disorder (Jansiewicz et al., 2004; Massa and O'Desky, 2012) and autism-spectrum disorders (Bruno et al., 2014; Lovelace et al., 2016; Tam et al., 2017). Drosophila shows habituation to electric shock, and normal attenuation of the response requires functional mushroom bodies (MBs) (Acevedo, Froudarakis et al. 2007). However, the molecular components of this process have not been

identified yet. In this study we investigated the role of serotonergic receptors in habituation to electric shock in *Drosophila melanogaster*.

Results and Discussion

The serotonergic 5HT1A receptor is necessary for normal habituation.

Studies in mammals have linked 5HT1A receptor with anxiety, since reduced 5HT1A levels result in increased levels of anxiety-related behavior in mice (Heisler, Chu et al. 1998, Ramboz, Oosting et al. 1998, Gordon and Hen 2004). In addition, 5-HT1A receptors function in stress response and locomotor activity (Li, Holmes et al. 2004). In Drosophila, 5-HT1A receptor has a sleep-regulatory role in the MBs, while 5-HT1A mutant flies present short and fragmented sleep (Yuan, Joiner et al. 2006).

To study the role of 5HT1A receptor in habituation we used the 5-HT1A^{MI04464} mutants. Repetitive stimulation with 15 electric shocks resulted in habituation in control y^1w^* flies, while 5HT1A mutants did not show attenuation of their response to the stimulus (Fig 1), indicating that 5HT1A receptor is necessary for normal habituation after repetitive stimulation with 15 electric shocks.

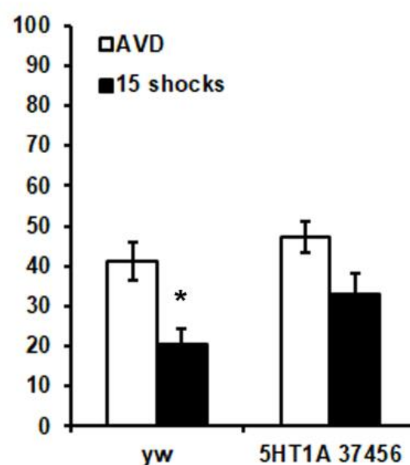


Figure 1. 5HT1A receptor is necessary for normal habituation after 15 electric shocks. Mean Performance Indices ± SEM are shown in all figures. Stars indicate significant differences from the

naive response. Mutation in the 5HT1A receptor results in abrogated habituation after 15 electric shocks (p=0.0405) while yw controls habituated normally (p=0.003). (n ≥ 10 for all groups)

The serotonergic 5HT1B receptor is necessary for normal habituation.

The serotonergic 5HT1B receptor is expressed in the mushroom bodies, the pars intercerebralis (PI) neurons, a subgroup of dorsal neurons, the ventral lateral neurons, the optic lobes, and a neuronal cluster located in the posterior lateral subesophageal ganglion (Yuan, Lin et al. 2005, Yuen, Jiang et al. 2005). 5HT1B receptor has an important role in circadian rhythmicity, since it is expressed in clock neurons and the alteration of its level of expression affects the molecular and behavioral responses to light (Yuan, Lin et al. 2005). In addition, 5HT1B has been found to regulate anxiety in Drosophila, in a similar manner as 5HT1A receptor does in mice (Mohammad, Aryal et al. 2016).

To test whether 5HT1B receptors are important in habituation, we used Drosophila with the 5-HT1B^{MB05181} mutation and accessed their response attenuation after exposure to 15 electric shocks. In contrast to the control line w^{1118} flies, 5HT1B mutants did not habituate after pre-exposure to the stimulus, proposing a role for this receptor in habituation (Fig 2).

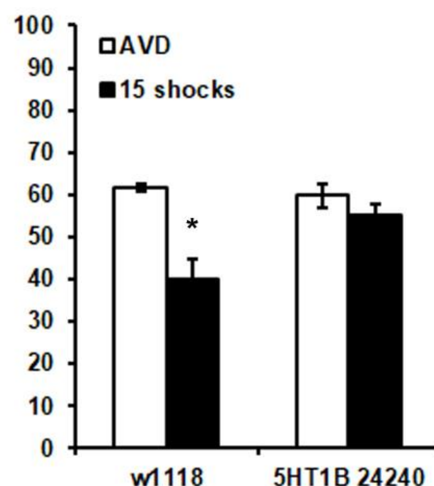


Figure 2. 5HT1B receptor is necessary for normal habituation after 15 electric shocks. Mean Performance Indices ± SEM are shown in all figures. Stars indicate significant differences from the naive response. Mutation in the 5HT1B receptor results in abrogated habituation after 15 electric shocks (p=0.2639) while w^{1118} controls habituated normally (p=0.0006). (n ≥ 10 for all groups)

The serotonergic 5HT2B receptor is necessary for normal habituation.

The 5HT2B serotonergic receptor is the most recently identified receptor in Drosophila (Blenau, Daniel et al. 2017). This receptor was shown to be important in sleep-wakefulness and respiratory control in mice (Popa, Lena et al. 2005) and it has been linked to anxiety in rats (Kennett and Curzon 1988) and Drosophila (Mohammad, Aryal et al. 2016), but its function in the brain remains largely unexplored.

To examine the role of this receptor in habituation we used two lines, with mutations in the 5HT2B gene. Both $5HT2B^{MI06500}$ (40810) and $5HT2B^{MB11858}$ (29257) mutants retained their response to the stimulus after pre-exposure to 15 electric footshocks, in contrast to control flies that habituated normally (Fig 3A-B). These results indicate that 5HT2B function is crucial for normal habituation to the electric shock.

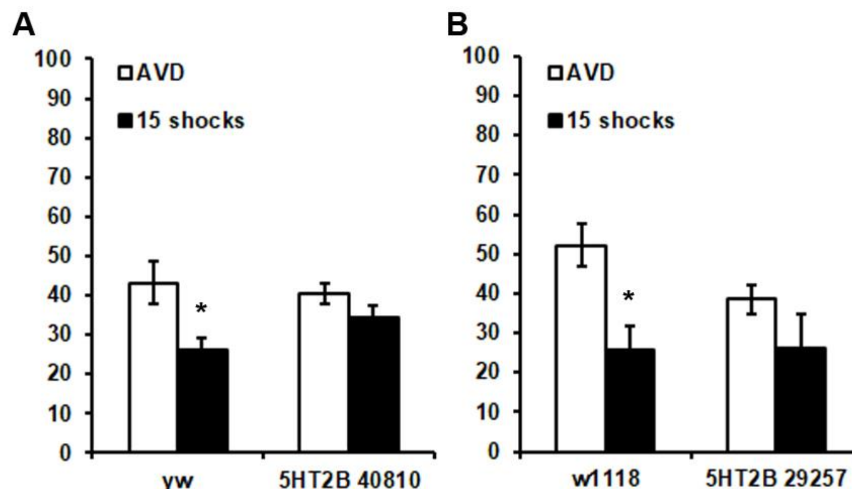


Figure 3. 5HT2B receptor is necessary for normal habituation after 15 electric shocks. Mean Performance Indices ± SEM are shown in all figures. Stars indicate significant differences from the naive response. **(A)** Mutation in 5HT2B disrupts habituation after 15 electric shocks (p=0.1532) while yw control line habituates normally (p=0.008). (n ≥ 10 for all groups) **(B)** Mutation in 5HT2B disrupts habituation after 15 electric shocks (p=0.1704), while the w^{1118} control line habituates normally (p=0.0041). (n ≥ 8 for all groups)

The serotonergic 5HT7 receptor is necessary for normal habituation.

The serotonergic 5HT7 receptor is expressed in ring neurons, the antennal love, and the ellipsoid body (Becnel, Johnson et al. 2011, Kahsai, Carlsson et al. 2012). 5-HT7 activity is

essential for normal courtship and mating behaviors in Drosophila, mediating the levels of interest in both males and females (Becnel, Johnson et al. 2011).

To investigate the role of 5HT7 receptor in habituation we examined two mutated lines, 5-HT7^{MB01344} and 5-HT7^{MB04445}. The first mutation, 5-HT7^{MB01344} (23066) resulted in disrupted habituation, in contrast to the y^{1}w* control line that presents normal habituation after 15 electric shocks (Fig 4A). However, the second mutation we have tested, 5-HT7^{MB04445} (24705) did not validate the previous result, and flies carrying this mutation habituated normally after 15 electric shock, similarly to control w^{1118} animals (Fig 4B). To further examine the role of 5HT7 we used RNA interference to downregulate the expression of the receptor panneuronally, expressing the 5HT7 RNAi under the elav-Gal4 driver. Abrogation of 5HT7 panneuronally disrupted habituation after 15 electric shock, while both control lines habituated normally (Fig 4C). Collectively, these results suggest that 5HT7 is necessary for normal habituation to electric shock.

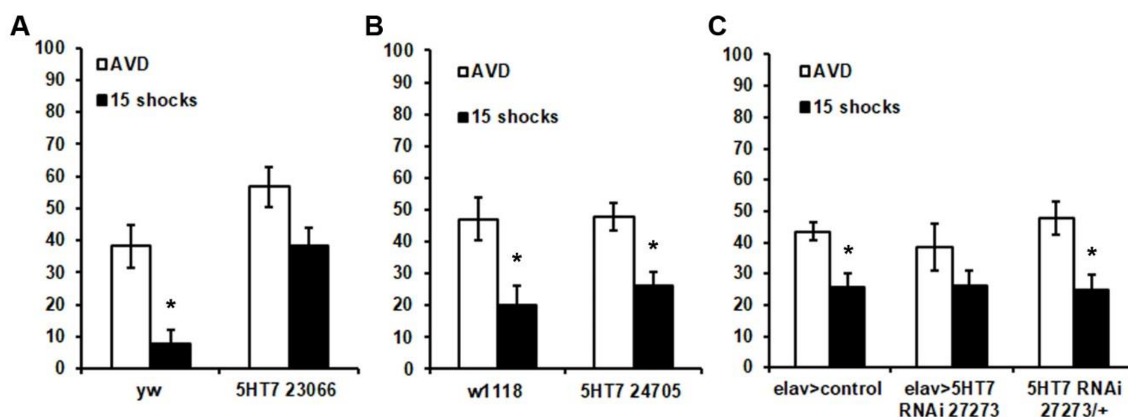


Figure 4. 5HT7 receptor is necessary for normal habituation after 15 electric shocks. Mean Performance Indices ± SEM are shown in all figures. Stars indicate significant differences from the naive response. **(A)** Mutation in 5HT7 disrupts habituation after 15 electric shocks ($p=0.048$) while yw control line habituates normally ($p=0.0018$). ($n \geq 6$ for all groups) **(B)** Mutation in 5HT7 does not affect habituation after 15 electric shocks ($p=0.0026$) w^{1118} control line habituates normally ($p=0.0078$). ($n \geq 8$ for all groups) **(C)** Panneuronal abrogation of 5HT7 via RNAi-mediating transgene expression under elavGal4 resulted in disrupted habituation after 4 min OCT exposure ($p=0.1694$) while both control lines presented normal habituation ($p=0.0056$ for elav>control and $p=0.0037$ for 5HT7 RNAi/+). ($n \geq 8$ for all groups)

In conclusion, our results indicate that serotonergic receptors are indispensable in response attenuation and propose a new role for serotonin in habituation. Additional investigation on the neuronal subsets where serotonergic neurons are expressed and the molecular pathways activated downstream of the receptors is necessary to comprehend the role of serotonin in this process and further elucidate the mechanisms underlying habituation. These results will further aid the understanding of a possible connection between abnormal serotonergic neurotransmission and habituation, learning, and locomotor activety in Drosophila and will provide insight on the role of serotonin in anxiety disorders and schizophrenia.

Materials and Methods

Drosophila strains

Drosophila were cultured in standard wheat-flour-sugar food supplemented with soy flour and CaCl2 at 25C. Control flies carrying the w1118 mutation and the y1w* mutations have been used in all experiments, depending on the background of each mutation. All lines with mutations were obtained from Bloomington (y^1 w^*; Mi{MIC}5-HT1A^{MI04464} #37456, w^{1118}; Mi{ET1}5-HT1B^{MB05181} #24240, y^1 w^*; Mi{MIC}5-HT2B^{MI06500} #40810, w^{1118}; Mi{ET1}5-HT2B^{MB11858} #29257, y^1 w^{67c23}; Mi{ET1}5-HT7^{MB01344} #23066, w^{1118}; Mi{ET1}5-HT7^{MB04445} CG31008^{MB04445} #24705). The RNAi for 5HT7 was obtained from Bloomington (y^1 v^1; P{TRiP.JF02576}attP2 #27273) as was the control line for TRiP RNAi lines (y^1 v^1; P{CaryP}attP2 #36303).

Behavioral analyses

All flies used in behavioral experiments were tested 3–5 d after emergence. For the RNAi experiment, males of the RNAi line and the control line for TRiP RNAi were crossed en masse to the elavGal4, while heterozygote RNAi controls were obtained by crossing w^{1118} females to males of the RNAi line. The progeny was collected and kept that 30C overnight, to induce the RNAi transgene expression. All experiments were performed under dim red light at 25°C and 60%–70% relative humidity.

Electric shock avoidance

Experiments were performed under the conditions described above. Approximately 50 flies were placed at the choice point of a T-maze to choose for 90 sec between an electrified and an otherwise identical inert standard copper grid. In the electrified grid, 45 V shocks were delivered every 4 s, each lasting 1.2 s. The performance index (PI) was calculated as the fraction of flies avoiding the electrified grid minus the fraction of flies that do not.

Habituation to electric shock

Habituation to electric shock experiments were performed under the conditions described above. For the training phase ~50 flies were sequestered in the upper arm of a standard T-maze lined with an electrifiable grid. They were exposed to fifteen 1.2-sec electric shocks at 45 V. Air was not drawn through the tube during training to avoid association of the shocks with air. After a 30-sec rest the flies were tested by choosing between an electrified and an inert grid. During the 90-sec choice period, 17–18 1.2-sec electric shocks of 45 V were delivered to the electrified arm of the maze. At the end of the choice period, the flies in each arm were trapped and counted, and a performance index was calculated as above.

Statistical analysis

Untransformed (raw) data were analyzed parametrically with the JMP3.1 statistical software package (SAS Institute Inc.). If significant, initial ANOVA tests were followed by Dunnett's test.

Conclusion

Habituation, the attenuated response to a repeated inconsequential stimulus, allows information filtering and is considered as the simplest form of learning. Habituation is crucial for animal survival and deficits in this process have been linked with various disorders. Even though the first studies of habituation date back to the beginning of the previous century, the neuronal circuits and molecular mechanisms that underlie habituation have not been elucidated yet. In this study I focused on olfactory habituation in *Drosophila melanogaster,* taking advantage of its very well described olfactory system to study the neuronal subsets implicated in habituation.

I have established a novel paradigm to study olfactory habituation in Drosophila and demonstrated a temporally dynamic response, composed of an initial period described as habituation latency during which the odor response is maintained, followed by habituation after four minutes of repetitive or continuous odor exposure. The response attenuation occurs after exposure to aversive and attractive odorants, depends on stimulus intensity and conforms to the characteristics of habituation, including spontaneous recovery and dishabituation.

My results indicate that mechanisms driving latency and habituation occur in distinct neuronal subsets to modulate the output of the lateral horn neurons, a neuropil significant for innate responses in Drosophila. More specifically, habituation latency requires the antennal lobe inhibitory local interneurons to modify the antennal lobe output, and excitatory projection neurons that project their axons to the mushroom bodies (MBs) and lateral horn (LH) to transfer the odor information to higher order neurons. In addition, neurotransmission from the αβ and α'β' MB lobes is essential for response maintenance during the initial period of odor exposure. I propose that activation of the MBs and the Mushroom Body Output Neurons (MBONs) activates the LH, which drives innate aversion and attraction responses, and the ventrolateral protocerebrum (vlpr), implicated in olfactory aversion, ensuring maintenance of the initial olfactory response. Securing the response to the odor during habituation latency is crucial, since it permits associations between the experienced stimulus and other cues in the environment necessary to establish associative learning and memory.

In contrast to habituation latency, response attenuation requires the activation of inhibitory projection neurons which specifically innervate the LH. Prolonged activation of the excitatory projection neurons during the extended odor exposure activates these inhibitory projection neurons which modulate the LH output, thus reducing the response to the odor. Interestingly, the MBs are dispensable during odor habituation, a result suggesting that their role is to maintain the response to a stimulus, as was previously proposed for shock habituation as well.

Interestingly, my results on response attenuation indicate that the neuronal subsets underlying habituation are differentially recruited, depending on the duration of the odor exposure. In this study I demonstrated that habituation after short odor exposure (4 min) relies on LH inhibition by antennal lobe projection neurons, while response attenuation after longer odor exposure (30 min) was previously shown to be centralized in the antennal lobe, depending on inhibition of projection neurons by the local interneurons. In addition, different molecular pathways are involved in short and long-exposure habituation, with the first being cAMP-independent, while the later apparently relies on cAMP levels within the inhibitory local interneurons for response attenuation. Similar results on timescale habituation were previously shown in mice, rats and primates, highlighting the evolutionary conservation of habituation.

Although habituation is crucial for salience filtering, fast recovery of the naïve response is of equal importance, since animals live in ever-changing environments and the need to re-evaluate stimuli when experiencing a change in their surroundings is crucial for their survival. Dishabituation allows instant recovery of the naïve response after exposure to a novel stimulus. However, our knowledge of the neuronal circuits and the molecular pathways that govern dishabituation is limited, and the few studies that focused on aspects of this behavior were mainly conducted in Aplysia. Here, using a more elaborate whole animal system, I defined the neuronal subsets implicated in dishabituation and proposed the circuit recruited to recover the naïve response after habituation has been established. Dishabituation of the odor response with mechanical stimuli activates neuronal subsets that are not involved in habituation, but feedback onto the habituated neurons to modulate their response. The results demonstrate that dishabituation after electric shock stimulation requires activation of the octopaminergic APL neurons which densely innervate the MBs and the PPL1 dopaminergic neurons which project their axons onto the α' lobe of the MBs.

Remarkably, activation of the MB neurons necessary for habituation latency, is also crucial for recovery of the response in dishabituation. The mushroom body output neurons (MBONs) which innervate the LH are in their majority cholinergic, and I propose that their activation restores the naïve response, by re-activating the LH neurons inhibited during habituation. In addition, both the MBONs and the dopaminergic PPL1 neurons recruited by electric shock stimulation innervate regions of the superior lateral protocerebrum (SLP), which is connected to the LH and might modulate its response. In contrast, activation of the MB γ lobe or the MBONs that inhibit the αβ lobes by PPL1 neurons is gratuitous for dishabituation. Additional experiments will discern the role of the α'β' MB neurons in dishabituation, providing further data to support the proposed neuronal circuit.

In conclusion, the above-mentioned results highlight the fundamental role of the LH both in habituation and dishabituation. However, this study has focused on the neuronal circuits that underlie habituation latency, habituation and dishabituation to aversive odor stimuli. It is possible that the same circuits are implicated in responses to attractive stimuli, although further experiments are necessary to verify this hypothesis. Furthermore, additional research on the molecular aspects of these processes is necessary, to identify the key molecules that are expressed in these neurons and the signaling pathways they mediate.

This study elucidates the functional organization of the brain that directs behavior and modulates innate responses regarding to changes in the environment. Elucidating the neuronal circuits and mechanisms of habituation and dishabituation is crucial for deeper understanding of these processes, deficits of which likely affect associative learning and are linked to various disorders in humans, including autism spectrum disorder, schizophrenia, and attention deficit and hyperactivity disorder. Understanding how the brain evaluates information and orchestrates behavior is essential to better comprehend these disorders, and that is one reason behind the investigation of the role of serotonin in electric shock habituation. Serotonin is implicated in schizophrenia, anxiety disorders, and depression and I have demonstrated that mutations in serotonergic receptors in Drosophila disturb habituation. Additional studies that will identify the downstream effectors of these receptors and the neurons where they function are necessary to define the role of serotonin in habituation. These results, combined with the results on the molecular pathways of olfactory habituation will provide necessary information for the deeper understanding of these processes, and how their perturbation leads to disease.

Figure 1. Schematic representation of the neuronal circuits implicated in (A) Habituation Latency (B) Habituation (C) Dishabituation.

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Additional projects

Interference of the complex between NCS-1 and Ric8a with phenothiazines regulates synaptic function and is an approach for fragile X syndrome.

*Mansilla A, Chaves-Sanjuan A, Campillo NE, **Semelidou O**, Martínez-González L, Infantes L, González-Rubio JM, Gil C, Conde S, Skoulakis EM, Ferrús A, Martínez A, Sánchez-Barrena MJ.*

Abstract

The protein complex formed by the Ca^{2+} sensor neuronal calcium sensor 1 (NCS-1) and the guanine exchange factor protein Ric8a coregulates synapse number and probability of neurotransmitter release, emerging as a potential therapeutic target for diseases affecting synapses, such as fragile X syndrome (FXS), the most common heritable autism disorder. Using crystallographic data and the virtual screening of a chemical library, we identified a set of heterocyclic small molecules as potential inhibitors of the NCS-1/Ric8a interaction. The aminophenothiazine FD44 interferes with NCS-1/Ric8a binding, and it restores normal synapse number and associative learning in a *Drosophila* FXS model. The synaptic effects elicited by FD44 feeding are consistent with the genetic manipulation of NCS-1. The crystal structure of NCS-1 bound to FD44 and the structure–function studies performed with structurally close analogs explain the FD44 specificity and the mechanism of inhibition, in which the small molecule stabilizes a mobile C-terminal helix inside a hydrophobic crevice of NCS-1 to impede Ric8a interaction. Our study shows the drugability of the NCS-1/Ric8a interface and uncovers a suitable region in NCS-1 for development of additional drugs of potential use on FXS and related synaptic disorders.

Introduction

The fragile X syndrome (FXS) is the most common inherited neurological disorder causing intellectual disability and autism. FXS affects ~1 in 2,500–5,000 men and 1 in 4,000–6,000 women and remains without effective pharmacological treatment (1–4). Thus, the

discovery of new targets and drugs that could normalize mental abilities is a great current challenge. The causative mutation of almost all known cases of FXS is a trinucleotide cytosine–guanine–guanine (CGG) expansion in the 5′ UTR of the fragile X mental retardation gene (*fmr1*), resulting in loss of the fragile X mental retardation protein (FMRP). FMRP is an RNA binding protein that regulates the transport and translation of mRNAs. FMRP interacts directly with about one-third of the mRNAs that encode the synaptic proteome (5). However, this scenario of pleiotropy has been challenged recently by suggesting that the primary target of FMRP is the Diacylglycerol kinase kappa (Dgkκ), which would trigger a subsequent cascade of synaptic effects, in particular, on the glutamatergic type (6). Although the issue would require additional analysis, independent of whether the effects are direct or indirect, the FMRP transcriptome has identified mRNA targets associated with autism spectrum disorders (ASDs), mood disorders, and schizophrenia, which suggest potential common pathways for these clinically different diseases.

A recurrent feature in all of these diseases is a deviation in the synaptic equilibrium that defines normalcy. Either excess or deficit in the number of synapses can lead to pathology. In the case of FXS, cortical neurons of patients or *fmr1* KO mice show postsynaptic spines at increased density and with long-neck morphology, which have been interpreted as defects in synapse maturation or pruning (7–10). Similar spine effects are reported for ASDs (11). Benefiting from the conservation of the *fmr1* gene, work in *Drosophila*has shown that loss of function mutations increase synapse number, whereas the excess of function condition yields the opposite effect, synapse loss (12, 13). As for human subjects, *fmr1* mutants in *Drosophila* are deficient in associative learning and memory (14, 15).

During the past two decades, intense efforts have been made to understand the molecular and cellular events underlying synaptic dysfunction in FXS. Studies in animal models have revealed defects in multiple neurotransmitter systems and related signaling pathways (1–4). These studies have led to the development of potential therapeutic agents that target (*i*) neurotransmitter/neuromodulator systems (such as metabotropic glutamate receptors, mGluRs, or GABAergic receptors), (*ii*) signaling pathways downstream of neurotransmitter receptors (such as MAPKs, PI3K, mTOR, and GSK3), (*iii*) proteins regulated by FMRP (such as MMP9 or PAK), and (*iv*) the endocannabinoid system (1–4). It is likely that additional therapeutic targets will be designed, if feasible, against Dgkκ (6). Targeting FMRP

or perhaps, even Dgkκ may not be a convenient strategy, because FMRP is also involved in the differentiation of neurons in the adult olfactory bulb and the hippocampus (16). Likewise, targeting the excess of postsynaptic mGlu receptors may also lead to secondary effects from the unbound excess of toxic glutamate and the imbalance between synapse number and amount of neurotransmitter released.

Under the current knowledge, it seems advisable to design potential therapies targeting the down-regulation of synapse number rather than the reduction of the number of neurons or neurotransmitter receptors (17). However, normal neuronal function requires tight control of probability of neurotransmitter release per synapse in addition to control of synapse number. Indeed, both neuronal properties are coregulated in an antagonistic manner (18, 19). Neurons with a high number of release sites usually manifest a low probability of release per site and vice versa. Thus, a potentially effective approach should target the signaling mechanism of this coregulation. Recently, we described the mechanism by which the Ca^{2+} sensor neuronal calcium sensor 1 (NCS-1; originally named Frequenin in *Drosophila*) interacts with the guanine exchange factor protein Ric8a to activate Gα proteins and coregulate synapse number and activity (18, 19). Interestingly, vertebrate NCS-1 mRNA has been identified as one of the FMRP targets (5), and Frequenin mRNA expression is decreased in the *Drosophila fmr1* mutant (20).

The available structural information on NCS-1/Ric8a recognition (19) and the function of the complex in synapse number control and probability of release led us to search for small compounds that could dock into the NCS-1/Ric8a interface to inhibit complex formation. We hypothesized that such compounds will decrease synapse number in FXS animal models and eventually, patients. Targeting protein–protein interactions (PPIs) has emerged as a viable approach in modern drug discovery. However, the identification of small molecules that effectively interrupt PPIs presents significant challenges (21). Here, we present biochemical, structural, and functional data showing that the aminophenothiazine derivative FD44 binds NCS-1 and inhibits its interaction with Ric8a. Furthermore, this compound mediates recovery of normal synapse number and improves associative learning in a *Drosophila* fragile X model. The crystal structure of NCS-1 bound to FD44 and the structure–function relationship study developed with close analogs explain the mechanism of action and the biological activity of this compound. This study shows the drugability of the NCS-1/Ric8a interface and the

potential use of the aminophenothiazine FD44 as a drug candidate to treat FXS and related synaptic disorders.

Results

We reported previously that *Drosophila* NCS-1 (dNCS-1; also known as Frq2) and Ric8a interact to regulate synapse number and neurotransmitter release and that this interaction is reproduced by the human homologs (19). Although the structure of the NCS-1/Ric8a complex is unknown, our structural data on Frq2 followed by a mutagenesis study shed light on the molecular mechanism of Ric8a recognition by NCS-1 (19). We found that R94, located at the N-terminal edge of a hydrophobic crevice, is essential for the interaction with Ric8a (Fig. 1*A*). In addition, T138 proved important for Ric8a recognition. T138 is located in a mobile loop between helices H7 and H8 (Fig. 1*B*), 40 Å opposite to R94, at the C-terminal edge of the crevice (19). Thus, the NCS-1/Ric8a interaction requires this crevice, in particular the two amino acids located at each end. Furthermore, we showed the key role that helix H10 plays in this protein–protein binding, functioning as a built-in competitive inhibitor. Deletion of this mobile helix, which is found inserted into the crevice or solvent-exposed in the ligand-free dNCS-1 structure (Fig. 1*A*), increases the affinity for Ric8a (19). R94 is located in helix H5 (Fig. 1*B*; Fig. 3), and its guanidinium group interacts with E26, located in helix H2, thus attaching helix H2 to the rest of the structure and contributing to the shape of the upper sidewall of the hydrophobic crevice. Because R94 is essential for Ric8a binding and reasoning that it might provide the key for developing targeted PPI inhibitors (22), we focused the search for small molecule candidates able to interrupt the NCS-1/Ric8a interaction at the R94 region.

Figure 1. VS calculations and validation of the inhibitory properties of the small molecule hits. (*A*) Predicted complex between human NCS-1 and FD44 by VS. FD44 molecule is displayed as sticks. Diagram shows the molecular surface of human NCS-1 [PDB ID code 1G8I, molecule A (23)], with the hydrophobic crevice in green; residues R94 and E26 in orange and blue, respectively; and helix H10 in lilac ribbon with D187 in sticks. The *Drosophila* ligand-free NCS-1 structures [4BY4 and 4BY5 (19)] have been superimposed, and helix H10, with its three different orientations inside or outside the crevice (orange and yellow ribbons, respectively), is shown to indicate its mobility. (*B*) Sequence alignment of human and *Drosophila* NCS-1 proteins. Secondary structure elements and EF-hand location of the crystallographic dNCS-1/FD44 structure are indicated. The amino acids implicated in FD44 recognition are shown as green circles. R94 and T138 are highlighted with yellow rectangles, and E26 is with a blue rectangle. EF, EF hand; H, helices; S, strands. (*C*) Structure of the aminophenothiazine molecules with high score in the VS. (*D*) Co-IP binding assay of human NCS-1 and V5-tagged Ric8a in transfected HEK cells in the presence of the small compounds and the vehicle DMSO. IP, immunoprecipitation; WB, Western blot.

The virtual screening (VS) through our in-house chemical library, composed of over 1,000 small heterocyclic compounds, was centered at the R94 environment of human [Protein Data Bank (PDB) ID code 1G8I (23)] and *Drosophila* [PDB ID code 4BY4, molecule B (19)] NCS-1 structures. Because of the motility of helix H10, which has been found inside the crevice (19, 24, 25), solvent-exposed (19), or parallel to the crevice (23) (Fig. 1*A*), it was not considered in the VS. It is interesting to note that the orientation of helix H10 in human NCS-1 (hNCS-1), caused by the crystallization conditions and the entrance of PEG molecules inside

the crevice, permits the interaction of D187 with R94 (Fig. 1*A*) (23). This interaction occludes R94, an essential amino acid for Ric8a recognition, which should be solvent-exposed to interact with its target (19). In this case, deletion of helix H10 was necessary to allow better recognition of the small molecules by the R94 region. Results from VS were similar with either the human or the *Drosophila* structures and showed aminophenothiazine derivatives as candidate hits (Fig. 1 *A* and *C* and Table S1). We selected for additional studies only those molecules that showed a good score value together with a plausible distance to R94 (Table S1): FD35, FD16, FD44, and chlorpromazine (CPZ). These compounds, sharing the same tricyclic phenothiazine central scaffold, were previously known for their butyrylcholinesterase inhibition in vitro and neuroprotective activity in cell assays (26, 27). The main structural differences among them are in the substituent attached to the heterocyclic nitrogen atom and in the presence or not of chlorine atoms in some of the benzene moieties. Although FD35, FD16, and FD44 are only research tools that may or may not become a human drug, the discovery of the Food and Drug Administration-approved drug CPZ in our VS encouraged us to go further with this study.

Table S1. Poses ranking during the postdocking analysis

Ranking	Ligand	GScore	Distance to R94 (Å)
1*	SC34	−6.39	8.51
2*	SC37	−6.28	4.47
3†	FD35	−6.17	3.97
4	SC33	−6.16	5.28
5	SC39	−6.07	4.03
6†	FD16	−6.03	1.85
7*	SC36	−5.92	4.15
8*	SC188	−5.83	4.49
9	SC40	−5.43	6.77
10†	FD44	−5.26	2.98
11†	CPZ	−5.03	3.61

*Not available or not purified.

†The molecules that were subsequently tested in the inhibition assays.

Binding and Toxicity Assays of Candidate Small Molecules.

To assay the interaction between human Ric8a and NCS-1 in the presence of the selected candidates, we carried out binding assays in HEK cell cultures (Materials and Methods and Fig. 1D). Cells were cotransfected with human NCS-1– and Ric8a-expressing constructs. Each compound was tested separately at a final concentration of 20 µM. The data show that FD16, FD44, and CPZ inhibit the NCS-1/Ric8a interaction, whereas FD35 does not seem to affect complex formation. During preparation of the corresponding solutions, it was evident that the compounds could be ranked CPZ, FD44, FD16, and FD35 in decreasing order of solubility. The lack of efficacy observed after treatment with FD35 may result from its low solubility.

To quantify the binding interference of FD44 and FD16, we carried out dose–response assays (Fig. 2 A *and* B). Throughout the range of drug concentrations applied to the transfected HEK cells, it became evident that FD44 is a more effective inhibitor of NCS-1/Ric8a binding than FD16. In addition, toxicity assays in HEK cell cultures showed that FD44 and FD16 are much better tolerated than CPZ (Fig. 2C), a feature required for an eventual use in therapy.

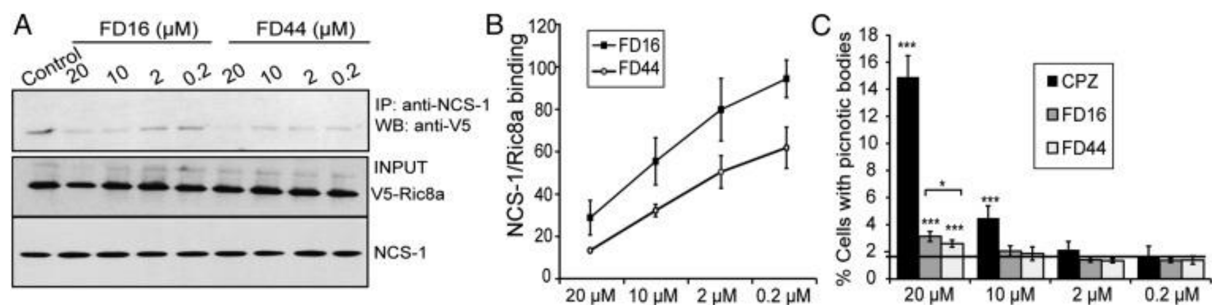


Figure 2. Comparative binding and toxicity assays for FD44 and related molecules. (*A*) Binding assay of HEK cells cotransfected with human NCS-1 and V5-tagged Ric8a. IP, immunoprecipitation; WB, Western blot. (*B*) Graph representing the densitometry of bands from the co-IP of three independent assays. The binding interference dosage–response assay over a range of drug concentrations shows that FD44 is a more effective inhibitor compared with FD16. Data ± SEM. (*C*) Quantification of cell toxicity as HEK cells with picnotic bodies treated with FD44, FD16, or CPZ over the same range of concentrations. FD44 is the least toxic. The horizontal line marks the basal apoptosis in untreated HEK cell cultures. Data ± SD from three independent experiments.

CPZ and related commercialized drugs are well-known in pharmacy because of their effects as antipsychotic agents. They show a strong affinity for dopamine D1 and D2 and

adrenergic α_1 and $\alpha_2$receptors, and they function as their antagonists (28). To determine if FD44 is different from CPZ, radio-ligand displacement assays were performed with the receptors (Table S2) (www.cerep.fr/Cerep/Users/index.asp). These experiments provide an in vitro measurement of the interactions that occur between a receptor and a small molecule, FD44 in this case, by analyzing the ability of the molecule to inhibit the binding of the receptors to specific control compounds. At 10 µM, FD44 does not inhibit the binding of control antagonists (Table S2), indicating that FD44 activity is completely different to that shown by its structural analog CPZ (28).

Table S2. Binding of FD44 to CPZ-targeting neuronal receptors: Ligand displacement assays at 10 µM FD44

Receptor	Antagonist control ligand concentration (nM)	Inhibition of control specific binding (%)
Dopamine 1	SCH 23390 (0.3)	10.5
Dopamine 2	Methylspiperone (0.3)	2.8
Adrenergic α_1	Prazosin (0.25)	10.7
Adrenergic α_2	RX 821002 (0.5)	8

An inhibition power higher than 75% is considered a potential binding molecule.

The Structure of NCS-1 Bound to Ca^{2+} and the Aminophenothiazine FD44 or Close Analogs.

To better understand the binding blockade properties of the candidate molecules, we crystallized the complex of dNCS-1 bound to FD44, FD16, and CPZ and solved their corresponding structures at 1.60-, 1.80-, and 1.52-Å resolution, respectively, using molecular replacement methods (Table S3). Crystals belonged to space group $P2_12_12_1$, and one complex was found in the asymmetric unit. Despite that Ca^{2+} was not added to the crystallization solution and that the protein sample was extensively dialyzed against Milli-Q water, the quality of the electron density maps clearly showed that the three functional EF hands (EF2, EF3, and EF4) contained Ca^{2+}. The well-defined pentagonal–bipyramidal coordination (29) together with the anomalous signal observed at the three EF hands unambiguously showed the presence of Ca^{2+} and discarded the presence of Mg^{2+} (Fig. S1*A*). The overall structure of dNCS-1 is nearly identical in FD44, FD16, and CPZ complexes, with differences confined to the

binding site. The rmsd of FD44 and FD16 structures is 0.6 Å, whereas that of FD44 and CPZ is 0.9 Å.

Table S3. Diffraction data collection and refinement statistics

Dataset	FD44	FD16	CPZ
Data collection			
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions			
a, b, c (Å)	54.05, 54.96, 61.13	54.06, 63.00, 63.42	54.22, 54.92, 60.21
α, β, γ (°)	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0
Wavelength (Å)	0.978720	0.979236	0.97872
Resolution (Å)	40.87–1.60 (1.64–1.60)	44.70–1.80 (1.84–1.80)	40.57–1.52 (1.55–1.52)
R_{pim}^{*}	0.024 (0.677)	0.022 (1.548)	0.020 (1.411)
$CC_{1/2}^{\dagger}$	0.999 (0.557)	1.000 (0.393)	1.000 (0.463)
$\langle I/\sigma(I) \rangle$	14.9 (1.2)	16.7 (0.5)	20.5 (0.8)
Completeness (%)	100 (100)	99.4 (99.4)	100 (100)
Wilson B factor	27.32	47.7	26.88
Redundancy	12.2 (11.7)	13.0 (13.5)	12.4 (9.7)
Refinement			
Resolution (Å)	40.87–1.60 (1.66–1.60)	44.70–1.80 (1.85–1.80)	40.57–1.52 (1.58–1.52)
No. of reflections	24,656	20,513	28,231
R_{work}/R_{free}	0.185/0.208 (0.299/0.305)	0.228/0.252 (0.470/0.594)	0.193/20.8 (0.384/0.375)
No. of atoms			
Protein residues	183 (3–185)	167 (7–132/143–185)	179 (4–183)
Ligand	1	1	2
PEG molecules	1	0	0
GOL molecules	0	0	2
EDO molecules	0	0	1
MPD molecules	0	6	0
Calcium ions	3	3	3
Water molecules	159	91	266
Average B factors	36.8	54.9	42.1
Average B factors (ligand)	57.3	67.8	48.5
rmsd			
Bond lengths (Å)	0.014	0.004	0.045
Bond angles (°)	1.242	0.87	1.30
Ramachandran plot statistics, %			

In favored	100	98	99
Outliers	0.0	0.0	0.0

Highest-resolution shell is shown in parentheses. EDO, 1,2-ethanediol.

*$R_{p.i.m.}=\sum_{hkl}\sqrt{1/n-1}\sum_{j=1}^{n}|I_{hkl}-\langle I_{hkl}\rangle|/\sum_{hkl}\sum_{j}I_{hkl,j}$

†$CC_{1/2}$ is the correlation coefficient of the mean intensities between two random half-sets of data.

NCS-1 and the Role of Myristoylation.

NCS-1 is a myristoylated protein (31). There are a significant number of experimental observations that suggest that the human variant does not contain a Ca^{2+}/myristoyl switch mechanism. (*i*) The human protein is permanently bound to the cell membrane in the presence and absence of Ca^{2+} in vitro (31) and in vivo (32) and recently, also with synthetic membranes (33). (*ii*) O'Callaghan and Burgoyne (34) showed that hydrogen bonding between residues located in helix H1 (Fig. S3*A*) stabilizes the helix in a rigid conformation that could keep the N terminus of hNCS-1 in an open conformation with the myristoyl group exposed to the solvent, regardless the Ca^{2+} content. Interestingly, this hydrogen bonding is conserved in dNCS-1 (Fig. S3*B*), but it is not observed in the Ca^{2+} myristoyl switch NCS proteins (Fig. S3*A*). (*iii*) We had reported previously that the binding of *Drosophila* or human NCS-1 to Ric8a still occurs in the absence of Ca^{2+} (19). Those experiments were carried out in HEK cells, where proteins are myristoylated. Because we had also proved that the complete crevice in NCS-1 is the binding interface for Ric8a and that Ca^{2+} myristoyl switches use the myristoyl group to occlude the crevice in the absence of Ca^{2+}, our data do not favor a switch for NCS-1. (*iv*) Finally and related to the influence of myristoylation on FD44 binding, the crystal structure of NCS-1 bound to FD44 reveals many hydrophobic contacts with residues located in the N-terminal part of the crevice. Some proteins of the NCS family containing a Ca^{2+}/myristoyl switch use some of these amino acids to contact the myristoyl group (Fig. S3*A*). In this case, myristoylation could have an influence in FD44 binding.

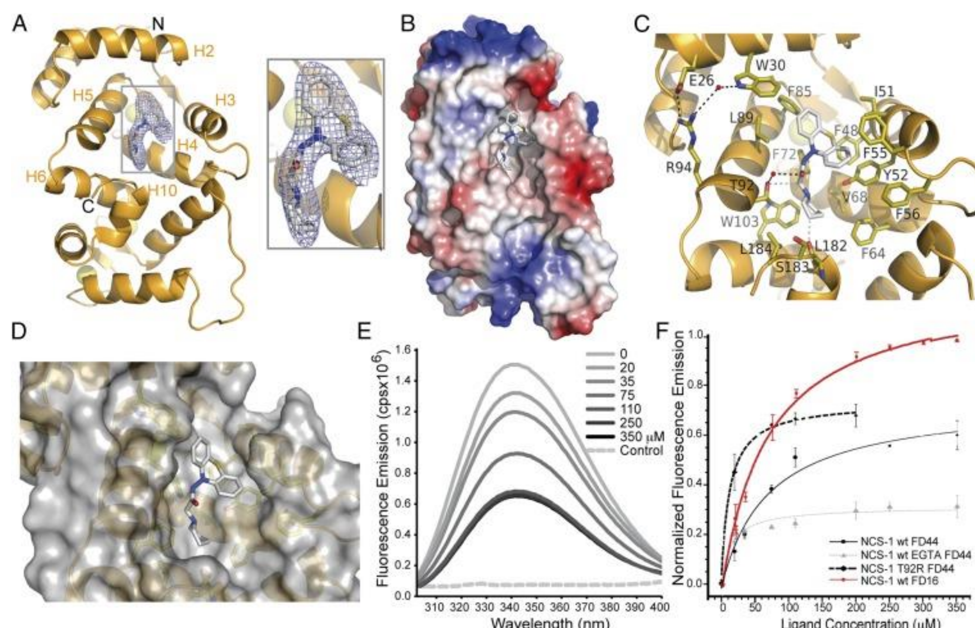
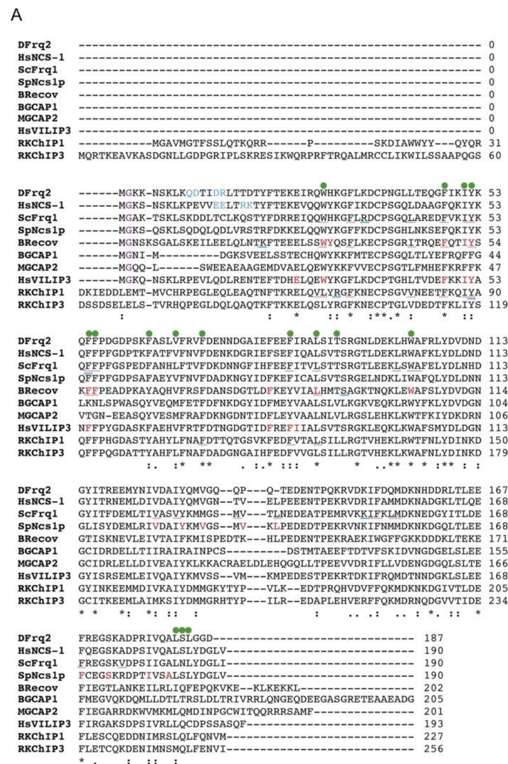


Figure 3. Structure of the Ca^{2+}-loaded NCS-1 bound to aminophenothiazine FD44. (*A*) Ribbon representation of dNCS-1 in complex with Ca^{2+} (yellow spheres) and FD44 (stick mode; C, N, O, and S atoms shown in white, blue, red, and yellow, respectively). The $2F_o$-F_c electron density map for the small molecule is shown at 1.0σ. (*Inset*) A zoomed-in view of the gray-squared region. Helices contributing to FD44 pocket formation are indicated. (*B*) Electrostatic molecular surface of dNCS-1 showing the hydrophobicity of the FD44 cavity. (*C*) A detailed view of the residues interacting with FD44 compound. Strong and weak H bonds are shown with black and gray dashed lines, respectively. Water molecules are displayed as spheres. (*D*) Molecular surface representation on FD44 cavity as shown in *C*. (*E* and *F*) Fluorescence assays of FD44 and FD16 binding to NCS-1 in solution. (*E*) Trp fluorescence emission spectra of WT fly NCS-1 at increasing concentrations of FD44. Control: fluorescence of 350 μM FD44 in the absence of the protein. (*F*) Representation of the Trp fluorescence quenching of WT and T92R fly NCS-1 proteins with the ligand concentration (FD44 or FD16) in the presence of Ca^{2+} or EGTA. Data ± SD. The curves represent the least squares fitting to the experimental data considering a 1:1 stoichiometry. To properly compare the different curves, intensities were normalized and represented as $(I_0 - I)/I_0$.



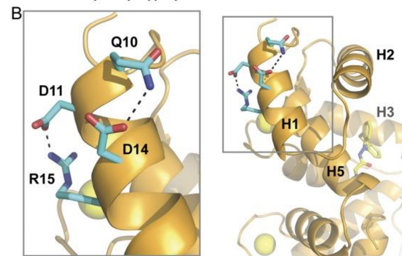


Figure S3. Sequence comparison of NCSs. (*A*) Sequence alignment of NCS proteins with 3D structures that are known: *Drosophila* NCS-1 (DFrq2), human NCS-1 (HsNCS-1), Budding yeast Frq1 (ScFrq1), Fission yeast Ncs1p (SpNcs1p), Bovine Recoverin (BRecov), Bovine GCAP1 (BGCAP1), Mouse GCAP2 (MGCAP2), human VILIP3 (HsVILIP3), and Rat KChiP1 and 2 (RKCHiP1 and RKCHiP2, respectively). Myristoylated Glycine 2 is in purple. The amino acids implicated in FD44 recognition are marked with green circles. The residues implicated in myristoyl group recognition are in red (48, 64–66). Residues contacting their corresponding targets are underlined in pink and blue [hydrophobic and polar contacts (H bonds and salt bridges), respectively] (Fig. 6). Conservation level is indicated at the bottom of the sequence alignment. Residues located in helix H1 of human and fly NCS-1, which contact with each other through H bonds and contribute to generating a rigid conformation of helix H1 (34), are shown in blue. (*B*) A detailed view of these hydrogen bonds found in dNCS-1/FD44 complex.

To investigate this possibility, we performed coimmunoprecipitation (co-IP) assays in HEK cells, where NCS-1 undergoes myristoylation, and found that FD44 inhibits Ric8a binding at 20 µM (Fig. 2*B*). In solution and with the recombinant unmyristoylated protein, affinity for

FD44 is 22 μM. These results suggest that myristoylation does not influence FD44 affinity. To test further the influence of myristoylation, we have found that a mutated form of NCS-1 that cannot be myristoylated binds Ric8a with the same affinity as the myristoylated version (Fig. S4). Also, FD44 shows interference of the NCS-1/Ric8 binding, irrespective of the myristoylation state of NCS-1 (Fig. S4). In conclusion, myristoylation does seem to have an effect on Ric8a binding or FD44 affinity, and these data, together with those previously reported, point to the absence of a Ca^{2+}/myristoyl switch in the fly and human NCS-1.

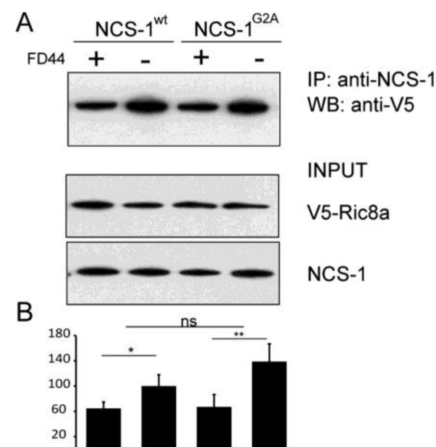


Figure S4. NCS-1/Ric8a complex formation and FD44-mediated inhibition are myristoylation-independent. (*A*) Co-IP assay from HEK293 cells cotransfected with humanV5-Ric8a and human NCS-1 (either the WT or a G2A mutated form that prevents myristoylation). Co-IP assays were performed under the presence of vehicle [DMSO (−)] or 10 μM FD44 (+). Input gels show protein levels before co-IP. IP, immunoprecipitation; WB, Western blot. (*B*) Comparative quantifications of each lane from three experiments expressed in arbitrary units ± SD. Student's *t* test. ns, not significant. *$P = 0.05$; **$P = 0.01$.

The Aminophenothiazine FD44 Modifies the Number of Synapses in a Genetic Model of Fragile X and Improves Learning Ability.

Because FD44 and FD16 prevent the NCS-1/Ric8a interaction (Figs. 1*D* and and2)2) and we had previously identified the antagonistic effects of these two proteins on synapse number (19, 35), we assayed whether the two drug candidates could reduce synapse number in a pathological condition in which synapses are in excess (10). Fragile X is a convenient case for this assay, because the synaptic phenotype is well-characterized and the genetic origin is conserved (1). Mutants in *fmr1* show essentially the same synaptic phenotype in humans,

mice, and flies (5, 11–13). Importantly, the fly homolog of NCS-1, Frq2, is among the mRNAs deficiently translated in *fmr1* mutants (20). We capitalized on these circumstances and the available structural and synaptic data about NCS-1 and Ric8a to directly test the potential effects of FD44 and FD16 on synapse number. To validate the specific tools for the planned experiments, we showed by Western blot that the mutant $fmr1^{\Delta 50}$ is null for protein expression (Fig. S5*A*), and we showed by RT-PCR assays that the $fmr1^{RNAi}$ effectively interferes with *fmr1* transcription (Fig. S5*B*).

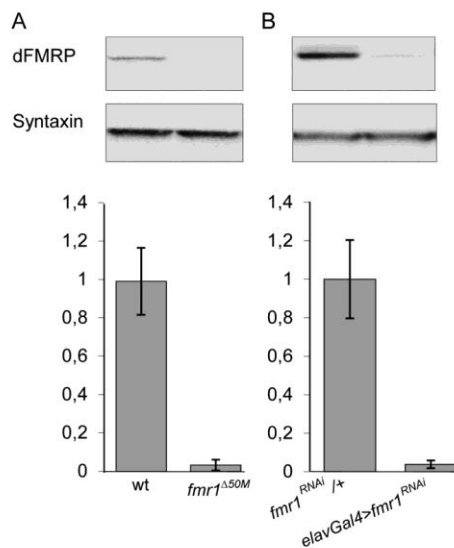


Figure S5. Western blot analysis of dFMRP levels in adult head lysates of (*A*) w^{1118} control flies (WT) and $fmr1^{\Delta 50M}$ mutants and (*B*) $fmr1^{RNAi}$/+ control flies and panneuronally expressing $fmr1^{RNAi}$ flies (*elavGal4 > $fmr1^{RNAi}$*). A representative blot of three repetitions is shown above, and the quantification below includes all replicates. (*A*) *Drosophila* FMRP levels relative to syntaxin loading control were arbitrarily set to one for the w^{1118} controls, and the mean relative levels ± SEM in the $fmr1^{\Delta 50M}$ mutant flies are plotted. dFMRP levels in the mutant animals were significantly different from those in controls (Student's *t* test; $P < 0.01$). (*B*) dFMRP levels relative to syntaxin loading control were arbitrarily set to one for the $fmr1^{RNAi}$/+ controls, and the mean relative levels ± SEM in the *elavGal4 >* $fmr1^{RNAi}$ flies were estimated and plotted. dFMRP levels in flies with panneuronal abrogation of dFMRP expression were significantly different from those in control animals (Student's *t* test; $P < 0.01$).

$fmr1^{\Delta 50}$ Mutant larvae and the corresponding genetic controls were fed with FD44, FD16, or the solvent, DMSO, throughout larval development (*Materials and Methods* and Fig. 5). Synapse number and neuron volume were determined in an identified larval motor neuron at the late third instar (Fig. 5 *A* and *B*). The data confirmed that neuron volume is increased in the mutant (13, 36). Furthermore, we provide direct counting of synapses, which are also

increased in the mutant (Fig. 5*A*). Notably, the pathological phenotype is largely suppressed by feeding FD44 to these larvae (Fig. 5 *A* and *B*). By contrast, FD44 or its solvent DMSO shows little or no effect on the controls. The result on the suppression of the aberrant number of synapses is fully consistent with the proposed function of the NCS-1/Ric8a interaction to control synapse number (19). The other drug candidate, FD16, proved less effective to reduce the aberrant number of synapses of the null *fmr1* mutant (Fig. 5 *A* and *B*).

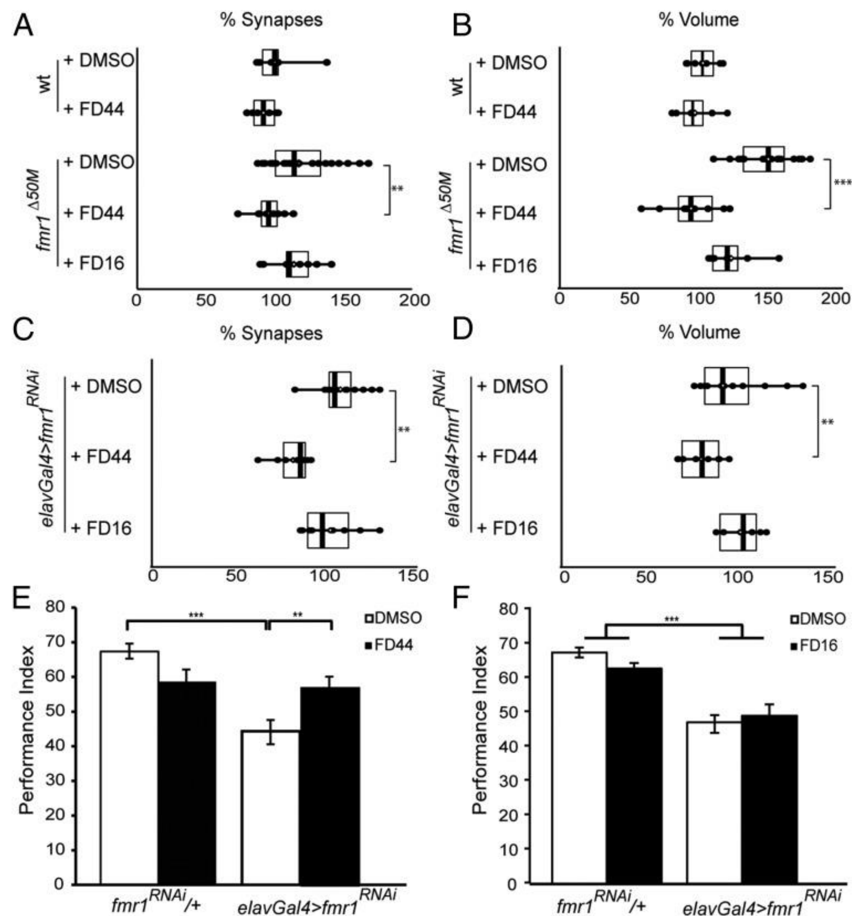


Figure 5. Effects of FD44 and FD16 in vivo. (*A–D*) *Drosophila* WT or *fmr1* mutants were fed with 200 µM FD44, FD16, or DMSO, and larva III NMJs were analyzed. (*A*) Synapse number (nc82-positive spots) and (*B*) synapse volume from 7 to 15 NMJs of different larva were determined using Imaris over the confocal images. Similar measurements were performed on animals with panneuronally abrogated *fmr1* via RNAi, in which (*C*) synapse number and (*D*) volume were determined. Data are plotted in whisker graphs, where each dot represents one value, boxes represent interquartile ranges, vertical lines are the medians, and white diamonds are the averages. Data are shown as percentages of the control WT larva fed with DMSO. (*E* and *F*) *Drosophila* olfactory associative learning assay. Olfactory learning in a negatively conditioned paradigm was performed with control flies (fmr1 RNAi/+) or animals with panneuronally abrogated *fmr1* (*elavGal4* > *fmr1^{RNAi}*) after feeding them with

250 µM FD44, 250 µM FD16, or DMSO for 12 h before the assay. The performance index is shown. (*E*) *fmr1*^{RNAi}-expressing flies present the anticipated learning deficits, and their performance is significantly improved when fed with FD44 for 12 h, showing a learning index indistinguishable from that of control flies. (*F*) In contrast to the results with FD44 treatment, *fmr1*^{RNAi}-expressing flies present no amelioration in their learning performance when fed with FD16. FD16, like FD44, does not improve the learning performance of control flies. Data ± SD from 8 to 19 replicates, with 100 flies per experiment. Student's *t* test. ***P* = 0.01; ****P* = 0.001.

Beyond the chronic effect of FD44 during development, we questioned if the drug could also have an acute effect. In addition, we questioned if the drug could ameliorate learning performance, a prominent feature of FXS syndrome. To these ends, we used the negatively reinforced associative olfactory learning paradigm in adult flies, in which FMRP levels had been abrogated because of the panneuronal expression of an interfering RNA-encoding transgene (Vienna *Drosophila* Resource Center; stockcenter.vdrc.at/control/main) and to which we administered the drug acutely. The use of an RNAi rather than a mutant allele in these experiments is justified because of the relatively low viability of adult mutant homozygotes. Also, driving the *fmr1*^{RNAi} to the CNS allows for assaying the specific contribution of this tissue to learning. The data from *fmr1*^{RNAi}-expressing adults fed with the solvent DMSO show a strong learning deficit that phenocopies the effects of FMRP loss and are consistent with the larval synapse phenotypes (14, 37) (Fig. 5*E*). Remarkably, the learning deficit was ameliorated by 12 h of treatment with FD44 before training (Fig. 5*E*). As in larvae, feeding DMSO or FD44 to adult controls had little or no effect on learning, and the statistical analysis of the data does not reveal a significant difference in the learning index of control flies fed with FD44 vs. DMSO (Fig. 5*E*). These results are congruent with those on FD44-mediated suppression of aberrant synapse number in mutant larvae and provide a readily measurable acute behavioral effect of the drug in fully differentiated adult fly CNS neurons. Finally, learning indexes in the *fmr1*^{RNAi}-expressing animals were not modified by FD16 treatment (Fig. 5*F*). These results confirm the specificity of FD44 vs. other related molecules.

Discussion

By combining structural and chemoinformatic methodologies, we have discovered a small molecule, the phenothiazine derivative FD44, as a PPI inhibitor.

FD44 is biologically active and can prevent the aberrant increase of synapse number and the learning disability of a *Drosophila fmr1* fragile X model. The structural study has revealed the mechanism of action of FD44 in its interaction with NCS-1 for NCS-1/Ric8a complex inhibition. We had previously shown that helix H10 is mobile and functions as a built-in competitive inhibitor. Specifically, this helix is extruded out of the crevice for proper Ric8a recognition (19). In this context, FD44 is able to trap NCS-1 in an inactive conformation. The substituent attached to the phenothiazine group interacts with the C-terminal helix H10 and stabilizes it inside the crevice blocking the contact surface with Ric8a (Fig. 3*C*). Therefore, FD44 prevents the conformational change that helix H10 has to suffer to accept Ric8a. The fact that FD16 does not block helix H10 as efficiently as FD44 explains why this other derivative is not biologically effective. This study shows a potential therapeutic use of an aminophenothiazine and shows the versatility of these compounds. The substituents in the phenothiazine group promote specificity and drive these molecules to their corresponding targets, thus determining their biological activity (28). In fact, it has been also shown that another derivative, Trifluoperazine, can target the cancer-related Ca^{2+} binding protein S100A4, representing a potential inhibitor of metastasis (38).

In addition to the X-ray crystallography data, using a fluorescence assay, we show that FD44 interacts with NCS-1 in solution (Fig. 3 *E* and *F*). Under Ca^{2+}-saturating conditions, NCS-1 affinity for FD44 is 71 µM. However, affinity increases up to 22 µM in the presence of EGTA. This increment is relevant, because as we showed previously, the NCS-1/Ric8a complex is more stable in the absence of Ca^{2+} (19). Thus, the conditions in which FD44 displays its inhibitory properties are exactly the same as those of its efficient interaction with NCS-1. In agreement with all of these results, cell-based dose–response studies show that the formation of the NCS-1/Ric8a complex is prevented at 20 µM FD44 (Fig. 2 *A* and *B*).

The in vivo experiments show that feeding FD44 is an effective method to suppress the excess of synapse in the *Drosophila* FXS model (Fig. 5 *A* and *C*). As previously reported, expression of NCS-1 is reduced and the number of synapses is increased in the

fly *fmr1* mutant (13, 20). However, simultaneous overexpression of NCS-1 and Ric8a suppresses the synaptic phenotypes of each genetic alteration and yields a normal number of synapses (19). Here, the blockade of NCS-1/Ric8a interaction by FD44 should be equivalent to the simultaneous excess of free NCS-1 and Ric8a. Thus, the observed normal number of synapses in the FD44-treated *fmr1* mutant shows the consistency between the genetic and pharmacological experiments. Additional consistency is also found with FXS pathology data. cAMP levels are low in FXS, and antagonizing the cAMP-dependent phosphodiesterase PDE-4 rescues several phenotypes in the fly model (14, 39, 40). In this context, changes in cAMP are expected if the NCS-1/Ric8a binding is prevented, because the complex has an impact on Gs activity (19). Actually, the genetic increase of fly NCS-1 in the nervous system does produce a significant increase of cAMP levels as measured in head extracts (Fig. S6). Therefore, FD44 would be expected to increase the cAMP levels reduced in FXS, because abrogation of NCS-1/Ric8a binding should raise the free (uncomplexed) levels of both proteins. In any event, it must be pointed out that changes in cAMP represent a downstream effect from the molecular mechanism reported here.

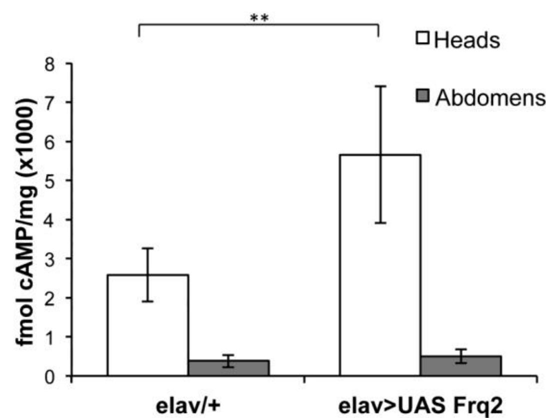


Figure S6. Regulation of total cAMP by Frq2 (dNCS-1). The neuronal overexpression of Frq2 (*elav Gal4 > UAS Frq2*) increases total cAMP levels compared with control genotype (*elav Gal4/+*), whereas cAMP levels in the internal control (abdomens) remain unaltered. Data ± SD from three independent experiments. Student's *t* test with ***P* = 0.01. In brief, 25 heads or abdomens from adult males were frozen in liquid nitrogen separately, homogenized to a fine powder, and resuspended in 0.1 M HCl to proceed with the cAMP measurements. Direct cAMP ELISA Kit (ENZO Life Science) was used following the manufacturer's instructions for the acetylated format.

Feeding FD44 to control flies did not affect viability or synapse number. In light of the available data, this innocuous effect is expected, because the number of synapses is determined by the balanced equilibrium between NCS-1 and Ric8a levels, and any treatments that maintain their ratio should lead to normalcy. Presumably, in the *fmr1* mutant, where NCS-1 levels are reduced, those of Ric8a would be relatively increased, and that condition leads to an increased number of synapses in accord with our prior report (19).

Aberrant synapse number and morphology have been shown to occur concurrently with learning and memory deficits (41, 42), but they were not directly linked to the behavior of the animals until now. FD44 restored the number of synapses in *fmr1* mutants (Fig. 5 *A* and *C*) and ameliorated the learning performance of adult flies (Fig. 5*E*), linking the number of synapses to the behavioral output of the mutant flies. Feeding control flies with FD44 or FD16 results in trends toward lower learning performance, but in all cases, they were not statistically different from controls (Fig. 5 *E* and *F*). It is also appropriate to point out that the parameter under evaluation is the learning index with variability that is well-known and documented. The effect of FD44 in adult flies under acute administration is relevant, because FXS diagnosis is typically achieved after the third year of life, although the synaptic aberration originates from embryonic stages.

Selectivity is an important issue in drug discovery. The 16 NCS family members show up to 60% sequence identity, all have similar topologies, and conserved hydrophobic residues within their crevice that participate in the recognition of their corresponding target also contact the myristoyl group or FD44 (Fig. 6and Fig. S3*A*). However, each protein generates a unique 3D fold and exhibits important structural features in their hydrophobic crevice that determine target specificity and function (43, 44) and equally, will influence FD44 selectivity.

- (*i*) The shape and size of the crevice, which are mainly determined by the ability of the protein to bind two or three Ca^{2+} ions, the positioning of the C-terminal helix H10, and the presence of a Ca^{2+}/myristoyl switch (43). The structure of the Recoverin/Rhodopsin kinase (RK25) (45) suggests that the aminophenothiazine ring of FD44 would not fit into the Recoverin crevice, because the different orientation of helix H5 generates a rather narrow crevice (Figs. 3 and and6).6). Regarding helix H10, it is important to note that it is one of the least conserved regions in sequence and

length (Fig. S3*A*). It can be inside the crevice and contribute to target recognition, like in Recoverin or KChIP1 complexes (45, 46), or it can be out, like in dNCS-1/Ric8a (19) or Frq1/Pik1 (47, 48) complexes, where the targets fully occupy the crevice (Fig. 6). The dNCS-1/FD44 complex structure illustrates how important helix H10 is to shape the FD44 cavity. Thus, the different orientation, conformation, and dynamics of helix H10 will determine FD44 selectivity (Fig. 6).

- (*ii*) Another important selectivity determinant is the presence of residues at the edge of the crevice that interact with the targets through strong polar contacts. For example, in the dNCS-1 isoforms, Frq1 and Frq2, an arginine at the edge of Frq2 crevice but not of Frq1 determines that Ric8a binds exclusively to Frq2 (19). These strong polar contacts are also found in other NCS/target complexes (Fig. 6) and the recognition of FD44 by T92 (Fig. 3*C*).
- (*iii*) To understand selectivity, it will also be important to consider the affinities of the NCS proteins toward their targets. The reported affinities in Recoverin and ScFrq1 complexes shown in Fig. 6 are 1.4 and 0.1 µM, respectively. The moderate affinity of FD44 to NCS-1, which is in the 20-µM range, suggests that deleterious side effects are unlikely with these NCS proteins.
- (*iv*) NCS-1 not only interacts with Ric8a (44). Although there is scarce information on the molecular mechanism of recognition and the relevance of the hydrophobic crevice for most of its targets (44), each interaction will have its unique structural requirement that would ensure selectivity. The structure solution of NCS-1 bound to Ric8a and other targets together with affinity calculations will be important to fully understand the mechanism of Ric8a inhibition by FD44 and rationalize, if necessary, the generation of FD44 derivatives with enhanced pharmacological properties toward the NCS-1/Ric8a or another NCS/target complex implicated in pathological processes.

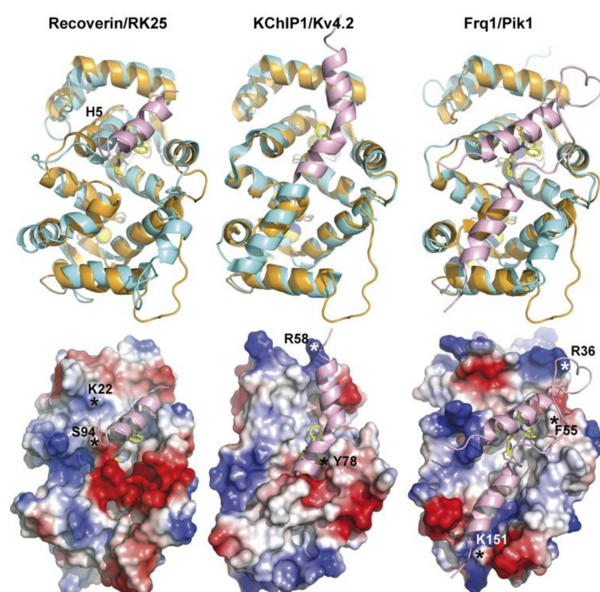


Figure 6. Structural comparison of NCSs in complex with their targets. (*Upper*) Structure and (*Lower*) electrostatic molecular surface of Recoverin/RK25 [657-Å^2 contact area; PDB ID code 2I94 (45)], KChIP1/Kv4.2 [854-Å^{2}contact area; PDB ID code 1S6C (46)], and Frq1/Pik1 [1,296-A^2 contact area; PDB ID code 2JU0 (47)] complexes. The structure of dNCS-1 (orange ribbons and yellow spheres representing Ca^{2+}) bound to FD44 (yellow sticks) was superimposed. NCSs and targets are shown as cyan and pink ribbons, respectively, and Ca^{2+} are shown as blue spheres. Asterisks indicate edge residues in NCSs interacting with their corresponding targets through H bonds or salt bridges (Fig. S3*A*). The comparison with NCS-1/FD44 complex (Fig. 3*B*) shows how different the hydrophobic grooves, their surroundings, and the orientation and fold of helix H10 are. Recoverin helix H5 is indicated to show its different orientation and consequences on crevice narrowness. In the KChIP1/Kv4.2 complex, helix H10 would clash with FD44, whereas in the Recoverin/RK25 complex, it would not reach the FD44 binding region.

Finally, small molecules are convenient for pharmacological therapy because of their ability to cross the blood–brain barrier and their easy administration. Although structurally related to CPZ, the derivative FD44 has revealed here unsuspected functions. The demonstration of its mechanism of action on the NCS-1/Ric8a interaction and the amelioration of synaptic and learning traits in FXS open a strategy to develop additional compounds that target this PPI for an eventual use in therapies against several forms of synaptopathies.

Materials and Methods

VS.

Ligand library preparation.

The molecules from our in-house chemical library were prepared in Schrödinger Ligprep Wizard (Schrödinger Software Modules). The ligands were subjected to additional predocking preparations, where hydrogens were added followed by minimization and optimization with OPLS_2005 force field. Finally, 10 conformations for each ligand were generated and ready for docking.

VS using GLIDE.

After preparing the ligand library and the protein, the grid was defined centering on R94. The library of small compounds was subjected to glide docking using the standard precision module.

Postdocking analysis.

The molecules were ranked based on the scoring function GlideScore (GScore) (Table S1). GScore is an empirical scoring function that approximates the ligand binding free energy. It has many terms, including force field (electrostatic and van der Waals) contributions and terms rewarding or penalizing interactions known to influence ligand binding. It has been optimized for docking accuracy, database enrichment, and binding affinity prediction. To analyze the interactions of docked protein–ligand complexes, the Liginteractions module implemented in Maestro (Maestro Version 9.4.047; Schrödinger Software Modules) was used to check interactions between receptor and ligand atoms within a range of 4 Å.

Co-IP Assays and Western Blotting.

The human NCS-1 construct (provided by R. D. Burgoyne, University of Liverpool, Liverpool, United Kingdom) was subcloned in pCDNA3.1, whereas the human Ric8a (a gift from G. Tall, University of Rochester Medical Center, Rochester, NY) was subcloned in nV5pCDNA3.1. *Drosophila* Ric8a, dNCS-1, and dNCS-1$^{\Delta H10}$ cloning and subcloning were previously described (19). The N-terminal G2A point mutation was performed with the Change-IT Directed Mutagenesis Kit (Affymetrix USB) following the manufacturer's instructions. These constructs were cotransfected into HEK293 cells using Lipofectamin

(Invitrogen). DMSO-diluted compounds at the indicated concentrations or DMSO as control were added at the culture cells 24 h after transfection; 48 h after transfection, cells were lysated (buffer = 150 mM sodium chloride, 1.0% Nonidet P-40, 50 mM Tris, pH 8.0), and the compounds were added at the indicated concentrations in the lysis buffer and maintained throughout the immunoprecipitation assay. Precleared lysates were incubated overnight (12 h) at 4 °C with mouse anti–NCS-1 (1:500; Cell Signaling) or mouse anti-V5 (Invitrogen) as indicated in each experiment. Samples were subsequently incubated overnight with Protein-G–Sepharose (Sigma-Aldrich). After washing, proteins were eluted from the Sepharose and analyzed by Western blot following standard procedures; 10% of the lysate before immunoprecipitation was run as input. Mouse anti-V5 (1:2,000; Invitrogen) and rabbit anti–NCS-1 (1:1,000; Cell Signaling) or mouse anti–c-Myc (1:2,000; Sigma-Aldrich) antibodies were used for Western blot, and blots were incubated with mouse or rabbit TrueBlot (Rockland) as secondary antibodies to avoid heavy-/light-chain antibody interference.

Drosophila Synapse Counting Studies.

FD44 and FD16 were dissolved in DMSO and added to the fly culture media before solidification to ensure a homogeneous distribution in the vial at the final concentration of 200 µM. Eggs were laid down in these media, and larvae were raised at 25 °C until late LIII stage, when they were processed for synapse counting and volume analysis.

We used the glutamatergic neuromuscular junction (NMJ) of the female third larval instar as the experimental system. Synapses were visualized under confocal microscopy by the mAb nc82 (DSHB Hybridoma Bank), which identifies the Bruch pilot protein, a constituent of the presynaptic active zone (49), located at the edge of the characteristic T-bar specialization of fly synapses (50). Also, presynaptic nc82 spots correlate with postsynaptic GluRII clusters (49, 51). Throughout the text, we refer to nc82-positive spots as mature synapses. The axon profile was revealed by rabbit anti-HRP antibody (Jackson ImmunoResearch). All counts were obtained from muscle fiber 6/7 of the abdominal segment 3. Serial 1-µm confocal images were acquired in a Leica TSC SP5 Confocal Microscope and quantified by Imaris software. Experimental and control genotypes were run in parallel, and quantifications were done blindly.

SI Materials and Methods

Fly Strains.

The mutant line for the *fmr1* gene, *fmr1*$^{\Delta 50M}$, corresponds to the Bloomington Stock Center 6930. We used Canton-S as WT control in larval NMJ studies, and for behavioral studies, the control strain was *w*^{1118}. The *fmr1*^{RNAi} strain was from Vienna *Drosophila* Resource Centre (110800KK). The *elavGal4*was used to drive expression in the nervous system for the larval and adult experiments.

Drosophila Behavioral Studies.

Drosophila crosses were set up in standard wheat–flour–sugar food supplemented with soy flour and $CaCl_2$ (52) and cultured at 18 °C and 50% humidity with a 12-h light/dark cycle. To drive expression of *fmr1*^{RNAi}, we used the binary Gal4/UAS system.

Behavioral tests were performed under dim red light at 24 °C to 25 °C and 73–80% humidity. All animals were raised at 18 °C, collected under light CO_2 anesthesia in groups of 50–70, and kept at 30 °C for 48 h to induce the expression of the transgene. For the pharmaceutical administration, all flies were transferred from food vials to plastic vials containing 0.7% yeast paste in water and DMSO/250 µM FD44 for 13 h before the experiment; 3- to 6-d-old animals were treated with FD44 for 12 h and then, kept in normal food vials for 1 h before conditioning. The experimenter was blind to the genotype in all experiments. Behavioral experiments were replicated at least once with flies from different crosses and a different time period (biological replicates). Olfactory learning in the negatively reinforced paradigm, coupling aversive odors as conditioned stimuli (CS; CS+ and CS−) with electric shock as unconditioned stimulus (US) (53), was performed essentially as described previously (14, 54). Six repetitions of 90-V electric shock were delivered with 4-s interstimulus intervals simultaneously with 30 s of odor presentation. The aversive odors used were benzaldehyde and 3-octanol. The performance index was calculated as described previously (55). Because the time between testing and US/CS coupling is 3 min, the initial performance assessment is that of 3-min memory, which we refer to as learning.

The statistical analysis was performed as follows. Untransformed (raw) data were analyzed parametrically with the JMP 7.1 statistical software package (SAS Institute Inc.) as described previously (14, 54). After initial ANOVA, planned multiple comparisons were performed

using a = 0.01. The level of significance was adjusted for the experimentwise error rate. Detailed results of all planned comparisons are mentioned in the figures.

Crystallization, Diffraction Data Collection, and Structure Solution.

dNCS-1 was purified as previously reported (56). The final protein was extensively dialyzed against MilliQ Water and concentrated to 0.46 µM using a concentrator with a 10-kDa cutoff membrane (Vivaspin).

Crystals of dNCS-1 in complex with FD44 or FD16 were obtained similarly; 0.3 mg FD44 or FD16 (0.88 or 0.8 µmol, respectively) was added to 250 µL protein sample (0.11 µmol) and incubated for 16 h at 4 °C. The insoluble fraction of each compound was removed by centrifugation. Preliminary crystallization screens were performed using an Innovadine Crystallization Robot and crystallization kits from Molecular Dimensions, Qiagen, Hampton Research, and Jena Bioscience. Conditions A11 from Proplex [0.1 M Hepes, pH 7.5, 25% (wt/vol) poly(ethylene glycol) methyl ether 2000 (PEG 2000 MME)] and D8 from JCSG [0.1 M Tris, pH 8, 40% (wt/vol) methyl pentanediol (MPD)] yielded to needle-shaped crystals with FD44 and FD16, respectively. Solubilizing 1.7 mM FD44 or 2.3 mM FD16 in the precipitant solution improved crystal quality. To do so, we previously prepared 7.5 or 10 mM stock solutions of FD44 or FD16 in ethanol. Then, crystallization experiments were set as follows: 0.3 µL stock solution was added to 1 µL reservoir solution. The mixture was sealed in the crystallization tray for 2 h to allow ethanol evaporation. Afterward, 1 µL protein was added to the mixture. Rod-like crystals appeared after 5–7 or 2–5 d using FD44 or FD16, respectively. The final precipitant composition for dNCS-1/FD16 diffracting crystals was scaled to 54% (wt/vol) MPD and 0.1 M Tris, pH 7.3.

Crystals of dNCS-1/CPZ complex were obtained differently. A solution containing 0.45 mM dNCS-1 and 31 mM CPZ was prepared. Pyramid-like multiple crystals appeared with the precipitant solution used for FD44 and by mixing equal volumes of protein/CPZ and reservoir solution. To obtain single crystals, we conducted several cycles of macroseeding using, in every cycle, the crystals obtained in the previous one.

Crystals with FD44 and CPZ were cryoprotected with a solution containing 15% glycerol (GOL), 0.1 M Hepes, pH 7.5, 25% (wt/vol) PEG 2000 MME, and 0.5 mM FD44 or CPZ, respectively. Crystals were mounted in fiber loops and then, flash-cooled in liquid nitrogen. Datasets for

the three complexes were collected at 100 K using the ALBA Synchrotron Radiation Source (BL13 Beamline). Data reduction was performed with XDS (57). The structures were solved by the molecular replacement method with Phaser (58) and using as search model the ligand-free protein structure (PDB ID code 4BY4, molecule A). The structure was refined with Phenix (59). FD44 and FD16 occupancy was refined, and the final value was 0.81. CPZ molecule was disordered, and a two-positions model with site occupation factor of 0.5 was refined. FD44 and FD16 dictionaries with geometric restraints were generated, taking into account the information included in the Cambridge Structural Database (60). The stereochemistry of the model was verified with MolProbity (61). Table S3 has additional details on the data processing and refinement. Analysis of the structure was done with CCP4i programs (58), and images were drawn with Pymol (62) and CCP4mg (58).

Fluorescence Emission Experiments.

Tryptophan fluorescence emission of WT and mutant dNCS-1 (18.4 µM concentration) was monitored at increasing concentrations of FD44 or FD16 at 5 °C in buffers containing 50 mM Tris, pH 7.9, 125 mM NaCl, 13.4% ethanol, and 0.5 mM $CaCl_2$ or alternatively, 20 mM EDTA and using a Jobin Yvon Floromax4 Spectrofluorimeter equipped with a Peltier Thermostat. The excitation wavelength was set to 295 nm, and emission spectra were collected over 300–400 nm. Two replicas were performed for each protein to ligand ratio. We previously verified that FD44 and FD16 do not emit any fluorescence when excited at 295nm (Fig. 3*E*, control). The apparent dissociation constant, K_d, was obtained by using a least squares algorithm to fit the following equation (63) to the recorded data:

$$I=\left(I_0+I_\infty[FD]K_d\right)\left(1+[FD]K_d\right).$$

I stands for the observed fluorescence emission at 340 nm (fluorescence maximum), I_0 is the observed fluorescence emission (340 nm) of dNCS-1 before addition of FD44/FD16, I_∞ is the emission when NCS-1 is fully bound to FD44/FD16, and [*FD*] is the concentration of the molecule in micromoles per liter. This model assumes a 1:1 stoichiometry, which was substantiated by the crystallographic structure reported in this work. The fitting was performed with KaleidaGraph Data Analysis Program.

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Drk-mediated signaling to Rho kinase is required for anesthesia-resistant memory in Drosophila.

Kotoula V[1], Moressis A[1], **Semelidou O**[1], Skoulakis EMC.

[1]V.K., A.M., and O.S. contributed equally to this work.

Abstract

Anesthesia-resistant memory (ARM) was described decades ago, but the mechanisms that underlie this protein synthesis-independent form of consolidated memory in *Drosophila* remain poorly understood. Whether the several signaling molecules, receptors, and synaptic proteins currently implicated in ARM operate in one or more pathways and how they function in the process remain unclear. We present evidence that Drk, the *Drosophila* ortholog of the adaptor protein Grb2, is essential for ARM within adult mushroom body neurons. Significantly, Drk signals engage the Rho kinase Drok, implicating dynamic cytoskeletal changes in ARM, and this is supported by reduced F-actin in the mutants and after pharmacological inhibition of Drok. Interestingly, Drk–Drok signaling appears independent of the function of Radish (Rsh), a protein long implicated in ARM, suggesting that the process involves at least two distinct molecular pathways. Based on these results, we propose that signaling pathways involved in structural plasticity likely underlie this form of translation-independent memory.

Introduction

Temporal coincidence of an odor (conditioned stimulus, CS) with electric footshocks (unconditioned stimulus, US) elicits different types of aversive short- (STM), intermediate- (ITM), and long-term (LTM) memories in *Drosophila* (1). Multiple forms of coincident memories contribute to posttraining selective avoidance of the CS. ITM, for example, which is measured 3 h posttraining with 12 US/CS pairings, has been dissected into a labile component, sensitive to cold-induced amnestic treatment called anesthesia-sensitive memory (ASM) and an anesthesia-resistant (ARM) form. ARM lasting at least 24 h can also be induced by 5–10 consecutive sessions of 12 US/CS pairings (massed training) (1–3). ARM,

unlike LTM, does not depend on protein synthesis but may involve modifications of preexisting proteins (1, 4) and is thought to be antagonistic to LTM (2, 4).

In contrast to ASM, the molecular pathways underlying ARM formation, storage, and recall remain poorly understood. Proteins with demonstrated roles in ARM formation include Radish (Rsh) (5); the constitutively active atypical PKC, PKM (6); the calcium channel Bruchpilot (Brp) (7); the d5HT1A serotonin and Oct2β2 octopamine receptors (8, 9); the Dop1R1, Dop2R dopamine receptors (10, 11); and Protein Kinase A (12). However, whether these molecules operate in one or more ARM-mediating signaling cascades is presently unclear.

ARM requires functional mushroom bodies (MBs), neurons essential for learning and memory in insects (13, 14). The MBs are bilateral neuronal clusters in the dorsal posterior brain extending dendrites ventrally to their somata and fasciculated axons projecting anteriorly and bifurcating to form the medial lobes (β/β′, γ) and dorsally to comprise the α/α′ lobes (15). Inhibition of synaptic output from α/β neurons impaired ARM (2), and this is consistent with the distribution of most proteins with known roles in this form of memory (5, 8), except for Oct2β2, which is required in α′ß′ (9). ARM formation appears to require octopaminergic input to the MB α′ß′ lobes from the Anterior Paired Lateral (APL) neurons (9, 16), whereas retrieval requires serotonergic input to the αβ lobes from the Dorsal Paired Medial (DPM) neurons. It appears then that at least two circuits and parallel molecular pathways contribute to ARM (16): an Oct2β2 receptor-mediated Rsh-independent in the α′ß′ lobes (9) and an Rsh-dependent, d5HT1A serotonin receptor-mediated in the αβ (8), which also receives Dop2R-mediated signals (11). Finally, Dop1R1 and Dop2R activities in the γ lobes have been suggested to contribute to ARM (10, 11). Drk, an SH2–SH3 domain adaptor protein orthologous to the mammalian Grb2, is also expressed preferentially in α/β neurons. Drk-mediated signaling to Ras and Raf is required for normal aversive learning signaling, whereas its role in ITM is independent of Ras activation (17). Because ITM comprises ASM and ARM (18), we investigated which of the two forms of memory is affected in *drk* mutant heterozygotes and revealed a specific role for the protein in ARM, mediated via the *Drosophila* homolog of Rho kinase, Drok, and apparently independent of the Rsh protein.

Results

Drk Reduction Selectively Affects ARM.

Heterozygotes for loss-of-function alleles of *drk* learn at a slower rate, a deficit reversible by Ras1 or Raf activation (17). However, memory of the association was significantly reduced even if these mutant heterozygotes were trained equivalently to controls and their memory deficit was independent of Ras1 and Raf activities (17). Because 3-h memory consists of ASM and ARM, we sought to identify which memory form is affected in the mutants. We capitalized on the fact that conditioning the mutants with 12 US/CS pairings results in immediate memory (learning) equivalent to that of controls (Fig. 1*A*). To differentiate between the two types of memory (1), animals trained with one round of 12 US/CS pairings were subjected to cold shock anesthesia 2 h posttraining, and their performance was assessed 1 h later along with similarly conditioned non-cold shocked flies. As expected, 3-h memory post-cold shock was significantly reduced in control animals (compare filled bars), indicating abrogation of the labile ASM but persistence of ARM (Fig. 1*B*). Notably, however, ARM appeared nearly absent in $drk^{\Delta P24}$ heterozygotes (Fig. 1*B*, open bars), indicating that Drk reduction may selectively affect this form of aversive olfactory memory. This was better illustrated after fragmentation of 3-h memory to its components by subtracting the performance after cold shock of controls and mutants from the respective scores of untreated animals (7, 11). This verified that 3-h memory comprised nearly equal parts of ASM and ARM for controls (2), and whereas ASM seemed largely unaffected, ARM was severely attenuated for $drk^{\Delta P24}/+$ (Fig. 1*C*). Therefore, 3-h memory in *drk* mutant heterozygotes consists nearly exclusively of ASM.

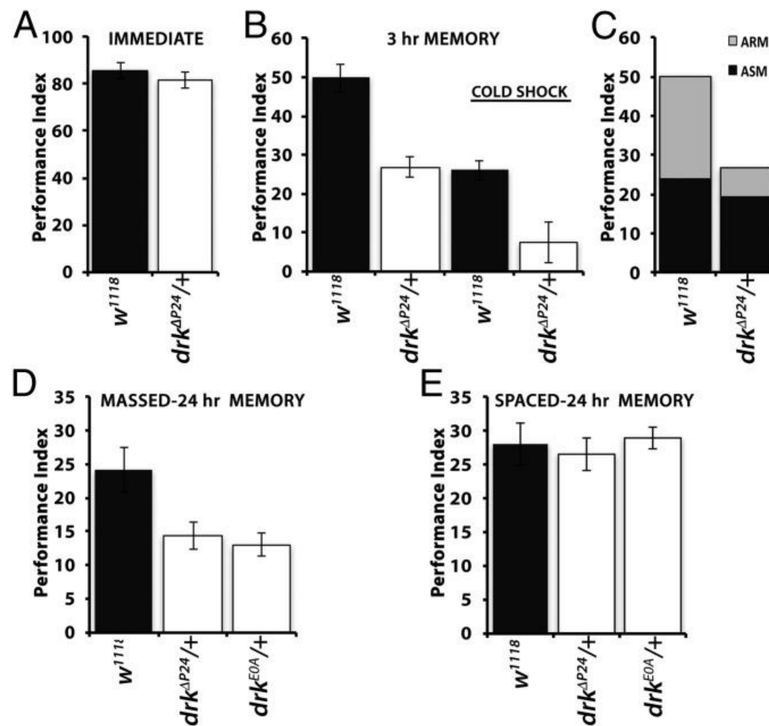

Fig. 1. ARM deficits in *drk* mutant heterozygotes. Performances of mutants are indicated by open bars and controls by black bars. The mean ± SEM are shown. Following ANOVA, potential differences among controls and mutants were assessed for significance with least square means contrast analyses. (*A*) Three-minute memory of $drk^{\Delta P24}$/+ animals after 12 US/CS conditioning once was not significantly different (P = 0.23, n = 8 each) than that of controls. (*B*) Three-hour memory (left side of the graph) of the null heterozygotes of single 12 US/CS session conditioning was significantly different from controls (P < 0.001, n = 7). Significant differences (P < 0.0013, n = 7) in 3-h ARM were also revealed following cold shock (right side of graph as indicated). (*C*) Partitioning the 3-h memory of $drk^{\Delta P24}$ heterozygotes and controls into ARM and ASM by subtracting from normal 3-h memory that after cold shock. ASM (black) is nearly identical, but ARM is highly reduced in $drk^{\Delta P24}$ heterozygotes. (*D*) ARM induced after 5× massed training and assessed 24 h later was significantly different in $drk^{\Delta P24}$/+ (P < 0.007, n = 9) and drk^{E0A}/+ (P < 0.005, n = 7) than controls. (*E*) In contrast, 24-h LTM induced by 5× spaced training was not affected (P = 0.9, n = 14 for $drk^{\Delta P24}$/+; P = 0.78, n = 12 for drk^{E0A}/+).

To independently verify this conclusion, we elicited ARM using a different conditioning protocol, massed training (1, 2), consisting of five consecutive cycles of 12 US/CS. Again, $drk^{\Delta P24}$/+ and heterozygotes for an additional mutant allele, drk^{E0A}, presented deficient ARM (Fig. 1*D*). However, when conditioned with five spaced training cycles, which yield protein synthesis-dependent LTM (1, 2), the performance of both $drk^{\Delta P24}$/+ and drk^{E0A}/+ was indistinguishable from that of controls. These results strongly suggest that Drk is specifically

required for normal ARM. Both these consolidated memory forms reside within the MBs (3, 8, 9) and have been hypothesized to be mutually exclusive and to engage distinct signaling pathways (2, 4). Although various signaling cascades and molecular pathways have been implicated in LTM formation, storage, and recall (19), such mechanisms remain largely elusive for ARM.

Drk Is Required in the α/β Lobes of the MBs for Normal ARM.

Drk is detected in many adult brain structures, including the antennal lobe (AL), ellipsoid body, and prominently the α, β, and γ but not the α'ß' lobes of the MBs (17), the anatomical site where the Rsh-dependent ARM trace is reported to reside (2, 5, 7). Therefore, we sought to determine whether Drk is required for ARM within the MBs or other adult brain structures. MB-specific Drk abrogation was achieved with transgenes (*drkR-1.2*), shown to effectively knock down its levels (17, 20) via RNA-mediated interference (RNAi).

Initially, we confirmed that 12 US/CS elicited normal immediate (3-min) memory in animals expressing *drkR-1.2* under two different MB αβ lobe-preferential Gal4 drivers (Fig. 2*A*), c772-Gal4 and c739-Gal4 (21). In contrast, expression of *drkR-1.2* under these drivers recapitulated the 3-h memory deficit of *drk*mutant heterozygotes (Fig. 2*B* and Fig. S1*A*), whereas cold shock appeared to abolish their 3-h memory (Fig. 2*B*), in accord with the ARM deficit of the mutants (Fig. 1*C*). The cold shock-induced ARM defect was further confirmed with the massed training protocol, which yielded highly compromised 24-h memory in *drkR-1.2*–expressing animals under both c772-Gal4 and c739-Gal4 (Fig. 2*C*). In agreement with the results for *drk*$^{\Delta P24}$*/+* (Fig. 1*D*), LTM was unaffected in *drkR-1.2*–expressing animals (Fig. 2*D*), in strong support of the notion that Drk is required specifically for ARM. Because our data indicate that both conditioning protocols yield nearly identical results, henceforth we used the more robust mass training method unless otherwise specified.

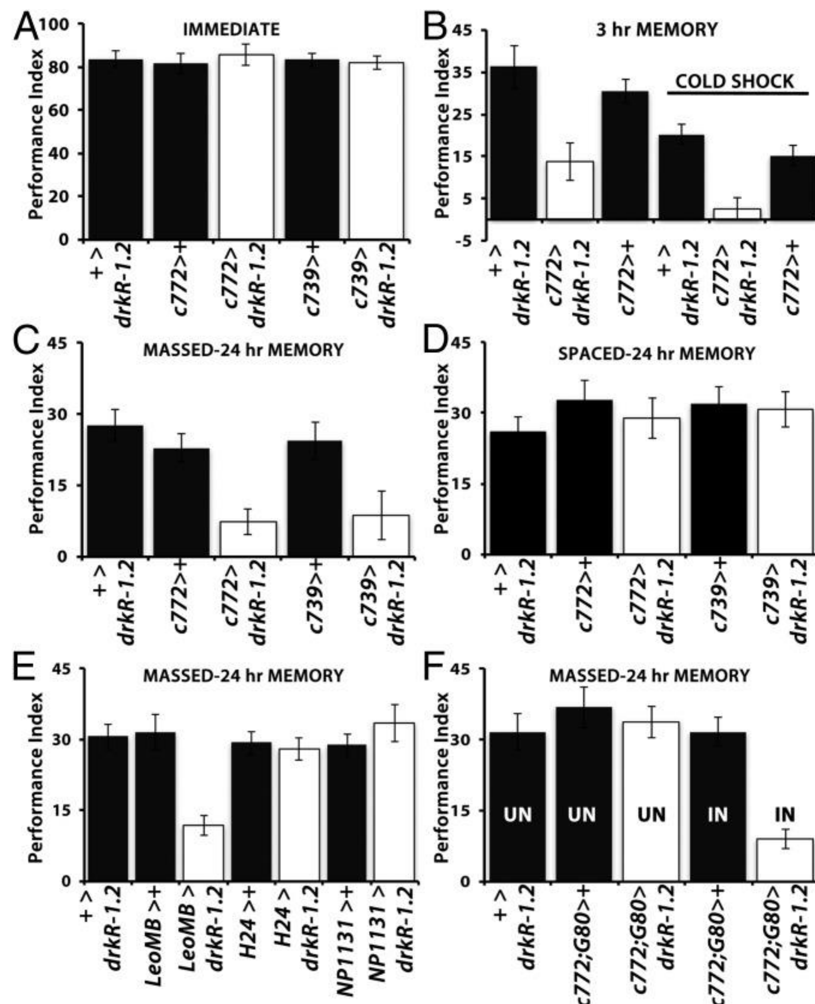

Fig. 2. Drk is required within the MB α/β lobes for normal ARM. The mean performance ± SEM is shown at the indicated times postconditioning. Controls are represented by black bars and animals with attenuated Drk via *UASdrkR1.2* (*drkR1.2*) expression by open bars. + denotes the presence of a w^{1118}-derived chromosome indicating either the lack of driver (+>) and heterozygosity for the transgene or heterozygosity for the Gal4 driver (i.e., c772>+). Potential differences were assessed with ANOVA and least square means contrast analyses. (*A*) Performance immediately after 12 US/CS conditioning of controls and animals with abrogated Drk in the indicated MB neurons. Loss of Drk in the MBs did not affect the performance relative to that of controls ($P = 0.634$, $n > 9$ per genotype). (*B*) Three-hour memory without and with cold shock of animals with abrogated Drk in 772 Gal4-marked neurons. The performance of *drkR1.2*-expressing animals was significantly different from that of both (+> *drkR1.*2 and c772>+) controls without ($P < 0.0011$, $n > 7$) and with cold shock ($P < 0.002$, $n > 8$). (*C*) Abrogation of Drk within α/β neurons precipitated deficits in 24-h ARM either under the c772 Gal4 ($P < 0.003$, $n > 6$) or the c739 Gal4 driver ($P < 0.001$, $n > 8$). (*D*) LTM induced with spaced conditioning was not affected in animals with abrogated Drk in α/β lobes relative to controls ($P = 0.82$, $n > 7$). (*E*) Abrogation of Drk in all MB neurons under LeoGal4 resulted in a highly significant ($P < 0.001$, $n > 14$) deficit in 24-h ARM, while abrogation in the AL and γ-neurons (H24) Gal4 or γ-neurons (NP1131) only,

did not yield significant differences from their respective controls (P = 0.894 and P = 0.900, respectively; n > 10). (*F*) Adult-specific abrogation of Drk in the MBs. Experimental flies (c772> *drkR1.*2) held under $Gal80^{ts}$-mediated suppression of Drk abrogation (UN) did not exhibit behavioral deficits compared with controls (P = 0.899, n > 9), whereas transgene induction (IN) precipitated deficient 24-h ARM (P < 0.007, n = 7).

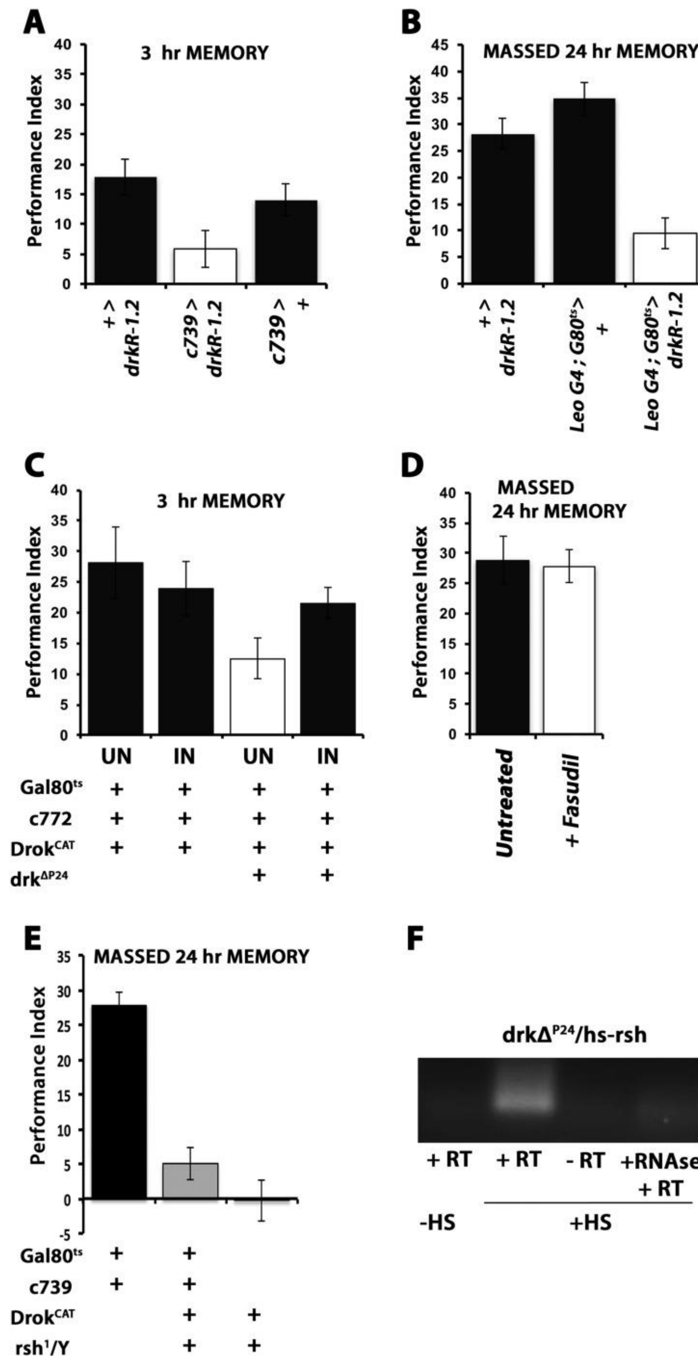

Fig. S1. (*A*) Cold shock-dependent 3-h memory is deficient upon drk abrogation within c739-marked MB neurons. The mean performance ± SEM is shown 3 h postconditioning for the indicated genotypes. All genotypes were given a 2-min cold shock 2 h posttraining. + denotes the presence of a w^{1118}-

derived chromosome indicating either the lack of driver (+>) and heterozygosity for the transgene or, conversely, heterozygosity for the Gal4 driver. The performance of *drkR1.2*-expressing animals was significantly different from that of both (+> *drkR1.*2 and c739>+) controls after cold shock ($P < 0.001$, $n > 6$). (*B*) Adult-specific abrogation of Drk throughout the MBs precipitated deficits in 24-h ARM. Expression of the Drk-attenuating transgene under LeoGal4 was suppressed under Gal80^{ts}-mediated suppression by raising these flies at 18 °C along with the two controls. The abrogating transgene was induced by incubation of experimental flies and controls at 30 °C for 24 h. The performance of LeoGal4:Gal80^{ts} > drkR-1.2 animals was significantly different from both controls ($P < 0.001$, $n > 6$). (*C*) Conditional expression (IN-induced) of $Drok^{CAT}$ in c772-marked MB neurons rescues the 3-h memory deficit of $drk^{\Delta P24}$ heterozygotes after cold shock, whereas the significant ARM deficit ($P < 0.002$, $n = 6$) of $drk\Delta^{P24}$ heterozygotes is apparent (open bar). (*D*) The Drok activity attenuator Fasudil was administered to control w^{1118} animals within 30–45 min after five cycles of massed training but did not yield detectable defects 24 h later (ANOVA $P = 0.5243$, $n > 5$). (*E*) Twenty-four–hour ARM of control (black bars) males was significantly different from that of rsh^1/Y; *c739 Gal4,G80*^{ts}*/+* ($P < 0.0001$) and from that of rsh^1/Y; *c739 Gal4,G80*^{ts}*/UASDrok*^{CAT} animals ($P < 0.0001$). In addition, the performance of rsh^1/Y; *c739 Gal4,G80*^{ts}*/+* was not significantly different from that of flies of the same genotype but expressing Drok^{CAT}($P = 0.1382$, $n > 11$). (*F*) Representative agarose electrophoresis of PCR products following Reverse Transcription (+RT) of RNAs isolated from heads of $drk\Delta^{P24}$*/hs rsh* animals after a 30-min heat shock (+HS). The PCR product was absent if RT was performed on RNA isolated from such animals not heat shocked to induce the transgene (−HS) or if RNA isolated from heat-shocked animals was treated with RNase A before RT (+RNase, +RT).

Furthermore, because the expression pattern of c772-Gal4 and c739-Gal4 overlaps within the α/β MB lobes (21), these results strongly indicate that Drk is required specifically within these neurons for normal ARM. Because both of these Gal4 drivers are also expressed in the AL, which has been implicated in ARM (22), we used H24-Gal4 and NP1131-Gal4 to specifically abrogate Drk in the AL and γ lobes. We utilized Leo-Gal4, which is an MB-specific driver expressed in all neurons (23), as the positive control. Clearly, whereas Drk abrogation within γ neurons and the AL did not precipitate deficits, ARM was compromised under Leo-Gal4 (Fig. 2*E*), which is also expressed in the α′β′ neurons. However, as Drk is not expressed in α′β′ neurons, RNAi-mediated Drk abrogation in all MB neurons (Fig. 2*E*) appeared quantitatively similar to that limited to αβ lobes (Fig. 2*C*). Therefore, Drk is required for ARM

within αβ neurons and appears to function independently of the octopaminergic signaling to the α’ß’ lobes.

Finally, to establish that deficient ARM does not result from developmental defects in the αβ lobes due to reduced Drk, we used TARGET (24, 25) to conditionally express *drkR-1.2* in adult MBs under c772-Gal4 (Fig. 2*F*) or Leo-Gal4 (Fig. S1*B*). Deficient 24-h ARM was observed only upon adult-specific induction of the Drk abrogating transgene, indicating that the ARM deficit does not originate from and underlie developmental deficits within MBs. Hence, Drk is specifically required within the postdevelopmental αβ MB neurons for normal ARM.

Rho Kinase Activation Restores ARM in *drk* Mutants.

Since Drk does not signal to Raf1 for its function in memory (17), we searched for potential involvement of alternative signaling cascades. We were guided by its vertebrate ortholog GRB2, which engages a Rho kinase to maintain fear memory in rodents (26) to investigate the possibility that a similar pathway is involved in Drk-mediated ARM. *Drosophila*possesses a single Rho kinase ortholog, Drok, which like Raf is a serine/threonine kinase activated by the GTPase Rho1 (27).

If Drk signals engage Drok to mediate normal ARM, then a transgenic constitutively active form of the kinase, $Drok^{CAT}$ (28), may rescue the ARM deficit of $drk^{\Delta P24}$/+. To avoid complications because of the reported aberrant MB development precipitated by continuous $Drok^{CAT}$ expression (27–29), we expressed it conditionally and exclusively within adult $drk^{\Delta P24}$/+ αβ neurons (25). Significantly, $Drok^{CAT}$ expression in the αβ neurons fully restored 24-h ARM in *drk* heterozygotes (Fig. 3*A*). Full rescue was also achieved with an independent $Drok^{CAT}$ transgene on a different chromosome (Figs. 3*B* and and4*C*).4*C*). In contrast, expression of the catalytically inactive transgenic protein $Drok^{CAT\text{-}KG}$ (28) in the same MB neurons did not rescue the deficient ARM of $drk^{\Delta P24}$ heterozygotes (Fig. 3*C*). Conditional expression of either $Drok^{CAT}$ or $Drok^{CAT\text{-}KG}$ in wild-type adult MBs did not suppress or enhance ARM (Fig. 3 *A*–*C*), indicating that rescue did not result because of nonspecific effects of transgene overexpression. In addition, acute $Drok^{CAT}$ expression in αβ neurons under the TARGET system also restored 3-h cold shock-dependent ARM to levels exhibited by control

animals (Fig. 3*D*), verifying that rescue was adult MB-specific. Similar results were obtained with the c772 driver (Fig. S1*C*).

Collectively, the results support a genetic Drk–Drok interaction within αβ neurons acutely required for normal ARM revealed after massed training or after cold shock.

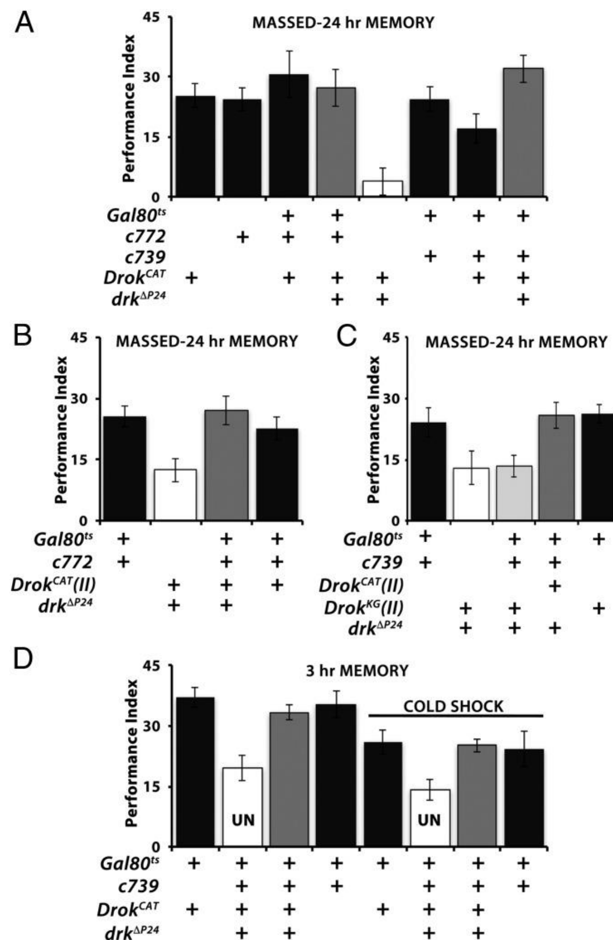

Fig. 3. Catalytically active Drok rescues the ARM deficits of *drk* mutant heterozygotes. Mean performances ± SEM are shown. Controls are represented by black bars, mutants by open bars, and mutant animals with potential behavioral rescue by gray bars. Following ANOVA, potential differences were assessed for significance with planned comparisons (least square means contrast analyses) as necessary. (*A*) Adult-specific expression of the constitutively active $UASDrok^{CAT}$ within α/β neurons rescued (gray bars) the ARM deficit of $drk^{\Delta P24}/+$ flies (open bar). The entire data shown are from animals induced in parallel. Whereas $drk^{\Delta P24}/+$; $Drok^{CAT}/Gal80^{ts}$ flies were significantly ($P < 0.0001$) deficient in ARM compared with any of the controls (black bars), $drk^{\Delta P24}/c772$; $Drok^{CAT}/Gal80^{ts}$ were not ($P = 0.9967$). Similarly, $drk^{\Delta P24}/c739$; $Drok^{CAT}/Gal80^{ts}$ flies performed equally well with controls ($P =$

0.688) but significantly different ($P < 0.0001$) from *drk*$^{\Delta P24}$*/+*; *Drok*^{CAT}*/Gal80*^{ts} animals. $n > 7$ for all. (*B*) Rescue of the ARM deficit of *drk*$^{\Delta P24}$*/+*; *Drok*^{CAT}*/Gal80*^{ts} animals (open bar) by induction of an independent *Drok*^{CAT} transgene on chromosome II (gray bar). Whereas the performance of *drk*$^{\Delta P24}$, *Drok*^{CAT}*/c772*; *+/Gal80*^{ts} was not different from that of controls ($P = 0.829$), it was significantly different from that of *drk*$^{\Delta P24}$, *Drok*^{CAT}*/+*; *+/Gal80*^{ts} ($P < 0.0007$). (*C*) The catalytically inactive *Drok*^{KG} transgene does not rescue (light gray bar) the ARM deficit of *drk*$^{\Delta P24}$, *Drok*^{KG}*/+*; *+/Gal80*^{ts} ($P = 0.774$) under the c739 driver, which recues ($P = 0.731$) the *drk*$^{\Delta P24}$ deficit with the *Drok*^{CAT} transgene (*drk*$^{\Delta P24}$, *Drok*^{CAT}*/c739*; *+/Gal80*^{ts}). $n > 7$. (*D*) Conditional expression of *Drok*^{CAT} in α/β neurons rescues (gray bars) the 3-h memory deficit of *drk*$^{\Delta P24}$ heterozygotes after cold shock. Uninduced (UN) *drk*$^{\Delta P24}$*/c739*; *Drok*^{CAT}*/Gal80*^{ts} animals were used as negative controls (open bars), and they performed significantly different from controls ($P < 0.0002$), while after induction they did not (gray bars, $P = 0.849$). Similarly, ARM after cold shock was significantly different in UN (open bars) *drk*$^{\Delta P24}$*/c739*; *Drok*^{CAT}*/Gal80*^{ts} from controls ($^{+/+}$; *Drok*^{CAT}*/Gal80*^{ts}, or *+/c739*; *+/Gal80*^{ts}; $P < 0.003$ for both) and from the same flies after transgene induction (gray bar; $P < 0.0015$). $n > 7$.

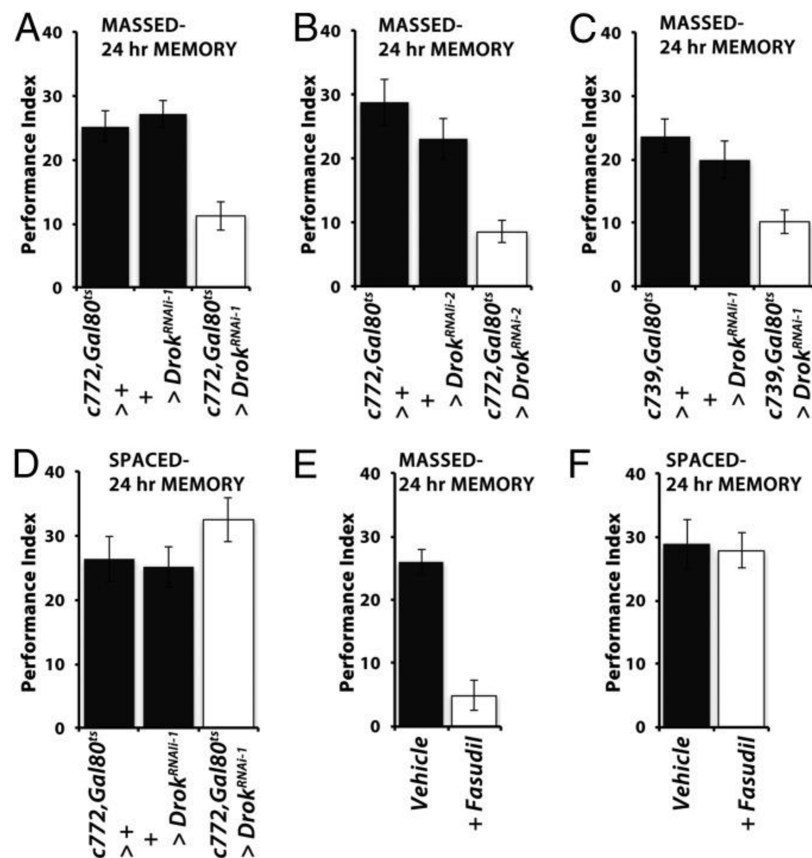

Fig. 4. Drok activity is required for ARM. Mean performances ± SEM are shown. Controls are represented by black bars and Drok attenuated flies by open bars. Following ANOVA, differences were

assessed for significance with planned comparisons (least square means contrast analyses) as necessary. (*A*) Adult-specific attenuation of Drok in c772Gal4-marked neurons precipitated a significant deficit in 24-h ARM after massed conditioning relative to both controls ($P < 0.0001$, $n > 10$). (*B*) Adult-specific attenuation of Drok in c772Gal4-marked neurons with an independent abrogating transgene (*UASDrok^{RNAi-2}*) precipitated a significant deficit in 24-h ARM relative to both controls ($P < 0.0002$, $n > 10$). (*C*) A similar ARM deficit was observed upon Drok abrogation under the c739Gal4 driver ($P < 0.0007$, $n > 12$). (*D*) Adult-specific Drok attenuation in αβ neurons did not affect 24-h LTM ($P = 0.2631$, $n > 8$). (*E*) Adult-specific Drok activity attenuation in control *w^{1118}*animals with Fasudil precipitated a highly significant 24-h ARM deficit (ANOVA $P < 0.0001$, $n = 12$ each). (*F*) Spaced conditioning-induced LTM was not affected by Fasudil treatment in *w^{1118}* animals (ANOVA $P = 0.7541$, $n > 10$).

Rho Kinase Activity Is Required for Normal ARM.

Drok activity can be specifically required for Drk-mediated ARM, or it could function in both forms of consolidated memory. To differentiate between these possibilities, we conditionally abrogated the kinase within adult αβ neurons by *Drok RNAi*-mediating transgenes. Adult-specific attenuation of Drok within 772 Gal4-marked neurons did not affect 3-min memory (71.6 ± 2.46 for c772 Gal4, Gal80^{ts}>+; 74.2 ± 1.16 for *UAS-Drok^{RNAi-1}*>+; and 77.8 ± 3.08 for c772-Gal4, Gal80^{ts} > *UAS-Drok^{RNAi-1}*; ANOVA $P = 0.2676$). However, it yielded significant deficits in ARM (Fig. 4 *A* and *B*) with two distinct RNAi-mediating transgenes. A similar deficit was also observed upon Drok abrogation within 739 Gal4-marked neurons (Fig. 4*C*). In contrast, LTM was not affected by Drok attenuation therein (Fig. 4*D*), strongly suggesting that the kinase plays a role specifically on the ARM form of memory.

In addition to its kinase activity, Drok contains a Rho GTPase binding site and a Pleckstrin domain (30), suggesting multiple ways that the protein could be involved in ARM. Because only constitutively active Drok rescued the deficit of *drk* mutants (Fig. 3), we hypothesized that ARM requires its kinase activity and not its Pleckstrin or GTPase domains. To differentiate between these possibilities and further validate the results with the kinase-dead transgene, we sought to inhibit the kinase activity without altering the levels of the protein itself and hence the dosage of these conserved domains. Because Rok family proteins are kinases of medical importance implicated in cancer, pulmonary hypertension, and

neurodegenerative diseases (31), specific inhibitors are commercially available. We opted for the potent selective Rok inhibitor Fasudil hydrochloride (HA-1077), because it had been used on *Drosophila* before without apparent ill effects (32). Adult 2–3-d-old w^{1118} flies were fed the inhibitor (200 μM) for 16 h before conditioning. As illustrated in Fig. 4*E*, flies treated with the inhibitor presented little 24-h ARM after massed conditioning, but the drug did not affect LTM. Exposing the flies to the inhibitor only after conditioning did not affect ARM (Fig. S1*D*). Collectively then, the kinase activity of Drok is required for Drk-mediated ARM formation within αβ MB neurons.

Rho Kinase Activation Does Not Restore ARM in *rsh* Mutants.

The requirement of Drk within αβ neurons for normal ARM is in agreement with the preferential expression and functional requirement of Rsh, which is specifically implicated in this form of consolidated olfactory memory (5) within these neurons (8). The exact function of Rsh is still unclear but appears to possess a GTPase-activating domain (Flybase Curators, 2008; flybase.org/reports/FBgn0265597.html). ARM is specifically impaired in *rsh*mutants (5, 18) and appears to be independent of octopaminergic (9) inputs to the MBs for ARM formation. Therefore, we asked whether Drk and Rsh act in the same molecular cascade by investigating whether they interact genetically. Hence, we tested 24-h ARM after massed training in males hemizygous for rsh^1 and heterozygous for $drk^{\Delta P24}$ (rsh^1; $drk^{\Delta P24}$/+) and in doubly heterozygous rsh^1/+; $drk^{\Delta P24}$/+females. Reducing Drk levels by 50% did not alter the ARM deficit of rsh^1 males (Fig. 5*A*), while ARM in the doubly heterozygous females was similar to those of $drk^{\Delta P24}$/+ females (Fig. 5*B*). Although the low performance levels of male flies potentially hindered resolution, the results suggest either that Drk is upstream of Rsh or the two proteins act in different molecular ARM-mediating pathways.

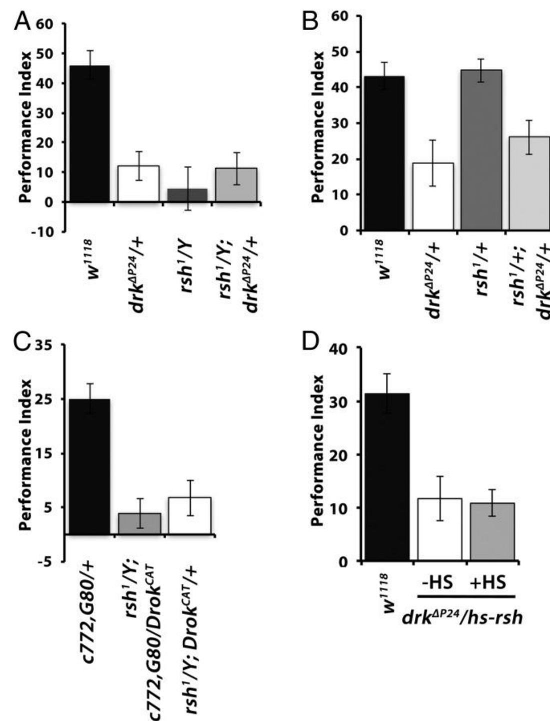

Fig. 5. Drk-mediated signaling to Drok for ARM does not engage Rsh. Mean performances ± SEM are shown for *A*–*D*. Following ANOVA, differences were assessed for significance least square means contrast analyses as necessary. (*A*) Twenty-four–hour ARM of control males (black bars) was significantly different ($P < 0.0001$) from that of *drk*$^{\Delta P24}$*/+* (open bars), *rsh*^{1} hemizygous males (dark gray bars), and *rsh*^{1}; *drk*$^{\Delta P24}$*/+* (light gray bars). However, the performance of *rsh*^{1} hemizygous males was not significantly different from that of *drk*$^{\Delta P24}$*/+* (P = 03428) or *rsh*^{1}; *drk*$^{\Delta P24}$*/+* ($P = 0.4023$). (*B*) Twenty-four–hour ARM of control females (black bars) was significantly different ($P < 0.0006$) from that of *drk*$^{\Delta P24}$*/+* and *rsh*^{1}/+; *drk*$^{\Delta P24}$*/+* ($P < 0.008$) but not from *rsh*^{1}/+ females ($P = 0.8318$, $n > 10$). (*C*) Drok^{CAT} expression in their MBs does not rescue the 24-h ARM deficit of *rsh*^{1} mutants; 24-h ARM of control (black bars) males was significantly different from that of *rsh*^{1}/Y; *c772 Gal4,G80*^{ts}*/+* ($P < 0.0001$) but also from *rsh*^{1}/Y; *c772-Gal4,G80*^{ts}*/UASDrok*^{CAT} flies ($P < 0.0001$). In addition, the performance of *rsh*^{1}/Y; *c772 Gal4,G80*^{ts}*/+* was not different from that of flies of the same genotype but was with those expressing Drok^{CAT} ($P = 0.4944$, $n > 9$). (*D*) Twenty-four–hour ARM for *drk*$^{\Delta P24}$heterozygotes carrying a conditional heat shock-inducible *rsh* transgene. Induction of the transgene (+HS) did not improve ($P = 0.8527$) their performance over that of their siblings without induction (−HS), which remained significantly different from that of (not heat shocked) controls ($P < 0.001$, $n > 9$).

If Drk, Drok, and Rsh were in the same cascade, Drok activation could reverse the deficient ARM of *rsh*^{1}males. This possibility is supported by the putative GTPase activator

function of Rsh, which could be involved in Drok activation. Therefore, we introduced the $Drok^{CAT}$ transgene that rescued the $drk^{\Delta P24}/+$ARM deficit into rsh^{1} mutant flies. However, conditional expression of $Drok^{CAT}$ within the MBs of adult rsh^{1} males under c772Gal4 or c739Gal4 failed to rescue their deficient ARM (Fig. 5*C* and Fig. S1*E*). The results suggest that Rsh does not act upstream of Drk or between Drk and Drok in a single signaling pathway. To test the alternative possibility that Rsh is downstream of Drk and Drok, we attempted to rescue the ARM deficit of $drk^{\Delta P24}/+$ flies with an inducible *rsh* transgene. Although the transgene was shown to rescue the deficient ARM of rsh^{1} mutants (5) and was highly induced by a brief heat shock (Fig. S1*F*), it was unable to reverse the deficit of $drk^{\Delta P24}$ heterozygotes (Fig. 5*D*). These data strongly suggest that Drk and Rsh operate in distinct, potentially parallel molecular pathways serving ARM within αβ neurons.

Because Drok-mediated signals likely engage actin and the actin cytoskeleton (26, 29, 31), we investigated whether changes in actin polymerization could be detected in animals with genetic or pharmacologically-induced attenuated ARM. We assessed filamentous actin (F-actin) levels in *drk* mutants and in control animals treated with the Drok inhibitor Fasudil. Brains were dissected from animals with strongly reduced Drk levels in the MBs [$drk^{\Delta P24}/LeoGal4$; *drkR-1.2/+* (17)], stained with phalloidin, and quantification of the signal within the calyces revealed a significant reduction in filamentous actin compared with controls (Fig. 6*A*). A similarly highly significant reduction in filamentous actin levels was observed upon treatment with Fasudil before dissection (Fig. 6*B*), a treatment that nearly abolishes ARM (Fig. 4*E*), which collectively with the results from the mutants strongly implicate actin cytoskeleton dynamics in the process.

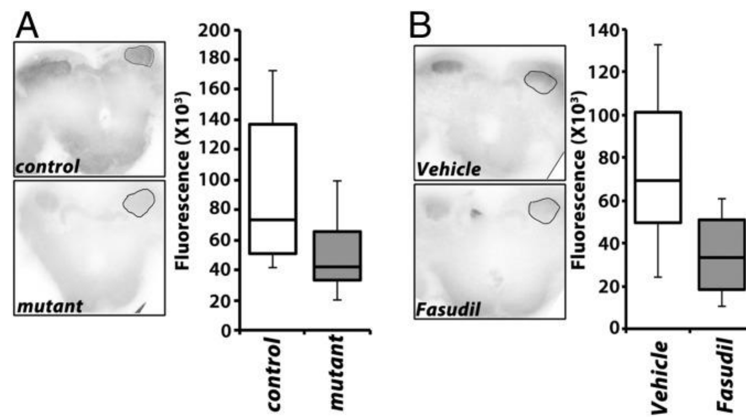

Fig. 6. Decreased filamentous actin in the MBs of *drk* mutants and upon acute pharmacological inhibition of Drok. (*A*) Representative confocal images of whole-mount brains at the level of the calyces used to quantify fluorescence from the marked regions of interest (ROI) after rhodamine-conjugated phalloidin staining. Control indicates *LeoGal4/+*, while mutant indicates the genotype *drk*$^{\Delta P24}$*/LeoGal4*; *drkR-1.2/+*. Quantification (*Right*) of multiple experiments revealed significant differences in fluorescence in the calyces control and mutant animals (Wilkoxon test, χ^2 = 12.4910, $P < 0.0004$, $n > 20$). (*B*) Representative confocal images of whole-mount brains at the level of the calyces used to quantify fluorescence from the marked ROIs after rhodamine-conjugated phalloidin staining of vehicle and Fasudil-treated w^{1118} animals. Quantification (*Right*) revealed significant differences in fluorescence in the calyces control and mutant animals (Wilkoxon test, χ^2 = 22.6722, $P < 0.0001$, $n > 28$).

Discussion

Elucidation of the molecular pathways specific to ARM is essential to understanding this translation-independent consolidated memory form, how it differs from LTM, and what underlies their apparent inverse relationship (2, 4). Our evidence indicates that the small adaptor protein Drk/Grb2 is essential for ARM in a Rsh-independent manner. Genetically, Drk signals to Drok, but it is unlikely that they also interact physically. Drok lacks significant polyproline stretches targeted by the SH3 domain of Drk, and none of its regulatory phosphorylations are on Tyrosines residues, which could engage the SH2 domain (30).

Drk and Rsh are both present within MB neurons (5, 8, 17), but our evidence indicates that Drk/Drok-dependent ARM does not require Rsh. This is consistent with two signaling pathways serving ARM within αβ neurons, where Rsh expression overlaps that of Drk. These two cascades may be independent or converging downstream of Drok in a coincidence

detection manner, but we favor the former based on the following: Serotonergic signals required for ARM formation in αβ neurons may engage Rsh (8), but not Drk/Drok required therein for structural plasticity as suggested below. Serotonergic signaling in Drk/Drok-mediated plasticity is currently under investigation. Another possibility consistent with independent cascades is that Rsh may only be involved in ARM retrieval within αβ neurons (16), while Drk/Drok in its formation or maintenance. In this model, *rsh* mutations will occlude rescue attempts with activated Drok, and Rsh overexpression will not rescue the *drk/+* ARM deficits, as we describe (Fig. 5).

The ubiquitous Serine/Threonine kinase Drok is activated by the Rho1 GTPase, whose activation in turn requires Rho GTPase Activating Proteins (Rho-GAPs), and Drk physically interacts with multiple Rho-GAPs (33), likely bringing it in proximity with Drok. Finally, the Pleckstin homology domain of Drok indicates its membrane association and potential interaction with the actin cytoskeleton. Drk attenuation or inhibition of Drok activity precipitated reduction in F-actin and deficient ARM, suggesting that the deficit results from reduced ability to establish or maintain structural changes. In accord with its known functions (31), we propose that Drok mediates polymerization or stabilization, possibly of cortical actin filaments. We further propose that this activity-dependent cytoskeletal remodeling alters synaptic strength or properties, which underlies ARM formation, a hypothesis currently under investigation.

The structural plasticity model for ARM we propose being dependent on Drk/Drok-mediated actin dynamics is supported by known functions of the Rho1/Rho-GAP/Drok module. Rho1, Rho-GAPp190, and Drok activities have been shown to transduce Integrin-originating signals to the cytoskeleton essential for axonal growth of MB α lobes during development (27). Interestingly, Integrins are known to regulate RTK signaling by recruiting adaptors such as Drk to the membrane (34). Upon RTK activation, Integrins and their associated signaling molecules colocalize at focal adhesion sites and signal to the cytoskeleton, mediating its remodeling (34), likely via the Rho1/Rho-GAP/Drok module. Rok/Rho-GAPp190 and Integrins are involved in neuroplasticity because they are essential for fear memory in the rat (26) and for fly olfactory learning (35), respectively. Furthermore, multiple reports detail neurotransmitter-mediated structural synaptic plasticity in adult vertebrate neurons via Rho and Ras GTPases (summarized in ref. 36). In addition, the reported

role of dopamine and serotonin in ARM (8, 10, 11) is consistent with the proposed structural plasticity model, as both neurotransmitters have been implicated in spine dynamics in both insects (37) and vertebrates (38, 39).

Collectively then, our results and the biochemical and interactome evidence detailed above lead us to propose that a molecular hallmark of ARM formation is activity-dependent localized structural and functional changes in the neuronal cytoskeleton that alter synaptic strength or properties, stable enough to last at least 24 h. Testing this hypothesis will provide essential insights into understanding not only the nature and function of the ARM form of consolidated memory and its relationship to LTM in flies but also its analogous process in vertebrates.

Materials and Methods

Drosophila culture, strains, genetics, and conditioning have been described before (17) and along with strains used are detailed in *SI Materials and Methods*. Fasudil (HA-1077; Sigma-Aldrich) dissolved in water was used to abrogate ARM. Confocal microscopy was performed using standard methods. Untransformed (raw) data were analyzed parametrically with the JMP 7.1 statistical software package (SAS Institute Inc.) as before (17). Detailed methods are presented in *SI Materials and Methods*.

SI Materials and Methods

Drosophila Culture, Strains, and Genetics.

Drosophila were raised and crosses set up in standard wheat–flour–sugar food supplemented with soy flour and $CaCl_2$ (17) and cultured at 25 °C and 50% humidity with a 12-h light/dark cycle. The $drk^{\Delta P24}$/CyO and drk^{E0A}/CyO as well as the rsh^{1} mutant have been described previously (5, 17). Transgenic lines used in this study were as follows: UAS-drk^{RNAi}*1.2*(*drkR-1.2*) (17), UAS-Drok-CAT, and UAS-Drok-CAT-KG (28). The Gal4 driver lines c772-Gal4, c739-Gal4, NP1131-Gal4, and H24-Gal4 and one stock carrying a Drok RNAi encoding transgene (#28797) were obtained from the Bloomington Stock Center, whereas an independent Drok RNAi-encoding transgene (# 9774R-3) was obtained from NIG-FLY. Leo-Gal4 has been described

previously (23). All transgenic lines were normalized to the w^{1118} genetic background with repeated backcrosses for at least six generations. The UAS-Drok-CAT and UAS-Drok-CAT-KG transgenes were introduced into the $drk^{\Delta P24}$mutant background by standard genetic crosses.

For behavioral experiments, non–balancer-bearing progeny from crosses of CyO-balanced *drk* males to w^{1118} females were used. To abrogate DRK levels, c772-Gal4, NP1131-Gal4, and H24-Gal4 males were crossed en masse to *drkR-1.2* virgin females. Progeny of the cross between w^{1118} females and *drkR-1.2*males were used as controls. Flies were collected, placed at 29 °C for 24 h for maximal transgene induction, and allowed 1 h recovery at 25 °C before training. For conditional abrogation of DRK in the adult MBs and rescue experiments with *UAS-Drok*^{CAT} transgenes, the TARGET system was used (25). To that end, c772-Gal4;Tub-Gal80^{ts} and c739-Gal4,Tub-Gal80^{ts} and control w^{1118} females were crossed en masse to $drk^{\Delta P24}$/CyO;*UAS-Drok*^{CAT} or $drk^{\Delta P24}$, *UAS-Drok*^{CAT}*/CyO* or *UAS-Drok*^{CAT} males. Progeny were reared at 18 °C, and the resultant nonbalanced 4–6-d-old flies were used for behavioral experiments. For conditional Drok abrogation, UAS-Drok RNAi encoding transgenes were crossed in the same manner to these drivers. To maximally induce the transgenes, flies were moved from 18 °C to 31 °C for 30–48 h, followed with a transfer to fresh vials and recovery at 25 °C for 1 h before conditioning. For the assessment of genetic interaction of *drk* and *rsh*, rsh^1 homozygous females were crossed to $drk^{\Delta P24}$*/CyO* males, and nonbalanced rsh^1 hemizygous males or *rsh1/+* heterozygous females for *rsh* were used. Male rsh^1; hs-rsh flies (9) were obtained from S. Waddell, Oxford University, Oxford, and were crossed with $drk^{\Delta P24}$/CyO females to obtain non–balancer-bearing males and rsh^1*/+* females for testing. The transgene was induced by incubation at 37 °C for 40 min, and such flies were tested behaviorally 40 min postinduction. Induction of the transgene was monitored by reverse transcription followed by PCR (RT-PCR) as described previously (17).

Drug Treatment.

Two- to 3-d-old adult w^{1118} flies were introduced in groups of 60–80 individuals to empty vials that contained ∼1 mL of Torula yeast in water (vehicle) or Fasudil (HA-1077; Sigma-Aldrich) dissolved in water at 200 μM. Flies were allowed to feed for 16 h and ∼30–40 min before conditioning were transferred to fresh normal media. Lower Fasudil concentrations were tested in pilot experiments but were ineffective.

Behavioral Analyses and Conditioning.

Behavioral experiments were performed under dim red light at 23–25 °C and 70–78% humidity as described before (17). All animals used were 3–6 d old and collected under light CO_2 anesthesia 1 d before testing and kept in food vials in groups of 50–70 at 23–25 °C or 18 °C as appropriate for strains with $Gal80^{ts}$ temporal restriction of transgene expression. In all drug experiments, the experimenter was blind to the treatment. One cycle of olfactory conditioning training consisted of 12 CS/US pairings as described previously (10). For assessment of 3-h ARM, trained flies were transferred back to their vials at 25 °C, and 2 h posttraining, they were cold-shocked in prechilled glass vials on ice for 2 min. Complete anesthesia judged by immobility of the flies in the bottom of the glass tube was ascertained. After 2 min, flies were transferred back to vials at 25 °C and maintained until testing. They recovered from anesthesia within 20–30 s.

To assess 24-h memory after spaced training, flies were subjected to five cycles of conditioning using 12 CS/US pairing as detailed before (40). After training, the flies were transferred back to food vials and kept at 18 °C in a dark box. One hour before testing, they were transferred to 25 °C in the behavior room. For massed training, the same procedure was followed, except that flies were subjected to five consecutive cycles of 12 CS/US.

Phalloidin Staining and Confocal Microscopy.

Adult brains were dissected in cold PBS, fixed in 4% paraformaldehyde for 15 min, and permeabilized with 0.3% Triton X-100 in PBS. The brains were incubated with Rhodamine-Phalloidin (#R415; Invitrogen Molecular Probes) for 45 min at 25 °C to stain for F-actin. Confocal laser microscopy was performed using the Leica TCS SP8 system, and images at 40× magnification were obtained. Before acquisition, laser parameters were adjusted to obtain nonsaturating conditions, and samples were processed simultaneously using identical confocal acquisition parameters (laserpower, gain, and pinhole settings), as previously described (41). Serial optical sections of 0.5 μm thickness were obtained from the fixed whole-mount adult brain samples. Quantification of fluorescent staining in the MB calyces was obtained from one z-stack of each calyx per brain, and each stack was taken approximately at the same depth. Image processing was performed with ImageJ software. To obtain flies with maximally reduced Drk levels, LeoGal4 virgins were crossed en masse with *drk*$^{\Delta P24}$*/CyO*; *drkR*-

1.2 males, which in addition to the deletion allele bear a strong RNAi transgene for *drk* (5). Progeny raised at 25 °C were kept for 14–16 h at 30 °C before dissection. Fasudil was administered as described above. Multiple biological and technical replicates were performed (see sample numbers in the figure legends).

Data Analysis.

Untransformed (raw) data were analyzed parametrically with the JMP 7.1 statistical software package (SAS Institute Inc.) as described before (17). Following initial ANOVA, planned multiple comparisons were performed, using $\alpha = 0.05$. The level of significance was adjusted for the experiment-wise error rate using Bonferroni correction. Detailed results of planned comparisons are mentioned in the figure legends. Data are shown as mean ± SEM. Imaging data were analyzed nonparametrically using Wilcoxon/Kruskal Wallis Rank Sums tests.

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Publications