Simultaneous activation of parallel sensory pathways promotes a grooming sequence in *Drosophila*.

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Abstract

A central model that describes how behavioral sequences are produced features a neural architecture that readies different movements simultaneously, and a mechanism where prioritized suppression between the movements determines their sequential performance. We previously described a model whereby suppression drives a *Drosophila* grooming sequence that is induced by simultaneous activation of different sensory pathways that each elicit a distinct movement (Seeds et al. 2014). Here, we confirm this model using transgenic expression to identify and optogenetically activate sensory neurons that elicit specific grooming movements. Simultaneous activation of different sensory pathways elicits a grooming sequence that resembles the naturally induced sequence. Moreover, the sequence proceeds after the sensory excitation is terminated, indicating that a persistent trace of this excitation induces the next grooming movement once the previous one is performed. This reveals a mechanism whereby parallel sensory inputs can be integrated and stored to elicit a delayed and sequential grooming response.

Introduction

A major question about nervous system function is how different movements are assembled to form behavioral sequences. One of the primary models of sequential behavior is reminiscent of how animals select among competing behavioral choices. Behavioral competition arises in situations where different mutually exclusive behaviors are appropriate, but they must be performed one at a time (Houghton and Hartley 1995; Redgrave, Prescott, and Gurney 1999). These conflicts can be resolved through the suppression of all but the highest priority behavior, as mollusks do to suppress their mating behavior while feeding (Davis 1979; Kupfermann and Weiss 2001; Kristan 2008). In the case of a behavioral sequence, it is proposed that the different movements to be performed are similarly readied in parallel and in competition for output, and a suppression hierarchy determines their priority order of execution (Lashley 1951; Houghton and Hartley 1995; Bullock 2004). Completion of the highest priority movement lifts suppression on movements of lower priority that are subsequently performed according to a new round of competition and suppression. This *parallel model* could drive behaviors across a range of complexity, from the sequential typing of letters on a keyboard in humans to the selection of which behavior to perform first in mollusks (Houghton and Hartley 1995). Thus, the identification of examples of simple parallel neural architectures that drive a prioritized selection of movements may inform a broad spectrum of sequential behaviors (Kristan 2014; Jovanic et al. 2016).

A *Drosophila melanogaster* grooming sequence provides one example of how conflicting stimuli can induce movement competition that is resolved through a suppression hierarchy. Coating the body of a fly with dust is thought to stimulate competition among different grooming movements that are each responsible for cleaning a particular body part (Phillis et al. 1993; Seeds et al. 2014). We previously presented evidence that the body grooming order is determined through a mechanism where earlier movements suppress later ones (Seeds et al.

2014). For example, removal of dust from the eyes occurs first because eye grooming suppresses cleaning of the other body parts. From a suppression hierarchy among the different grooming movements emerges a sequence that proceeds in the order: eyes > antennae > abdomen > wings > notum. We further proposed a computational model to describe this sequence that features parallel activation of the different grooming movements by dust to induce competition, and hierarchical suppression among the movements to determine their selection order (Seeds et al. 2014). The parallel activation of the movements was proposed based on evidence that stimulation to each body part induces site-directed grooming responses (Vandervorst and Ghysen 1980; Corfas and Dudai 1989; Seeds et al. 2014; Hampel et al. 2015). Thus, the simultaneous, or parallel stimulation of sensory neurons by dust would cause different grooming movements to compete for output because only one can be performed at a time. However, it was not confirmed that simultaneous activation of sensory neurons across the body indeed elicits the same prioritized grooming response that we observed using a dust stimulus.

Here, we reveal a neural basis for parallel activation of sensory inputs for a sequential behavior by identifying sensory neurons that stimulate different grooming movements, and by testing the hypothesis that activation of these neurons in parallel elicits a prioritized grooming response. We identify transgenic expression tools for visualizing and optogenetically activating sensory neurons on the body parts that elicit specific grooming movements. This enables the simultaneous activation of sensory neurons across the body to induce competition among their respective grooming movements. As we observed by coating the bodies of flies in dust, whole-body sensory activation elicits grooming that prioritizes the head and then proceeds to the other body parts. This provides direct evidence that the grooming sequence can be induced through simultaneous activation of sensory neurons across the body. These experiments also reveal that flies have a persistent trace of the body parts that were stimulated, which results in delayed

and sequential grooming of the stimulated parts. Work presented here lends neural-based evidence to the parallel model of hierarchical suppression among grooming movements and provides new insights into its underlying organization.

Results

GAL4 lines targeting sensory neurons across the body that elicit grooming

Our initial goal was to identify GAL4 transgenic lines expressing in sensory neurons across the body, to directly test whether simultaneous activation of these neurons leads to a prioritized grooming response. As an entry point, we examined a collection of previously identified enhancer-driven GAL4 lines that express in different neuronal populations whose activation drove grooming (Seeds et al. 2014). Confocal microscopy imaging of the *peripheral nervous system* (PNS) expression patterns of different lines from this collection revealed three that express in sensory neurons across the body (R52A06-, R30B01-, and R81E10-GAL4; **Figure 1A-F**, R52A06-GAL4 shown as an example, **Figure 1 – figure supplement 1A-D**). We classified the different sensory neuron types based on previous anatomical descriptions (Murphey et al. 1989; Cole and Palka 1982; Dickinson and Palka 1987; Smith and Shepherd 1998; Kays, Cvetkovska, and Chen 2014) and found that the lines express predominantly in mechanosensory neurons (**Figure 1G**). However, R30B01-GAL4 also showed expression in chemosensory neurons (**Figure 1G**).

We next tested whether local populations of sensory neurons on specific body regions can elicit individual grooming movements when focally activated. Site-directed grooming responses have previously been investigated using tactile stimulation to particular mechanosensory bristles on the body surface of decapitated flies (Vandervorst and Ghysen 1980; Corfas and Dudai 1989). Here, we used optogenetic activation with Channelrhodopsin, directing blue light via an optical fiber to particular body regions of the broad sensory GAL4 lines

to activate sensory neurons on either the dorsal anterior or posterior body regions of decapitated flies (**Figure 1 – figure supplement 2A,B**). Light directed to the posterior dorsal body surface elicited grooming of the wings, whereas illumination of the anterior dorsal surface elicited grooming of the notum (**Figure 1H**, **Video 1**, **Video 2**). This indicated that site-directed grooming responses can be elicited optogenetically, and that the GAL4 lines express in sensory neurons whose activation can elicit grooming movements for at least two parts of the body.

Simultaneous excitation of sensory neurons across the body induces a grooming sequence

The GAL4 lines described above were next used to test a prediction of the model of hierarchical suppression that simultaneous activation of sensory neurons across the body elicits head grooming preferentially (Seeds et al. 2014). Freely moving flies of each line expressing the red light-gated neural activator CsChrimson were exposed to whole body illumination to optogenetically activate their targeted sensory neurons, and grooming responses were subsequently measured. Each of the three GAL4 lines expresses in sensory neurons whose activation can elicit wing or notum grooming, as revealed by localized optogenetic activation (**Figure 1G,H**). Additionally, each line expresses in eye bristle mechanosensory neurons whose activation we hypothesized could elicit eye grooming, while two of the lines (R52A06- and R30B01-GAL4) also express in antennal Johnston's Organ neurons that were previously shown to elicit antennal grooming (Hampel et al. 2015). Although these GAL4 lines can elicit several movements from different body sensory neurons, we predicted that activating them simultaneously should elicit only the highest-priority movement, according to the hierarchical suppression model. Indeed, the simultaneous optogenetic activation of body sensory neurons targeted by each GAL4 line resulted in head rather than posterior (abdomen, wing, notum) grooming, consistent with the model of hierarchical suppression (**Figure 2A**, during red light-on period).

Optogenetic activation of sensory neurons across the body also elicited a grooming sequence reminiscent of dust-induced grooming. Flies groomed their heads at the onset of a five-second red light stimulus, and then transitioned to grooming their posterior bodies during the period after the light was turned off (**Figure 2A**). One trivial explanation for this sequence could be that optogenetic activation of sensory neurons on the posterior body elicited grooming with a latency, whereas there was no latency to groom with activation of the head sensory neurons. We tested for this latency to groom the posterior body using decapitated flies that no longer received a sensory drive to groom their heads. In contrast to intact flies, activation of the posterior body sensory neurons of decapitated flies elicited posterior grooming during the red light (**Figure 2B**). Thus, a latency does not explain the sequence because head and posterior grooming can be elicited on similar time scales. Instead, evidence that intact flies do not display posterior grooming with the light stimulation supports the hypothesis that it is suppressed by head grooming (discussed below). Notably, optogenetic activation of sensory neurons across the body causes flies to groom their bodies in the same order as when they were coated in dust (head > abdomen > wings > notum) (**Figure 2 – figure supplement 2A,B**, **Video 3**, **Video 4**, and **Video 5**). Further, the posterior body grooming sequence continued through the minute after the cessation of the red light, while the sensory neurons were no longer activated (**Figure 2A**, green histogram). This suggests a persistent trace of posterior sensory neurons that had been activated, which allowed each movement to be elicited once the previous grooming movement terminated.

The behavior resulting from simultaneous activation of sensory neurons across the body supports a role of suppression in establishing the grooming movement hierarchy. Evidence of suppression was found when sensory neurons were reactivated during the period when flies had transitioned to posterior grooming (**Figure 2A**). The hierarchical suppression model predicts

157 that switching the red light back on during this period to reactivate sensory neurons across the
158 body would result in head grooming, coupled with the termination of ongoing posterior
159 grooming. Indeed, in cases where flies were engaged in posterior grooming, delivery of the next
160 red light stimulus caused flies to terminate posterior grooming and switch to grooming their
161 heads. This is seen in **Figure 2A** (histogram plots on right, green traces) where the fraction of
162 flies grooming their posterior bodies drops to zero at the onset of the red light. Thus, we find
163 optogentic-based evidence consistent with the hypothesis that the grooming sequence is driven
164 by a hierarchical suppression mechanism, as was revealed from experiments using natural
165 stimulus such as dust (Seeds et al. 2014).
166
167 ***Identification of mechanosensory neurons that elicit specific grooming movements***
168 We next sought to test whether the hierarchy of grooming movements could be observed with
169 competing activation of defined sets of sensory neurons that elicit distinct movements. We first
170 acquired transgenic lines for manipulating sensory neurons on specific body parts. Eye
171 grooming is the most hierarchically superior, and is thus elicited first in competition with other
172 grooming movements (Seeds et al. 2014). Based on previous work implicating the
173 interommatidial bristle mechanosensory neurons in eye grooming in the praying mantis and
174 cricket (Honegger 1977; Honegger, Reif, and Müller 1979; Zack and Bacon 1981), we found
175 that these neurons elicit eye grooming in *Drosophila*. A search through an image database of
176 brain expression patterns from the Vienna *Drosophila* collection identified a LexA line
177 (VT17251-LexA) that expressed exclusively in the interommatidial bristle mechanosensory
178 neurons. The hundreds of bristles on the compound eyes each contains the dendrite of a
179 sensory neuron, which also projects an axon into an afferent tract that enters the CNS in the
180 *subesophageal zone* (SEZ) (**Figure 3A,B**). In contrast to the praying mantis and cricket, the fly
181 eye bristle afferents project only to the SEZ, and not also the prothoracic neuromeres (**Figure**
182 **3B**). We tested whether activation of eye bristle mechanosensory neurons would elicit grooming

by expressing CsChrimson using VT17251-LexA and exposing flies to red light. Indeed, optogenetic activation of the eye bristle mechanosensory neurons elicited eye grooming (**Figure 3C**).

We next acquired a transgenic driver line for manipulating sensory neurons that could elicit wing grooming, which is lower in the hierarchy than eye grooming. From our previous screen (Seeds et al. 2014), we identified a GAL4 line that expresses in neurons whose activation could elicit wing grooming and showed expression in sensory neurons on the wings (**Figure 4 – figure supplement 1A**, R31H10-GAL4, behavioral data not shown). However, the identities of those sensory neurons were obscured by expression in other cells (**Figure 4 – figure supplement 1B**). Therefore, we used the intersectional *Split GAL4* (spGAL4) technique to restrict expression to only the sensory neurons (Luan et al. 2006; Pfeiffer et al. 2010). spGAL4-mediated expression occurs only when the two GAL4 domains, the GAL4 *DNA binding domain* (DBD) and the *transcriptional activation domain* (AD), are expressed in the same cells. We generated spGAL4 flies that were anticipated to target the wing sensory neurons by expressing the DBD in the pattern of R31H10-GAL4 and the AD in the pattern of R30B01-GAL4 (**Figure 4 – figure supplement 1B,C**).

The R30B01-AD ∩ R31H10-DBD combination expresses in two main types of mechanosensory neurons on the wings and halteres (**Figure 4A**). The first type includes campaniform sensilla, which are dome-shaped structures on the fly cuticle that are each innervated by a mechanosensory neuron that responds to deformations of the cuticle (Dickinson and Palka 1987). Campaniform sensilla on the proximal part of the wing are largely clustered in fields, whereas individual sensilla are found along the distal wing (Palka, Lawrence, and Hart 1979; Cole and Palka 1982; Palka et al. 1986; Dickinson and Palka 1987). R30B01-AD ∩ R31H10-DBD flies show a sparse labeling of neurons in the proximal fields (5 to 10 out of ~77

209 neurons (median = 6.5), **Figure 4A,B**, white asterisks), and expression in the majority of the
210 distal campaniform sensilla (5 to 6 out of 8 neurons (median = 5), **Figure 4A,C**, yellow
211 asterisks). The spGAL4 line also expresses in campaniform sensilla on the halteres (7 to 10 out
212 of ~139 neurons, **Figure 4E**). The other type of sensory neurons targeted by R30B01-AD ∩
213 R31H10-DBD are mechanosensory bristle neurons on the distal wing (expression in 3-5 out of
214 ~221 neurons (median = 3.5), **Figure 4A,D**, white arrowheads) (Hartenstein and Posakony
215 1989). These different neurons on the wings and halteres send projections to the *ventral*
216 *nervous system* (VNS), where they follow diverse paths locally, with some further ascending to
217 the SEZ in the brain (**Figure 4F**). The ascending afferents are likely from campaniform sensilla
218 on the halteres and proximal wings, whereas afferents that remain in the VNS are likely from
219 wing mechanosensory bristle neurons and distal campaniform sensilla (Palka, Lawrence, and
220 Hart 1979; Ghysen 1980; Dickinson and Palka 1987).
221
222 Optogenetic activation of the neurons targeted by R30B01-AD ∩ R31H10-DBD
223 expressing CsChrimson elicited wing but not haltere grooming (**Figure 4G**). The parsimonious
224 explanation for this result is that the grooming was elicited by sensory neurons on the wing.
225 However, because the line also expresses in haltere campaniform sensilla, we cannot rule out
226 their involvement in the behavior. Nevertheless, the spGAL4 driver affords access to sensory
227 neurons for independent control of wing grooming.
228
229 ***Competition between eye and wing sensory neurons elicits prioritized grooming***
230 The hierarchical associations between eye and wing grooming were next examined by
231 activating their respective sensory pathways. We first compared the individual grooming
232 responses to acute activation of either the eye bristle mechanosensory neurons or the
233 wing/haltere sensory neurons. Flies were exposed to five-second pulses of red light, followed by

rest periods with no light. Activation of the eye bristle mechanosensory neurons elicited eye grooming during the period when the red light was on that decayed when it turned off (**Figure 5A**, top, magenta). In contrast, activation of the wing/haltere sensory neurons elicited grooming with the red light that persisted after light cessation (**Figure 5A**, middle, green). Importantly, activation of either the eye bristle mechanosensory neurons or the wing sensory neurons alone did not elicit the other corresponding grooming movement, or an anterior-to-posterior grooming sequence. Thus, activation of these specific sensory types only elicits grooming of its corresponding body part.

We next tested whether activation of the eye bristle mechanosensory neurons and wing/haltere sensory neurons at the same time would elicit a prioritized eye grooming response, as is predicted by the model. For this experiment, we identified a spGAL4 combination (R31H10-AD ∩ R34E03-DBD) that expressed both in the eye bristle mechanosensory neurons and the same three categories of sensory neurons on the wings and halteres that were expressed in the R30B01-AD ∩ R31H10-DBD combination (**Figure 5B**). Simultaneous optogenetic activation of these defined sensory neurons elicited prioritized grooming that started with the eyes and then proceeded to the wings (**Figure 5A**, bottom), like what we observed with activation of sensory neurons across the body (**Figure 2**). We also found evidence of suppression by eye grooming, as ongoing grooming of the wings terminated and all flies groomed their eyes with each red light stimulus (**Figure 5A**, bottom). These results demonstrate the prioritization between grooming movements through direct optogenetic activation of the sensory neurons that elicit grooming of specific body parts. This strengthens the conclusion of our previous work that the sequence occurs when the grooming movements are activated in parallel and then sequentially prioritized through hierarchical suppression (Seeds et al. 2014).

Discussion

The goal of this work was to test the prediction of the model of hierarchical suppression that simultaneous activation of sensory neurons on different body parts elicits a prioritized grooming response. Two lines of evidence led us to this prediction. The first was based on our previous finding that coating the body of the fly in dust elicits grooming that prioritizes head over posterior body grooming (Seeds et al. 2014). The second was based on data showing that local stimulation to the body surface elicits site-specific grooming responses (Vandervorst and Ghysen 1980; Corfas and Dudai 1989; Seeds et al. 2014; Hampel et al. 2015). Thus, we proposed that sensory neurons across the body are stimulated in parallel by dust to elicit competition among their respective grooming movements. Here, we test this by identifying transgenic driver lines for targeting and directly activating sensory neurons that elicit grooming, allowing us to bypass the dust stimulus and reveal the underlying sensory neurons. Using simultaneous optogenetic activation of sensory neurons across the body we observe the same anterior-to-posterior prioritization among the grooming movements that occurs when flies are coated in dust. This lends strong support to the hypothesis that the grooming movements are activated in parallel, and are thus selected in a hierarchically determined competition through suppression.

Sensory neurons involved in grooming behavior

One aim of this work was to identify sensory neurons that can induce grooming behavior. The bristles are canonically thought to be involved in insect grooming based on evidence that their tactile stimulation on different body parts induces site directed grooming responses (Vandervorst and Ghysen 1980; Corfas and Dudai 1989; Page and Matheson 2004). Here, we provide evidence that direct activation of the bristle mechanosensory neurons can elicit grooming. We identify the fruit fly interommatidial bristle mechanosensory neurons based on their anatomical similarity to those of the praying mantis and cricket (Honegger, Reif, and Müller 1979; Zack and Bacon 1981). Next, we use a transgenic driver line that expresses in these

neurons to show that their optogenetic activation elicits eye grooming. We also identified different spGAL4 lines that express in neurons whose activation elicits wing grooming. However, these lines express both in bristle mechanosensory neurons and campaniform sensilla, raising the question of whether one or both sensory types are involved. Given the wealth of data implicating the bristles in grooming (Tuthill and Wilson 2016), the parsimonious explanation is that the wing bristle mechanosensory neurons are involved. However, there is also a precedent for the involvement of non-bristle mechanosensory neurons such as the campaniform sensilla. For example, we previously showed that Johnston's Organ chordotonal neurons can detect displacements of the antennae to induce antennal grooming (Hampel et al. 2015), and others have shown that gustatory neurons on the wing can detect different chemicals to trigger grooming (Yanagawa, Guigue, and Marion-Poll 2014). Therefore, further work is required to resolve which sensory neurons are involved in wing grooming.

One outstanding question is whether the sensory neurons have a direct role in establishing hierarchical suppression. We previously proposed two mechanisms of hierarchical suppression (Seeds et al. 2014). One is that unidirectional inhibitory connections between the movements drive suppression, a mechanism not likely to involve the sensory neurons. The other is that differences in sensitivity to dust across the body establish a gradient of sensory drives among the grooming movements, leading to suppression through winner-take-all competition. One way that sensitivity differences could be established is through differing numbers of receptors on each body part. For example, if we assume that the bristle mechanosensory neurons on the different body parts detect dust to elicit grooming (which remains to be shown), a comparison of bristle numbers on different body parts gives mixed support for this hypothesis. There are 600, 221, and 235 bristles reported to be on the eyes, wings, and notum respectively (Hartenstein and Posakony 1989; Cadigan, Jou, and Nusse 2002). The eyes are the highest priority part to be groomed, and have 2.7 times more bristles

than the wings, which is consistent with the suppression hierarchy. In contrast, the lowest priority body part is the notum, which has more bristles than the wings, arguing against the hypothesis. Furthermore, given that other sensory neuron types elicit grooming (e.g. chordotonal and gustatory neurons), there may be multiple ways of detecting dust (Yanagawa, Guigue, and Marion-Poll 2014; Hampel et al. 2015). Alternatively, hierarchical suppression could be established at the level of sensory neurons by regulating their output through presynaptic inhibition (Blagburn and Sattelle 1987; Burrows and Matheson 1994; Clarac and Cattaert 1996; Rudomin and Schmidt 1999). For example, the feeding behavior of the medicinal leech causes presynaptic inhibition of mechanosensory neurons, which suppresses touch-induced behavioral responses (Gaudry and Kristan 2009). Future experiments will test such hypotheses about whether hierarchical suppression is established at the level of sensory neurons.

Persistent neural activity within grooming neural circuits

Emerging behavioral evidence indicates that neural circuits controlling *Drosophila* grooming movements have mechanism(s) for maintaining excitability. This was originally proposed from a study identifying a mechanosensory circuit that elicits persistent grooming of the antennae (Hampel et al. 2015). That is, neurons within this circuit elicit grooming that continues for tens of seconds beyond their optogenetic activation. Work presented here reveals that activation of wing sensory neurons similarly elicits persistent grooming. Interestingly, grooming responses that outlast their stimulus have also been described in vertebrates, suggesting that persistence is a common feature of grooming (Sherrington 1906; Stein 2005). Despite the prevalence of persistent grooming, its biological function remains unclear. One possibility is that persistence prevents unnecessary switches between behaviors (Redgrave, Prescott, and Gurney 1999); for example swimming responses can last beyond the initial stimulus so that an animal can safely

337 avoid a predator. In the case of grooming, persistence may ensure that a dirty body part is
338 thoroughly cleaned before switching to another behavior.
339
340 We also infer the maintenance of excitability within grooming neural circuits from the
341 observation that brief activation of sensory neurons across the body elicits a grooming
342 sequence. That is, flies groom their heads and then transition to their posterior bodies, even
343 during the period after the red light has turned off. This indicates that flies maintain a persistent
344 trace of which body parts are stimulated to elicit a delayed and sequential grooming response.
345 We postulate that this occurs when the simultaneous stimulation of sensory neurons across the
346 body activates each grooming movement in parallel. Eye grooming occurs first by suppressing
347 grooming movements occurring later (Seeds et al. 2014), however the circuitry for each later
348 movement remains active without requiring further sensory input. The next movement is then
349 elicited via this persistent neural activity once suppression from eye grooming ceases. If this is
350 the case, it raises the question of how the previous movement terminates to allow the next
351 movement to proceed. Further, it is unclear how circuits that drive later grooming movements
352 retain neural excitability. Such acquisition and maintenance of excitability is reminiscent of a
353 previously described feature of grooming called *temporal summation*, whereby successive
354 subthreshold stimuli are summed to elicit grooming (Sherrington 1906; Stein 2005; Guzulaitis,
355 Alaburda, and Hounsgaard 2013). Thus, both temporal summation and the grooming sequence
356 observed here point to a mechanism within the grooming neural circuitry that maintains a
357 persistent trace of the sensory stimulus.
358
359 How does a mechanism that maintains excitability within the grooming neural circuitry
360 affect our previously proposed model of grooming behavior? Our previous model indicated that
361 constant stimulation is necessary for each grooming movement to be active (Seeds et al. 2014).
362 That is, dust on a body part provides a constant drive to groom that is lessened through its

363 removal. Indeed, a computational model where the movements are driven entirely by the
364 presence of dust produces grooming that resembles dust-induced grooming. This indicates that
365 the model well describes grooming that occurs over relatively long time scales (~30 minutes).
366 However, based on observations that grooming persists after a brief stimulus, we now propose
367 that the circuitry contains a neural mechanism that allows grooming movements to remain
368 active on shorter time scales (tens of seconds). The ability to identify and manipulate the
369 sensory neurons that elicit grooming movements and their downstream circuits now enable
370 experiments to determine how persistent neural excitability is acquired and maintained.
371

372 **Methods**

373 ***Fly stocks and rearing conditions***

374 The GAL4 lines used in this study were produced by Gerald Rubin's lab at Janelia Research
375 Campus and are available from the Bloomington *Drosophila* stock center (Jenett et al. 2012).
376 The lines were identified in a screen for those that expressed GAL4 in neurons whose activation
377 could elicit grooming behavior (Seeds et al. 2014). In this work, we screened through the
378 images of the CNS expression patterns of these GAL4 lines (Jenett et al. 2012), searching for
379 those with expression in afferents from each of the different body parts (**Figure 1 – figure**
380 **supplement 1A-D**). These lines were selected for detailed behavioral and anatomical analysis
381 as described in the results section. The control used for the GAL4 lines was BDPGAL4U, which
382 contains the vector backbone used to generate each GAL4 line (including GAL4), but lacks any
383 enhancer to drive GAL4 expression (Seeds et al. 2014). The Split GAL4 stocks were produced
384 by Gerald Rubin's lab according to previously described methods (Pfeiffer et al. 2010).
385 VT17251-LexA was a gift from the lab of Barry Dickson. Controls for the Split GAL4 stocks were
386 produced in the same way as BDPGAL4U, but each spGAL4 half was used in place of GAL4
387 (Hampel et al. 2015).
388

Transgenic flies carrying the following UAS drivers were from the following citations: *UAS-dTrpA1* (Hamada et al. 2008), 20x*UAS-mCD8::GFP* (pJFRC7) (Pfeiffer et al. 2010), 13xLexAop-myr::GFP (pJFRC19) (Pfeiffer et al. 2010), *UAS-Channelrhodopsin-2* (Hwang et al. 2007), and 20x*UAS-CsChrimson* (attP18) (Klapoetke et al. 2014). GAL4, spGAL4, and LexA lines were crossed to their respective UAS or LexAop drivers, and the progeny were reared on cornmeal and molasses food at 21°C and 50% relative humidity using a 16/8-hour light/dark cycle. For optogenetic experiments using CsChrimson or ChR2, flies were reared in the dark on food containing 0.4 mM all-*trans*-retinal. All experiments were done with five to eight day old males.

Channelrhodopsin-mediated activation of sensory neurons using decapitated flies

Two different regions of the bodies of decapitated flies were illuminated to locally activate sensory neurons expressing ChR2. For decapitations, flies were cold-anesthetized, decapitated using a standard razor blade, and allowed to recover for 10-20 minutes. The flies were positioned on a slide for the experiment using a fine paint brush. A 473-nm blue light LED (Nichia Corp, Tokushima, Japan) was attached to an optical fiber (1 mm in diameter) to direct light to a specific region on the fly. The optical fiber was held approximately 1 mm from the target body region to deliver a blue light stimulus with a luminance of 0.075 mW/mm^2. The light was directed towards the dorsal posterior region, away from the anterior body, to activate sensory neurons primarily on the wings (**Figure 1 – figure supplement 2A)**. Alternatively, the light was directed towards the dorsal anterior region, away from the posterior body, to activate sensory neurons primarily on the notum (**Figure 1 – figure supplement 2B)**. The LED stimulus was controlled using a Grass SD9 stimulator (Astro-Med Inc., Warwick, RI) that delivered 10 Hz pulses that were 20 milliseconds in duration, with 8-millisecond delays between pulses. Each fly was subjected to stimulation on each body region in random order; however, in some cases the flies would jump during the experiment and could not be used further. A grooming response to

the illuminated body region within a ten-second time frame was scored as a positive response. The fraction of flies that responded was plotted. The number of trials for each dorsal body region for each line were: R52A06-GAL4, anterior (n = 100), posterior (n = 100); R30B01-GAL4, anterior (n = 40), posterior (n = 61); R81E10-GAL4, anterior (n = 86), posterior (n = 89). Statistical significance was addressed using Chi-Square tests and Bonferroni correction.

CsChrimson-mediated activation of sensory neurons using freely moving flies

The camera and behavioral setups used for recording freely moving flies with optogentic activation were described previously (Seeds et al. 2014; Hampel et al. 2015). Flies were cold anesthetized, loaded into behavioral chambers, and allowed to recover for at least ten minutes. R52A06-, R30B01-, and R81E10-GAL4 were used to express the light-gated channel CsChrimson. The red light used for gating CsChrimson readily penetrates the fly cuticle (Inagaki et al. 2014), allowing for uniform activation of sensory neurons across the body. Our initial experiments using optogenetic activation of the neurons targeted by these GAL4 lines revealed that high levels of red light activation caused defects in motor coordination. This was likely caused by the strong activation of sensory neurons across the body, some of which are known to be involved in proprioception (e.g. femoral chordotonal organs). Therefore, it was necessary to reduce the red-light power to the point where it elicited grooming without causing coordination defects. The light power that met these requirements for each GAL4 line are: R52A06-GAL4 (0.066 mW/mm^2), R30B01-GAL4 (0.066 mW/mm^2), and R81E10-GAL4 (0.077 mW/mm^2). The light power used for each LexA and spGAL4 line was: VT17251-LexA (0.382 mW/mm^2), R30B01-AD ∩ R31H10-DBD and R31H10-AD ∩ R34E03-DBD (0.135 mW/mm^2). The red light frequency was 5 Hz (0.1 seconds on/off) for 5 seconds, followed by 30 second intervals where the red light was off. The experiment consisted of a total of three photostimulation periods with 30 second intervals between each stimulation. The experiment was recorded for manual annotation of the grooming movements performed.

441

442 The recorded grooming movements of flies were manually annotated as described
443 previously (Seeds et al. 2014). For the ethogram and histogram plots in **Figure 2**, the different
444 head grooming movements (e.g. eye, antennal, and proboscis grooming) were binned (1
445 second time bins) and plotted as head grooming. Similarly, all movements that were directed
446 towards the body (e.g. abdomen, wings, notum) were binned and plotted as posterior body
447 grooming. Statistical analysis and display of the data were previously described (Hampel et al.
448 2015).

449

450 ***Analysis of CNS and PNS expression patterns***

451 Dissection and staining of the CNS was performed using a previously reported protocol (Hampel
452 et al. 2011). The head stain shown in **Figure 3A** was done as follows. Fine scissors were used
453 to cut off part of the proboscis and part of the eyes to improve antibody penetration. Heads were
454 fixed in phosphate buffered saline (PBS) containing 2% paraformaldehyde and 0.1% Triton for 2
455 hours at 4°C, and stained with primary antibodies: rabbit anti-GFP (1:500, Thermo Fisher
456 Scientific, Waltham, MA, #A11122) and mouse anti-nc82 (1:50, Developmental Studies
457 Hybridoma Bank, University of Iowa) followed by secondary antibodies: goat anti-rabbit DyLight
458 594 (Thermo Fisher Scientific #35560) and goat anti-mouse DyLight 633 (Thermo Fisher
459 Scientific #35512), with Calcofluor White to stain the cuticle (a few grains in 300 μl volume,
460 Sigma #F3543). Images were collected using a Zeiss LSM710 confocal microscope using a
461 Plan-Apochromat 20x/0.8 M27 objective (Carl Zeiss Corporation, Oberkochen, Germany).

462

463 Dissection of the different body parts and imaging of the PNS expression patterns of the
464 different GAL4, LexA, and Split GAL4 lines were performed as follows. The lines were crossed
465 to 20x*UAS-mCD8::GFP* (JFRC7) or 13xLexAop-myr::GFP (pJFRC19). The progeny were
466 anesthetized using CO_2, decapitated, dipped in 70% ethanol, transferred to PBS, and each

body part was dissected as described below. The unfixed body parts were imaged immediately in PBS or Vectashield (Vector Laboratories, Burlingame, CA). We used both PBS and Vectashield and did not notice a difference in the cell morphology or expression pattern when using either reagent. The use of Vectashield had the advantage of resulting in fewer air bubbles between the coverslip and sample.

Head: Flies were decapitated using a standard razor blade. Heads were then placed "face up" on a slide in a small well that was made by stacking six reinforcement labels (Avery Dennison Corporation, Brea, CA) and filled with PBS or Vectashield. A cover slip was then placed over the well. *Abdomen:* The abdomen was severed from the rest of the body just posterior to the scutellum. Abdomens were then placed on a slide in a well created as described above. The abdomens were placed either ventral or dorsal side up so that each side could be imaged. *Notum:* A scalpel was used to slice longitudinally between the legs and the dorsal side of the notum. The notum was imaged in the same well preparation described above. *Wing:* A scalpel was used to remove the left wing from the body of the fly. To ensure that the entire wing was obtained, part of the body wall was also cut with the wing. The wing was then placed on a drop of Vectashield and then covered with a coverslip. *Leg:* The left prothoracic leg was dissected in the same way as the wing. All body parts were imaged using a Zeiss 710 confocal microscope using 10x and 20x air objectives. Native GFP fluorescence was imaged using an excitation wavelength of 488 nm, whereas autofluorescence from cuticle was imaged using 568 nm. Body parts from at least three flies were imaged from separate crosses on different days. In some cases, the body parts were imaged at 20x and then stitched using a FIJI plugin (Preibisch, Saalfeld, and Tomancak 2009).

The different sensory neuron types on each body part were classified based on previous descriptions (Ghysen 1980; Cole and Palka 1982; Dickinson and Palka 1987; Murphey et al.

1989; Smith and Shepherd 1998). The numbers of campaniform sensilla and mechanosensory bristle neurons on the wings were previously counted (Cole and Palka 1982; Dickinson and Palka 1987; Hartenstein and Posakony 1989). Proximal campaniform sensilla described in this work include ANWP, Teg, d.Rad.A, d.Rad.B, d.Rad.C, d.Rad.D, d.Rad.E, d.HCV, v.Rad.A, v.Rad.B, v.Rad.C, v.HCV, and vL.III. Distal campaniform sensilla described in this work include GSR, p.TSM, d.TSM, L3-V, ACV, L3-1, L3-2, and L3-3. We classified neurons on the wings as bristle mechanosensory rather than chemosensory given that their dendrites appear to terminate at the base of the bristle rather than projecting to the bristle tip.

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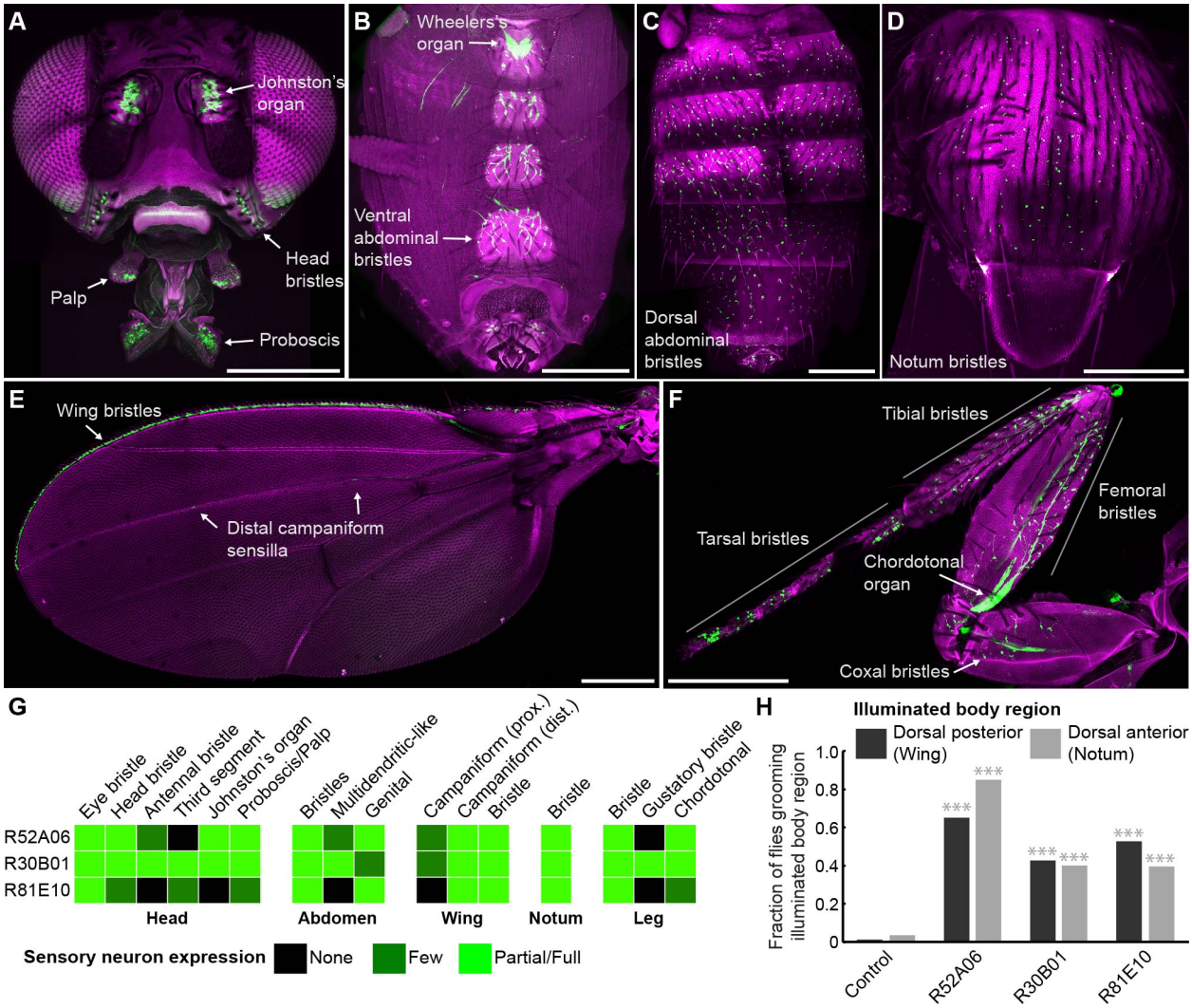

Figure 1. GAL4 lines expressing in sensory neurons whose activation elicits grooming. (**A-E**) Peripheral expression pattern of R52A06-GAL4 expressing *green fluorescent protein* (GFP). Confocal maximum projections are shown. Sensory neurons are in green and autofluorescence from the cuticle is in magenta. Body parts shown are: (**A**) head, (**B**) ventral abdomen, (**C**) dorsal abdomen, (**D**) notum, (**E**) wing, and (**F**) prothoracic leg. Labeled arrows indicate specific sensory classes. In (**C**) and (**D**) all GFP positive cells are bristle mechanosensory neurons. Scale bars, 250 μm. (**G**) Summary table of the expression patterns of R52A06-, R30B01-, and R81E10-GAL4 in sensory neurons on each indicated body part. (**H**) Grooming responses to optogenetic activation of sensory neurons targeted by different GAL4

706 lines expressing ChR2. An optical fiber connected to an LED was used to direct light to the
707 dorsal surface of the anterior or posterior body (**Figure 1 – figure supplement 2**). The fraction
708 of flies that showed a grooming response to the blue light-illuminated body region is plotted ($n \geq$
709 40 trials for each body part). Grey shades and labels indicate the region that was illuminated.
710 Chi-squared test, Asterisks: $p < 0.0001$. See **Video 1** and **Video 2** for representative examples.
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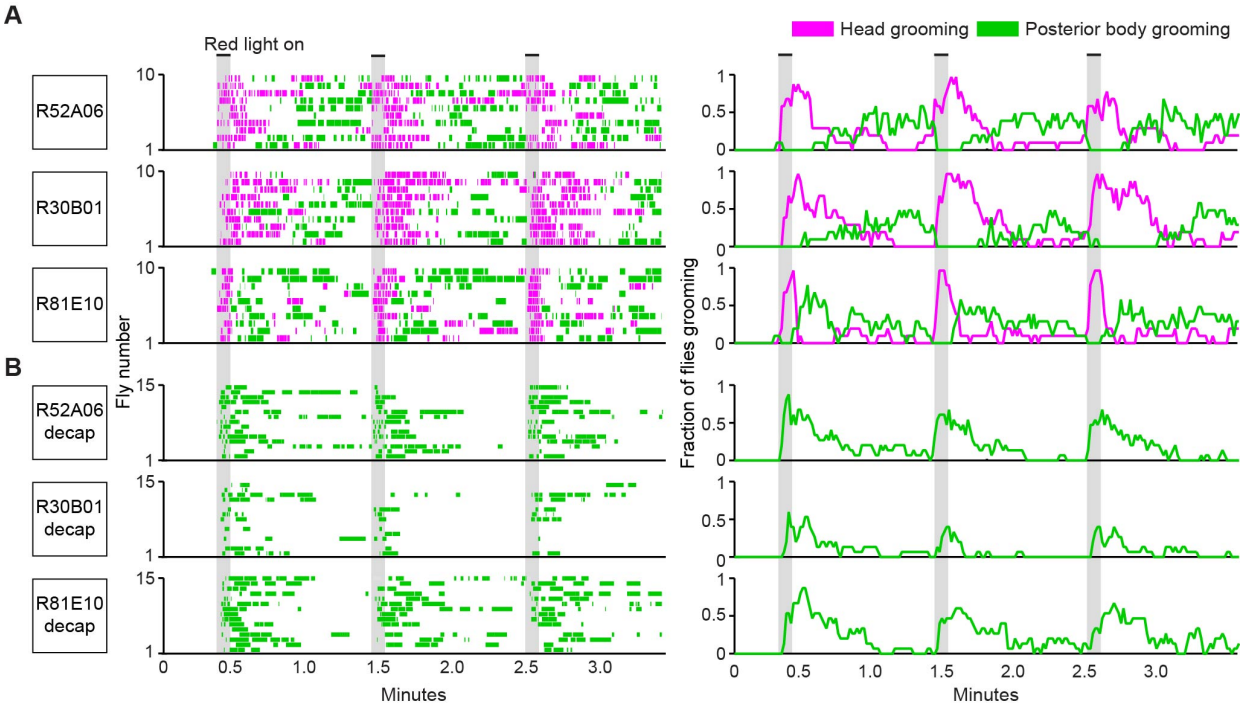

Figure 2. Simultaneous optogenetic activation of body sensory neurons elicits sequential grooming. (**A**-**B**) Head (magenta) or posterior body grooming movements (green) elicited with red light-illumination of R52A06-, R30B01-, and R81E10-GAL4 flies expressing CsChrimson. The movements are mutually exclusive. Ethograms of ten individual flies are stacked for each line (left). Histograms show the fraction of flies that were performing specific grooming movements within one-second time bins (right). Gray bars indicate five second presentations of red light. (**A**) Grooming movements performed by intact flies. (**B**) Grooming movements performed by decapitated flies. See **Video 3**, **Video 4**, and **Video 5** for representative examples. Red light illumination of control flies did not elicit grooming (**Figure 2 – figure supplement 1**).

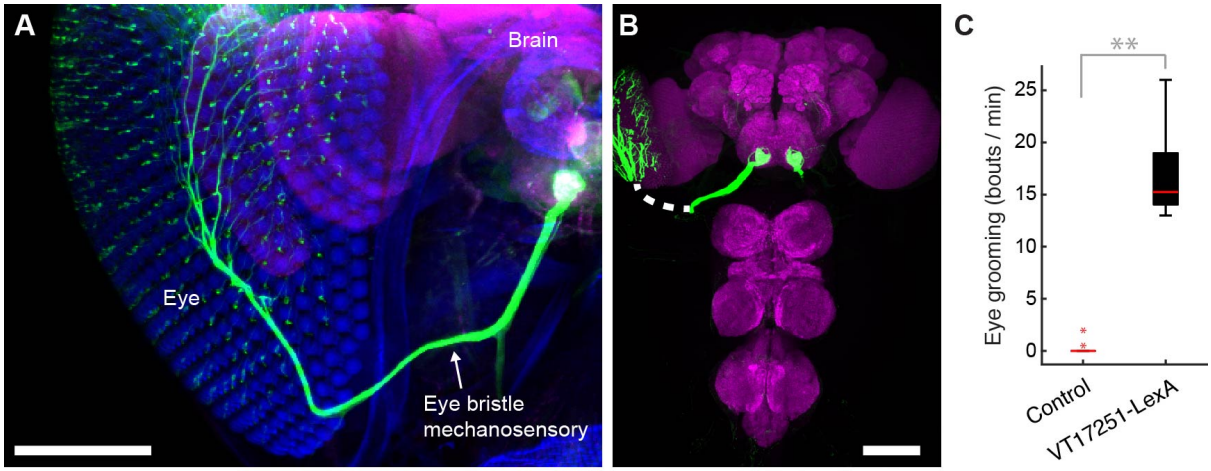

Figure 3. Interommatidial bristle mechanosensory neurons elicit eye grooming. (**A-B**) The expression pattern of VT17251-LexA in eye bristle mechanosensory neurons. The neurons were stained with anti-GFP (green) and the brain neuropile is stained with anti-Bruchpilot (magenta). Both images are maximum intensity projections. Scale bars, 100 μm. (**A**) Expression pattern shown in the semi-intact head. The eye and head cuticle is shown in blue. (**B**) Expression pattern in the CNS. White dashed line indicates the trajectory of eye bristle mechanosensory neuron axons found from the more intact preparations in (A). (**C**) Eye grooming bout rate with optogentic activation of neurons targeted by VT17251-LexA. Bottom and top of the boxes indicate the first and third quartiles respectively; median is the red line; whiskers show the upper and lower 1.5 IQR; red dots are data outliers (n = 10 for each box; asterisks show $p < 0.001$, Kruskal-Wallis and post hoc Mann-Whitney U pairwise test).

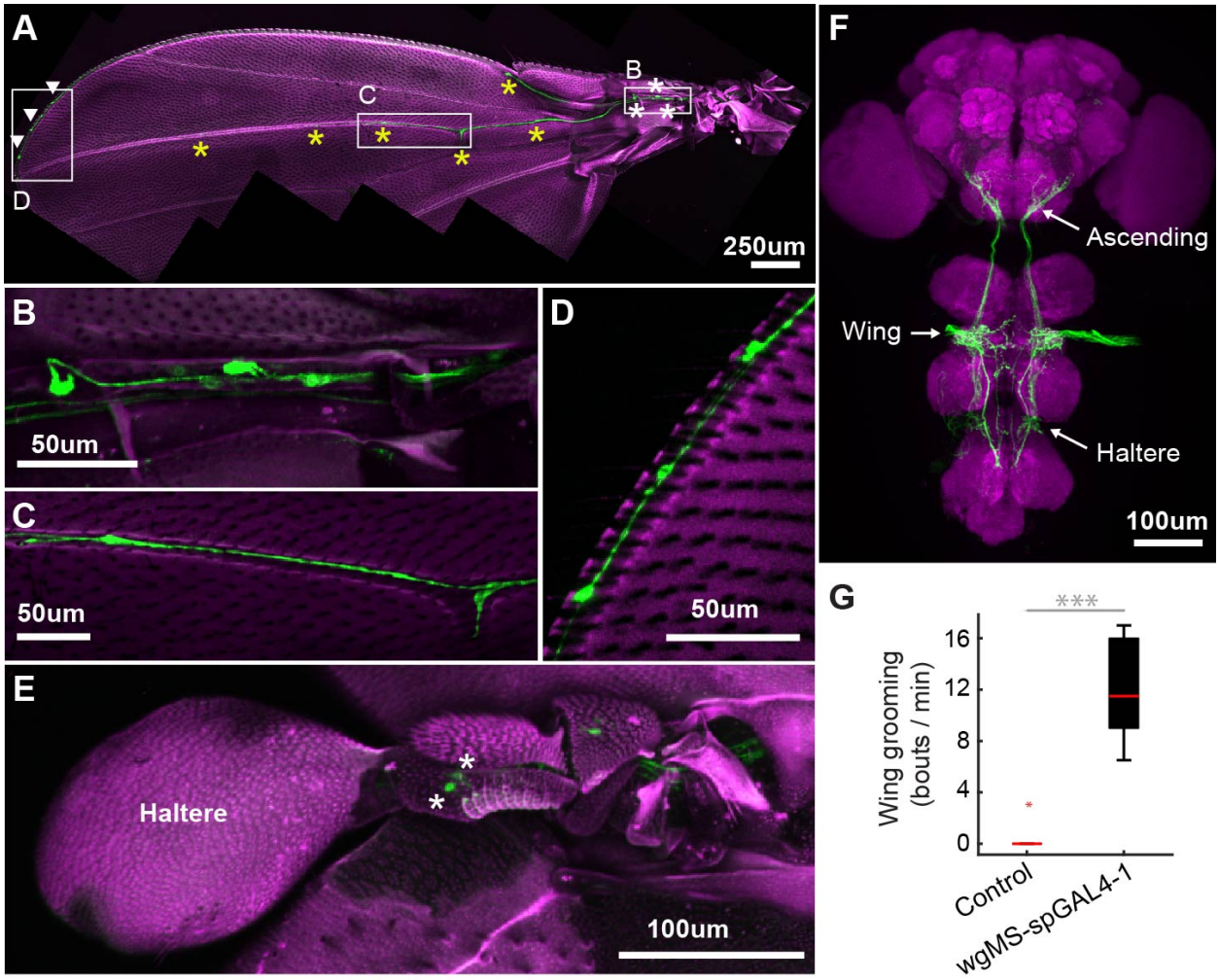

Figure 4. spGAL4 driver that expresses in wing and haltere sensory neurons whose activation elicits wing grooming. (**A-E**) The expression pattern of R30B01-AD ∩ R31H10-DBD in sensory neurons of the wings and halteres. Native GFP fluorescence is shown in green and autofluorescence from the cuticle is in magenta. Maximum intensity projections are shown. The proximal wing is to the right and the distal wing is to the left. (**A**) Sensory neurons on the wing. White boxes and letters indicate the regions shown in **B-D**. The different symbols indicate the sensory neuron types on the wing as proximal campaniform sensilla (white asterisks), distal campaniform sensilla (yellow asterisks), or bristle mechanosensory (white arrowheads). Scale bar, 250 μm. (**B-D**) Larger images of the regions shown in **A**. Scale bars, 50 μm. Shown are the proximal campaniform sensilla (**B**), distal campaniform sensilla (**C**), and bristle mechanosensory

757 neurons (**D**). (**E**) Expression in the haltere campaniform sensilla (asterisks). Scale bar, 100 μm
758 (**F**) CNS expression visualized by co-stain with anti-GFP (green) and anti-Bruchpilot (magenta).
759 Arrows indicate the CNS entry points of afferents from the wings and halteres, and the location
760 of ascending projections from some of these afferents. Scale bar, 100 μm. (**C**) Wing grooming
761 bout rate with optogentic activation of neurons targeted by R30B01-AD ∩ R31H10-DBD. Data
762 are displayed as described for **Figure 3C**. Asterisks: $p < 0.0001$.
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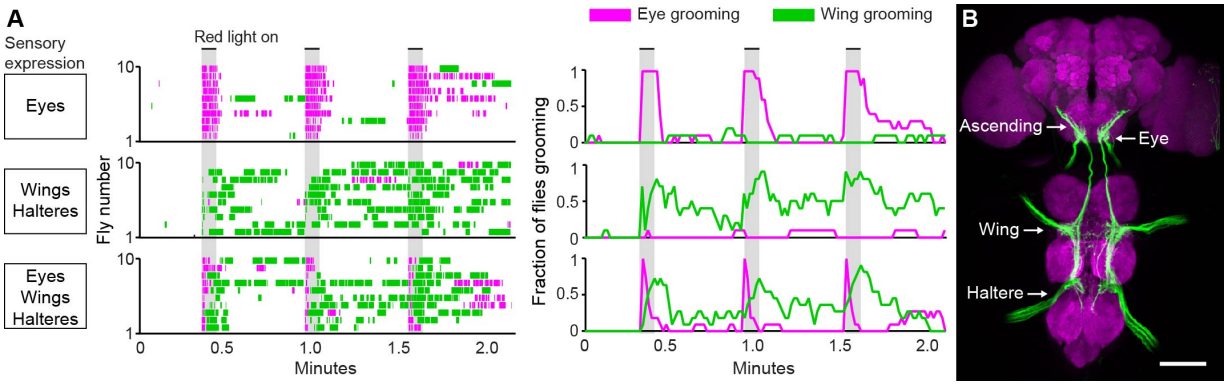


Figure 5. Simultaneous excitation of eye and wing/haltere sensory neurons produces sequential grooming. (**A**) Ethograms (left) and histograms (right) showing eye grooming (magenta) or wing grooming (green) elicited with red light-activated CsChrimson expressed in different transgenic lines. The lines express in sensory neurons on the eyes (VT17251-LexA (top row)), wings and halteres (R30B01-AD ∩ R31H10-DBD (middle row)), or eyes, wings, and halteres (R31H10-AD ∩ R34E03-DBD (bottom row)). Data is plotted as described in **Figure 2**. See **Video 6**, **Video 7**, **and Video 8** for representative examples. (**B**) GFP expression pattern of R31H10-AD ∩ R34E03-DBD in the CNS. Image shows a maximum intensity projection of a co-stain with anti-GFP (green) and anti-Bruchpilot (magenta). Arrows indicate the body part each sensory projection is from, and the location of ascending projections from the wings and halteres. Scale bars, 100 μm.

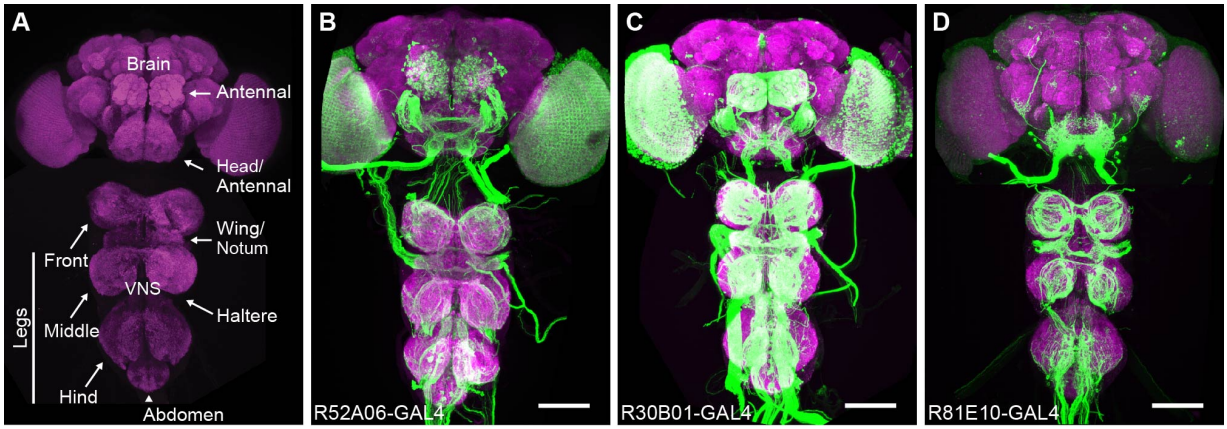


Figure 1 – figure supplement 1. Anatomy of sensory GAL4 lines. (**A**) Sensory neurons project their axons (afferents) to specific regions of the *central nervous system* (CNS) depending on which body part they are from. The neuropile of the CNS visualized with anti-Bruchpilot (magenta). A confocal image maximum projection is shown. (**B-D**) Three different GAL4 lines expressing GFP in afferent projections from the different body parts. GFP was visualized with anti-GFP antibodies (green). Scale bar, 100 µm. GAL4 lines shown are R52A06-GAL4 (**B**), R30B01-GAL4 (**C**), and R81E10-GAL4 (**D**).

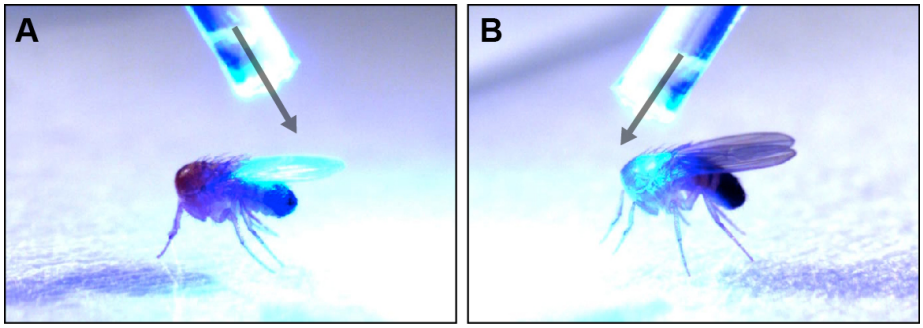


Figure 1 – figure supplement 2. Optogenetic illumination of sensory neurons on different body regions. (**A-B**) A fiber optic probe that was connected to a blue light LED was used to direct light to specific body regions. Images show illumination of the posterior (**A**) or anterior (**B**) dorsal surfaces of decapitated flies. See **Video 1** and **Video 2** for representative examples.

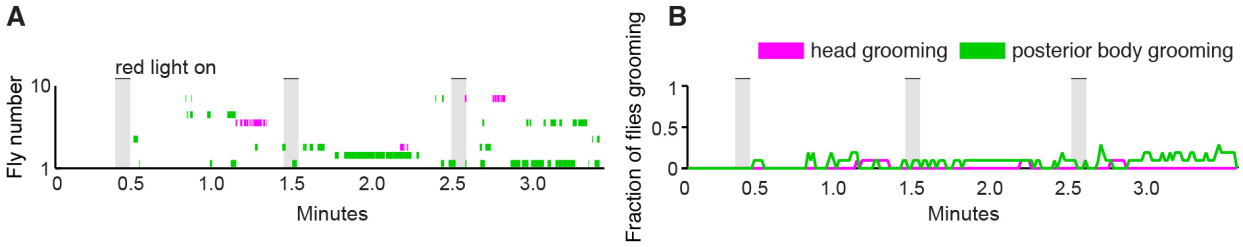
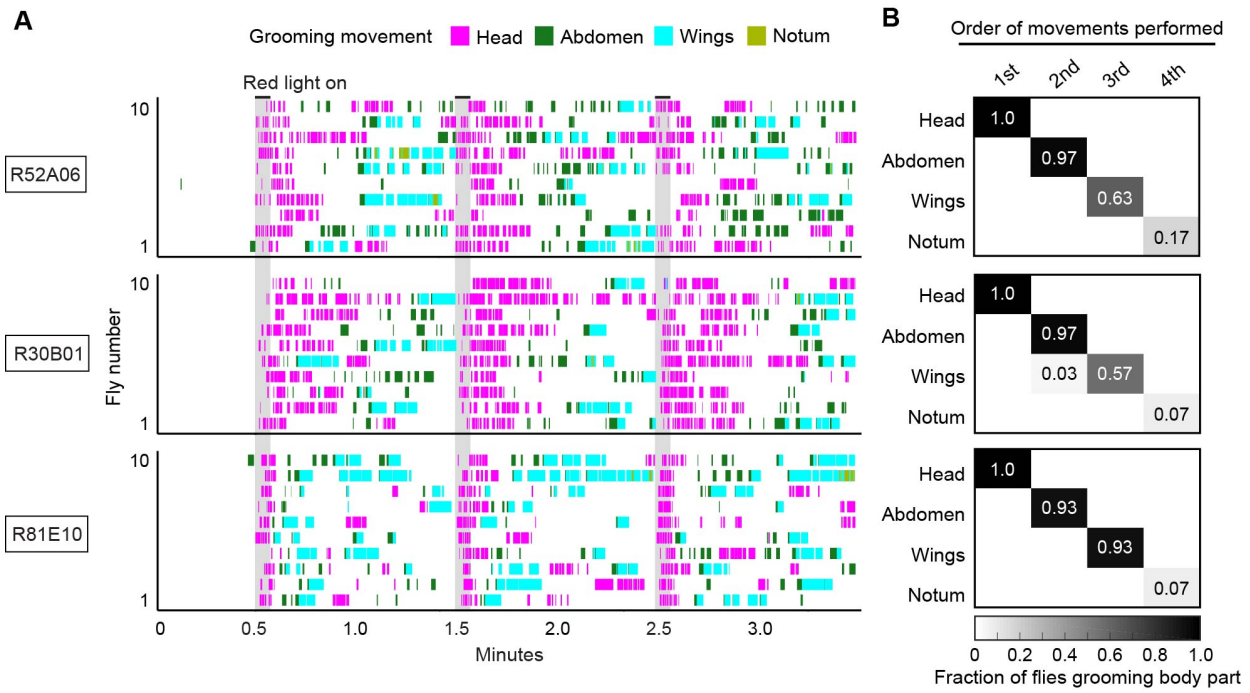


Figure 2 – figure supplement 1. Illumination of control flies does not elicit grooming. (**A**) Ethograms showing head (magenta) or posterior body grooming (green) with red light-illumination of control flies. Ethograms of individual flies are stacked on top of each other. (**B**) Histogram shows the fraction of flies that were performing each grooming movement within one-second time bins. Gray bars indicate a five second presentation of red light. This is the control for the experiment shown in **Figure 2**.



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841 **Figure 2 – figure supplement 2. Simultaneous optogenetic activation of body sensory**
842 **neurons elicits a grooming sequence.** (**A**) Ethograms showing different grooming movements
843 elicited with red light-illumination of R52A06-, R30B01-, or R81E10-GAL4 flies expressing
844 CsChrimson. Ethograms of ten individual flies are stacked for each GAL4 line. Gray bars
845 indicate five-second presentations of a red light stimulus. Colors indicating the grooming
846 movements are shown above the ethograms. The same ethograms with binned head and
847 posterior grooming movements are shown in **Figure 2A**. (**B**) Grids show the fraction of flies
848 performing a specific grooming movement as their first, second, third, or fourth novel movement
849 from the onset of the red light stimulation until the beginning of the next red light stimulus. The
850 fraction of flies performing notum grooming is low because most flies did not perform that
851 grooming movement. Notum grooming was similarly rare with dust induced grooming (Seeds et
852 al. 2014).

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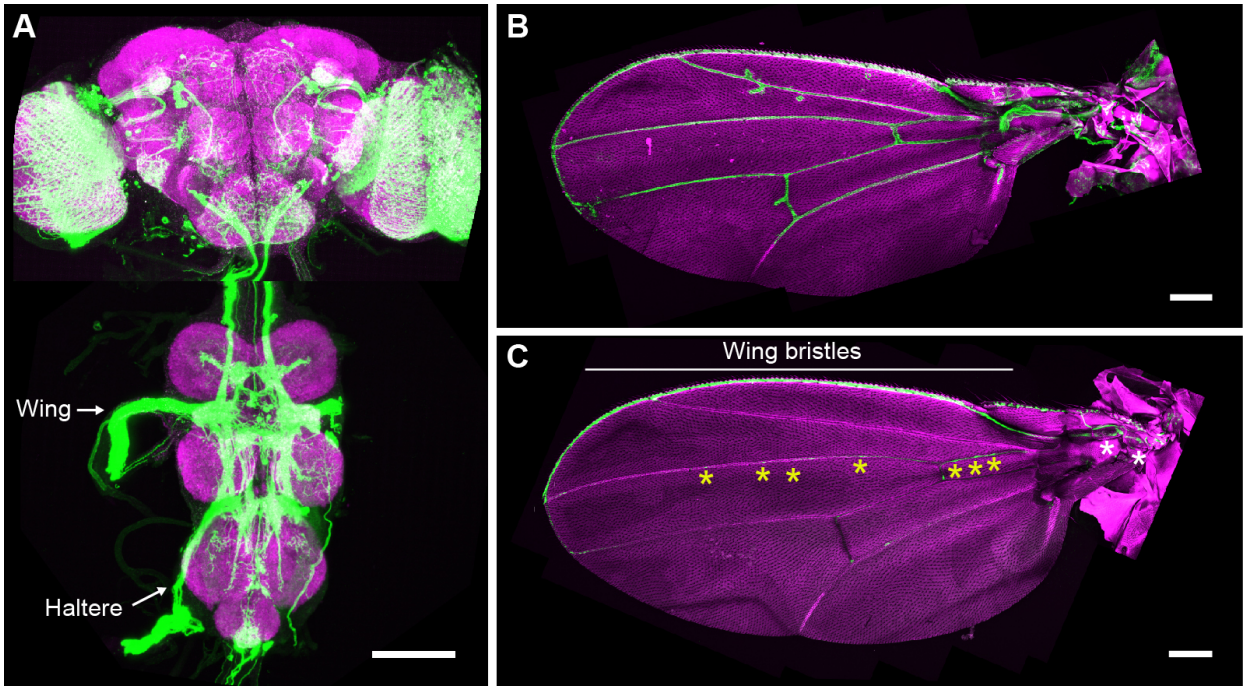

Figure 4 – figure supplement 1. GAL4 lines that express in wing sensory neurons. CNS (**A**) and wing (**B**) expression patterns of R31H10-GAL4 that targets neurons whose activation elicits wing grooming. Maximum intensity projections are shown. (**A**) The CNS expression is visualized by co-staining with anti-GFP (green) and anti-Bruchpilot (magenta). Arrows indicate the CNS entry points of afferents from the wings and halteres. Scale bar, 100 μm. (**B**) Wing expression pattern visualized using the native GFP fluorescence in green and autofluorescence from the cuticle in magenta. Scale bar, 250 μm. (**C**) Wing expression pattern of R30B01-GAL4. The different symbols indicate the sensory neuron types on the wing as proximal campaniform sensilla (white asterisks), distal campaniform sensilla (yellow asterisks), or bristle mechanosensory (white line). Scale bar, 250 μm.