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

2   ***Drosophila*.**

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12   **Abstract**

13   A central model that describes how behavioral sequences are produced features a neural

14   architecture that readies different movements simultaneously, and a mechanism where

15   prioritized suppression between the movements determines their sequential performance. We

16   previously described a model whereby suppression drives a *Drosophila* grooming sequence that

17   is induced by simultaneous activation of different sensory pathways that each elicit a distinct

18   movement (Seeds et al. 2014). Here, we confirm this model using transgenic expression to

19   identify and optogenetically activate sensory neurons that elicit specific grooming movements.

20   Simultaneous activation of different sensory pathways elicits a grooming sequence that

21   resembles the naturally induced sequence. Moreover, the sequence proceeds after the sensory

22   excitation is terminated, indicating that a persistent trace of this excitation induces the next

23   grooming movement once the previous one is performed. This reveals a mechanism whereby

24   parallel sensory inputs can be integrated and stored to elicit a delayed and sequential grooming

25   response.

26

27 **Introduction**

28 A major question about nervous system function is how different movements are assembled to

29 form behavioral sequences. One of the primary models of sequential behavior is reminiscent of

30 how animals select among competing behavioral choices. Behavioral competition arises in

31 situations where different mutually exclusive behaviors are appropriate, but they must be

32 performed one at a time (Houghton and Hartley 1995; Redgrave, Prescott, and Gurney 1999).

33 These conflicts can be resolved through the suppression of all but the highest priority behavior,

34 as mollusks do to suppress their mating behavior while feeding (Davis 1979; Kupfermann and

35 Weiss 2001; Kristan 2008). In the case of a behavioral sequence, it is proposed that the

36 different movements to be performed are similarly readied in parallel and in competition for

37 output, and a suppression hierarchy determines their priority order of execution (Lashley 1951;

38 Houghton and Hartley 1995; Bullock 2004). Completion of the highest priority movement lifts

39 suppression on movements of lower priority that are subsequently performed according to a

40 new round of competition and suppression. This *parallel model* could drive behaviors across a

41 range of complexity, from the sequential typing of letters on a keyboard in humans to the

42 selection of which behavior to perform first in mollusks (Houghton and Hartley 1995). Thus, the

43 identification of examples of simple parallel neural architectures that drive a prioritized selection

44 of movements may inform a broad spectrum of sequential behaviors (Kristan 2014; Jovanic et

45 al. 2016).

46

47 A *Drosophila melanogaster* grooming sequence provides one example of how conflicting

48 stimuli can induce movement competition that is resolved through a suppression hierarchy.

49 Coating the body of a fly with dust is thought to stimulate competition among different grooming

50 movements that are each responsible for cleaning a particular body part (Phillis et al. 1993;

51 Seeds et al. 2014). We previously presented evidence that the body grooming order is

52 determined through a mechanism where earlier movements suppress later ones (Seeds et al.

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53   2014). For example, removal of dust from the eyes occurs first because eye grooming

54   suppresses cleaning of the other body parts. From a suppression hierarchy among the different

55   grooming movements emerges a sequence that proceeds in the order: eyes > antennae >

56   abdomen > wings > notum. We further proposed a computational model to describe this

57   sequence that features parallel activation of the different grooming movements by dust to

58   induce competition, and hierarchical suppression among the movements to determine their

59   selection order (Seeds et al. 2014). The parallel activation of the movements was proposed

60   based on evidence that stimulation to each body part induces site-directed grooming responses

61   (Vandervorst and Ghysen 1980; Corfas and Dudai 1989; Seeds et al. 2014; Hampel et al.

62   2015). Thus, the simultaneous, or parallel stimulation of sensory neurons by dust would cause

63   different grooming movements to compete for output because only one can be performed at a

64   time. However, it was not confirmed that simultaneous activation of sensory neurons across the

65   body indeed elicits the same prioritized grooming response that we observed using a dust

66   stimulus.

67

68   Here, we reveal a neural basis for parallel activation of sensory inputs for a sequential

69   behavior by identifying sensory neurons that stimulate different grooming movements, and by

70   testing the hypothesis that activation of these neurons in parallel elicits a prioritized grooming

71   response. We identify transgenic expression tools for visualizing and optogenetically activating

72   sensory neurons on the body parts that elicit specific grooming movements. This enables the

73   simultaneous activation of sensory neurons across the body to induce competition among their

74   respective grooming movements. As we observed by coating the bodies of flies in dust, whole-

75   body sensory activation elicits grooming that prioritizes the head and then proceeds to the other

76   body parts. This provides direct evidence that the grooming sequence can be induced through

77   simultaneous activation of sensory neurons across the body. These experiments also reveal

78   that flies have a persistent trace of the body parts that were stimulated, which results in delayed

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79   and sequential grooming of the stimulated parts. Work presented here lends neural-based

80   evidence to the parallel model of hierarchical suppression among grooming movements and

81   provides new insights into its underlying organization.

82

83   **Results**

84   ***GAL4 lines targeting sensory neurons across the body that elicit grooming***

85   Our initial goal was to identify GAL4 transgenic lines expressing in sensory neurons across the

86   body, to directly test whether simultaneous activation of these neurons leads to a prioritized

87   grooming response. As an entry point, we examined a collection of previously identified

88   enhancer-driven GAL4 lines that express in different neuronal populations whose activation

89   drove grooming (Seeds et al. 2014). Confocal microscopy imaging of the *peripheral nervous*

90   *system* (PNS) expression patterns of different lines from this collection revealed three that

91   express in sensory neurons across the body (R52A06-, R30B01-, and R81E10-GAL4; **Figure**

92   **1A-F**, R52A06-GAL4 shown as an example, **Figure 1 – figure supplement 1A-D**). We

93   classified the different sensory neuron types based on previous anatomical descriptions

94   (Murphey et al. 1989; Cole and Palka 1982; Dickinson and Palka 1987; Smith and Shepherd

95   1998; Kays, Cvetkovska, and Chen 2014) and found that the lines express predominantly in

96   mechanosensory neurons (**Figure 1G**). However, R30B01-GAL4 also showed expression in

97   chemosensory neurons (**Figure 1G**).

98

99   We next tested whether local populations of sensory neurons on specific body regions

100 can elicit individual grooming movements when focally activated. Site-directed grooming

101 responses have previously been investigated using tactile stimulation to particular

102 mechanosensory bristles on the body surface of decapitated flies (Vandervorst and Ghysen

103 1980; Corfas and Dudai 1989). Here, we used optogenetic activation with Channelrhodopsin,

104 directing blue light via an optical fiber to particular body regions of the broad sensory GAL4 lines

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105   to activate sensory neurons on either the dorsal anterior or posterior body regions of

106   decapitated flies (**Figure 1 – figure supplement 2A,B**). Light directed to the posterior dorsal

107   body surface elicited grooming of the wings, whereas illumination of the anterior dorsal surface

108   elicited grooming of the notum (**Figure 1H**, **Video 1**, **Video 2**). This indicated that site-directed

109   grooming responses can be elicited optogenetically, and that the GAL4 lines express in sensory

110   neurons whose activation can elicit grooming movements for at least two parts of the body.

111

112   ***Simultaneous excitation of sensory neurons across the body induces a grooming***

113   ***sequence***

114   The GAL4 lines described above were next used to test a prediction of the model of hierarchical

115   suppression that simultaneous activation of sensory neurons across the body elicits head

116   grooming preferentially (Seeds et al. 2014). Freely moving flies of each line expressing the red

117   light-gated neural activator CsChrimson were exposed to whole body illumination to

118   optogenetically activate their targeted sensory neurons, and grooming responses were

119   subsequently measured. Each of the three GAL4 lines expresses in sensory neurons whose

120   activation can elicit wing or notum grooming, as revealed by localized optogenetic activation

121   (**Figure 1G,H**). Additionally, each line expresses in eye bristle mechanosensory neurons whose

122   activation we hypothesized could elicit eye grooming, while two of the lines (R52A06- and

123   R30B01-GAL4) also express in antennal Johnston's Organ neurons that were previously shown

124   to elicit antennal grooming (Hampel et al. 2015). Although these GAL4 lines can elicit several

125   movements from different body sensory neurons, we predicted that activating them

126   simultaneously should elicit only the highest-priority movement, according to the hierarchical

127   suppression model. Indeed, the simultaneous optogenetic activation of body sensory neurons

128   targeted by each GAL4 line resulted in head rather than posterior (abdomen, wing, notum)

129   grooming, consistent with the model of hierarchical suppression (**Figure 2A**, during red light-on

130   period).

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132   Optogenetic activation of sensory neurons across the body also elicited a grooming

133   sequence reminiscent of dust-induced grooming. Flies groomed their heads at the onset of a

134   five-second red light stimulus, and then transitioned to grooming their posterior bodies during

135   the period after the light was turned off (**Figure 2A**). One trivial explanation for this sequence

136   could be that optogenetic activation of sensory neurons on the posterior body elicited grooming

137   with a latency, whereas there was no latency to groom with activation of the head sensory

138   neurons. We tested for this latency to groom the posterior body using decapitated flies that no

139   longer received a sensory drive to groom their heads. In contrast to intact flies, activation of the

140   posterior body sensory neurons of decapitated flies elicited posterior grooming during the red

141   light (**Figure 2B**). Thus, a latency does not explain the sequence because head and posterior

142   grooming can be elicited on similar time scales. Instead, evidence that intact flies do not display

143   posterior grooming with the light stimulation supports the hypothesis that it is suppressed by

144   head grooming (discussed below). Notably, optogenetic activation of sensory neurons across

145   the body causes flies to groom their bodies in the same order as when they were coated in dust

146   (head > abdomen > wings > notum) (**Figure 2 – figure supplement 2A,B**, **Video 3**, **Video 4**,

147   and **Video 5**). Further, the posterior body grooming sequence continued through the minute

148   after the cessation of the red light, while the sensory neurons were no longer activated (**Figure**

149   **2A**, green histogram). This suggests a persistent trace of posterior sensory neurons that had

150   been activated, which allowed each movement to be elicited once the previous grooming

151   movement terminated.

152

153   The behavior resulting from simultaneous activation of sensory neurons across the body

154   supports a role of suppression in establishing the grooming movement hierarchy. Evidence of

155   suppression was found when sensory neurons were reactivated during the period when flies

156   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

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183   by expressing CsChrimson using VT17251-LexA and exposing flies to red light. Indeed,

184   optogenetic activation of the eye bristle mechanosensory neurons elicited eye grooming (**Figure**

185   **3C**).

186

187   We next acquired a transgenic driver line for manipulating sensory neurons that could

188   elicit wing grooming, which is lower in the hierarchy than eye grooming. From our previous

189   screen (Seeds et al. 2014), we identified a GAL4 line that expresses in neurons whose

190   activation could elicit wing grooming and showed expression in sensory neurons on the wings

191   (**Figure 4 – figure supplement 1A**, R31H10-GAL4, behavioral data not shown). However, the

192   identities of those sensory neurons were obscured by expression in other cells (**Figure 4 –**

193   **figure supplement 1B**). Therefore, we used the intersectional *Split GAL4* (spGAL4) technique

194   to restrict expression to only the sensory neurons (Luan et al. 2006; Pfeiffer et al. 2010).

195   spGAL4-mediated expression occurs only when the two GAL4 domains, the GAL4 *DNA binding*

196   *domain* (DBD) and the *transcriptional activation domain* (AD), are expressed in the same cells.

197   We generated spGAL4 flies that were anticipated to target the wing sensory neurons by

198   expressing the DBD in the pattern of R31H10-GAL4 and the AD in the pattern of R30B01-GAL4

199   (**Figure 4 – figure supplement 1B,C**).

200

201   The R30B01-AD ∩ R31H10-DBD combination expresses in two main types of

202   mechanosensory neurons on the wings and halteres (**Figure 4A**). The first type includes

203   campaniform sensilla, which are dome-shaped structures on the fly cuticle that are each

204   innervated by a mechanosensory neuron that responds to deformations of the cuticle (Dickinson

205   and Palka 1987). Campaniform sensilla on the proximal part of the wing are largely clustered in

206   fields, whereas individual sensilla are found along the distal wing (Palka, Lawrence, and Hart

207   1979; Cole and Palka 1982; Palka et al. 1986; Dickinson and Palka 1987). R30B01-AD ∩

208   R31H10-DBD flies show a sparse labeling of neurons in the proximal fields (5 to 10 out of ~77

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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

234   rest periods with no light. Activation of the eye bristle mechanosensory neurons elicited eye

235   grooming during the period when the red light was on that decayed when it turned off (**Figure**

236   **5A**, top, magenta). In contrast, activation of the wing/haltere sensory neurons elicited grooming

237   with the red light that persisted after light cessation (**Figure 5A**, middle, green). Importantly,

238   activation of either the eye bristle mechanosensory neurons or the wing sensory neurons alone

239   did not elicit the other corresponding grooming movement, or an anterior-to-posterior grooming

240   sequence. Thus, activation of these specific sensory types only elicits grooming of its

241   corresponding body part.

242

243   We next tested whether activation of the eye bristle mechanosensory neurons and

244   wing/haltere sensory neurons at the same time would elicit a prioritized eye grooming response,

245   as is predicted by the model. For this experiment, we identified a spGAL4 combination

246   (R31H10-AD ∩ R34E03-DBD) that expressed both in the eye bristle mechanosensory neurons

247   and the same three categories of sensory neurons on the wings and halteres that were

248   expressed in the R30B01-AD ∩ R31H10-DBD combination (**Figure 5B**). Simultaneous

249   optogenetic activation of these defined sensory neurons elicited prioritized grooming that started

250   with the eyes and then proceeded to the wings (**Figure 5A**, bottom), like what we observed with

251   activation of sensory neurons across the body (**Figure 2**). We also found evidence of

252   suppression by eye grooming, as ongoing grooming of the wings terminated and all flies

253   groomed their eyes with each red light stimulus (**Figure 5A**, bottom). These results demonstrate

254   the prioritization between grooming movements through direct optogenetic activation of the

255   sensory neurons that elicit grooming of specific body parts. This strengthens the conclusion of

256   our previous work that the sequence occurs when the grooming movements are activated in

257   parallel and then sequentially prioritized through hierarchical suppression (Seeds et al. 2014).

258

259   **Discussion**

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260 The goal of this work was to test the prediction of the model of hierarchical suppression that

261 simultaneous activation of sensory neurons on different body parts elicits a prioritized grooming

262 response. Two lines of evidence led us to this prediction. The first was based on our previous

263 finding that coating the body of the fly in dust elicits grooming that prioritizes head over posterior

264 body grooming (Seeds et al. 2014). The second was based on data showing that local

265 stimulation to the body surface elicits site-specific grooming responses (Vandervorst and

266 Ghysen 1980; Corfas and Dudai 1989; Seeds et al. 2014; Hampel et al. 2015). Thus, we

267 proposed that sensory neurons across the body are stimulated in parallel by dust to elicit

268 competition among their respective grooming movements. Here, we test this by identifying

269 transgenic driver lines for targeting and directly activating sensory neurons that elicit grooming,

270 allowing us to bypass the dust stimulus and reveal the underlying sensory neurons. Using

271 simultaneous optogenetic activation of sensory neurons across the body we observe the same

272 anterior-to-posterior prioritization among the grooming movements that occurs when flies are

273 coated in dust. This lends strong support to the hypothesis that the grooming movements are

274 activated in parallel, and are thus selected in a hierarchically determined competition through

275 suppression.

276

277 ***Sensory neurons involved in grooming behavior***

278 One aim of this work was to identify sensory neurons that can induce grooming behavior. The

279 bristles are canonically thought to be involved in insect grooming based on evidence that their

280 tactile stimulation on different body parts induces site directed grooming responses

281 (Vandervorst and Ghysen 1980; Corfas and Dudai 1989; Page and Matheson 2004). Here, we

282 provide evidence that direct activation of the bristle mechanosensory neurons can elicit

283 grooming. We identify the fruit fly interommatidial bristle mechanosensory neurons based on

284 their anatomical similarity to those of the praying mantis and cricket (Honegger, Reif, and Müller

285 1979; Zack and Bacon 1981). Next, we use a transgenic driver line that expresses in these

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286   neurons to show that their optogenetic activation elicits eye grooming. We also identified

287   different spGAL4 lines that express in neurons whose activation elicits wing grooming. However,

288   these lines express both in bristle mechanosensory neurons and campaniform sensilla, raising

289   the question of whether one or both sensory types are involved. Given the wealth of data

290   implicating the bristles in grooming (Tuthill and Wilson 2016), the parsimonious explanation is

291   that the wing bristle mechanosensory neurons are involved. However, there is also a precedent

292   for the involvement of non-bristle mechanosensory neurons such as the campaniform sensilla.

293   For example, we previously showed that Johnston's Organ chordotonal neurons can detect

294   displacements of the antennae to induce antennal grooming (Hampel et al. 2015), and others

295   have shown that gustatory neurons on the wing can detect different chemicals to trigger

296   grooming (Yanagawa, Guigue, and Marion-Poll 2014). Therefore, further work is required to

297   resolve which sensory neurons are involved in wing grooming.

298

299   One outstanding question is whether the sensory neurons have a direct role in

300   establishing hierarchical suppression. We previously proposed two mechanisms of hierarchical

301   suppression (Seeds et al. 2014). One is that unidirectional inhibitory connections between the

302   movements drive suppression, a mechanism not likely to involve the sensory neurons. The

303   other is that differences in sensitivity to dust across the body establish a gradient of sensory

304   drives among the grooming movements, leading to suppression through winner-take-all

305   competition. One way that sensitivity differences could be established is through differing

306   numbers of receptors on each body part. For example, if we assume that the bristle

307   mechanosensory neurons on the different body parts detect dust to elicit grooming (which

308   remains to be shown), a comparison of bristle numbers on different body parts gives mixed

309   support for this hypothesis. There are 600, 221, and 235 bristles reported to be on the eyes,

310   wings, and notum respectively (Hartenstein and Posakony 1989; Cadigan, Jou, and Nusse

311   2002). The eyes are the highest priority part to be groomed, and have 2.7 times more bristles

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312   than the wings, which is consistent with the suppression hierarchy. In contrast, the lowest

313   priority body part is the notum, which has more bristles than the wings, arguing against the

314   hypothesis. Furthermore, given that other sensory neuron types elicit grooming (e.g.

315   chordotonal and gustatory neurons), there may be multiple ways of detecting dust (Yanagawa,

316   Guigue, and Marion-Poll 2014; Hampel et al. 2015). Alternatively, hierarchical suppression

317   could be established at the level of sensory neurons by regulating their output through

318   presynaptic inhibition (Blagburn and Sattelle 1987; Burrows and Matheson 1994; Clarac and

319   Cattaert 1996; Rudomin and Schmidt 1999). For example, the feeding behavior of the medicinal

320   leech causes presynaptic inhibition of mechanosensory neurons, which suppresses touch-

321   induced behavioral responses (Gaudry and Kristan 2009). Future experiments will test such

322   hypotheses about whether hierarchical suppression is established at the level of sensory

323   neurons.

324

325   ***Persistent neural activity within grooming neural circuits***

326   Emerging behavioral evidence indicates that neural circuits controlling *Drosophila* grooming

327   movements have mechanism(s) for maintaining excitability. This was originally proposed from a

328   study identifying a mechanosensory circuit that elicits persistent grooming of the antennae

329   (Hampel et al. 2015). That is, neurons within this circuit elicit grooming that continues for tens of

330   seconds beyond their optogenetic activation. Work presented here reveals that activation of

331   wing sensory neurons similarly elicits persistent grooming. Interestingly, grooming responses

332   that outlast their stimulus have also been described in vertebrates, suggesting that persistence

333   is a common feature of grooming (Sherrington 1906; Stein 2005). Despite the prevalence of

334   persistent grooming, its biological function remains unclear. One possibility is that persistence

335   prevents unnecessary switches between behaviors (Redgrave, Prescott, and Gurney 1999); for

336   example swimming responses can last beyond the initial stimulus so that an animal can safely

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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

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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).

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389   Transgenic flies carrying the following UAS drivers were from the following citations:

390   *UAS-dTrpA1* (Hamada et al. 2008), 20x*UAS-mCD8::GFP* (pJFRC7) (Pfeiffer et al. 2010),

391   13xLexAop-myr::GFP (pJFRC19) (Pfeiffer et al. 2010), *UAS-Channelrhodopsin-2* (Hwang et al.

392   2007), and 20x*UAS-CsChrimson* (attP18) (Klapoetke et al. 2014). GAL4, spGAL4, and LexA

393   lines were crossed to their respective UAS or LexAop drivers, and the progeny were reared on

394   cornmeal and molasses food at 21°C and 50% relative humidity using a 16/8-hour light/dark

395   cycle. For optogenetic experiments using CsChrimson or ChR2, flies were reared in the dark on

396   food containing 0.4 mM all-*trans*-retinal. All experiments were done with five to eight day old

397   males.

398

399   ***Channelrhodopsin-mediated activation of sensory neurons using decapitated flies***

400   Two different regions of the bodies of decapitated flies were illuminated to locally activate

401   sensory neurons expressing ChR2. For decapitations, flies were cold-anesthetized, decapitated

402   using a standard razor blade, and allowed to recover for 10-20 minutes. The flies were

403   positioned on a slide for the experiment using a fine paint brush. A 473-nm blue light LED

404   (Nichia Corp, Tokushima, Japan) was attached to an optical fiber (1 mm in diameter) to direct

405   light to a specific region on the fly. The optical fiber was held approximately 1 mm from the

406   target body region to deliver a blue light stimulus with a luminance of 0.075 mW/mm^2. The light

407   was directed towards the dorsal posterior region, away from the anterior body, to activate

408   sensory neurons primarily on the wings (**Figure 1 – figure supplement 2A)**. Alternatively, the

409   light was directed towards the dorsal anterior region, away from the posterior body, to activate

410   sensory neurons primarily on the notum (**Figure 1 – figure supplement 2B)**. The LED stimulus

411   was controlled using a Grass SD9 stimulator (Astro-Med Inc., Warwick, RI) that delivered 10 Hz

412   pulses that were 20 milliseconds in duration, with 8-millisecond delays between pulses. Each fly

413   was subjected to stimulation on each body region in random order; however, in some cases the

414   flies would jump during the experiment and could not be used further. A grooming response to

16

415   the illuminated body region within a ten-second time frame was scored as a positive response.

416   The fraction of flies that responded was plotted. The number of trials for each dorsal body

417   region for each line were: R52A06-GAL4, anterior (n = 100), posterior (n = 100); R30B01-GAL4,

418   anterior (n = 40), posterior (n = 61); R81E10-GAL4, anterior (n = 86), posterior (n = 89).

419   Statistical significance was addressed using Chi-Square tests and Bonferroni correction.

420

421   ***CsChrimson-mediated activation of sensory neurons using freely moving flies***

422   The camera and behavioral setups used for recording freely moving flies with optogentic

423   activation were described previously (Seeds et al. 2014; Hampel et al. 2015). Flies were cold

424   anesthetized, loaded into behavioral chambers, and allowed to recover for at least ten minutes.

425   R52A06-, R30B01-, and R81E10-GAL4 were used to express the light-gated channel

426   CsChrimson. The red light used for gating CsChrimson readily penetrates the fly cuticle (Inagaki

427   et al. 2014), allowing for uniform activation of sensory neurons across the body. Our initial

428   experiments using optogenetic activation of the neurons targeted by these GAL4 lines revealed

429   that high levels of red light activation caused defects in motor coordination. This was likely

430   caused by the strong activation of sensory neurons across the body, some of which are known

431   to be involved in proprioception (e.g. femoral chordotonal organs). Therefore, it was necessary

432   to reduce the red-light power to the point where it elicited grooming without causing coordination

433   defects. The light power that met these requirements for each GAL4 line are: R52A06-GAL4

434   (0.066 mW/mm^2), R30B01-GAL4 (0.066 mW/mm^2), and R81E10-GAL4 (0.077 mW/mm^2). The

435   light power used for each LexA and spGAL4 line was: VT17251-LexA (0.382 mW/mm^2),

436   R30B01-AD ∩ R31H10-DBD and R31H10-AD ∩ R34E03-DBD (0.135 mW/mm^2). The red light

437   frequency was 5 Hz (0.1 seconds on/off) for 5 seconds, followed by 30 second intervals where

438   the red light was off. The experiment consisted of a total of three photostimulation periods with

439   30 second intervals between each stimulation. The experiment was recorded for manual

440   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

18

467   body part was dissected as described below. The unfixed body parts were imaged immediately

468   in PBS or Vectashield (Vector Laboratories, Burlingame, CA). We used both PBS and

469   Vectashield and did not notice a difference in the cell morphology or expression pattern when

470   using either reagent. The use of Vectashield had the advantage of resulting in fewer air bubbles

471   between the coverslip and sample.

472

473   *Head:* Flies were decapitated using a standard razor blade. Heads were then placed

474   "face up" on a slide in a small well that was made by stacking six reinforcement labels (Avery

475   Dennison Corporation, Brea, CA) and filled with PBS or Vectashield. A cover slip was then

476   placed over the well. *Abdomen:* The abdomen was severed from the rest of the body just

477   posterior to the scutellum. Abdomens were then placed on a slide in a well created as described

478   above. The abdomens were placed either ventral or dorsal side up so that each side could be

479   imaged. *Notum:* A scalpel was used to slice longitudinally between the legs and the dorsal side

480   of the notum. The notum was imaged in the same well preparation described above. *Wing:* A

481   scalpel was used to remove the left wing from the body of the fly. To ensure that the entire wing

482   was obtained, part of the body wall was also cut with the wing. The wing was then placed on a

483   drop of Vectashield and then covered with a coverslip. *Leg:* The left prothoracic leg was

484   dissected in the same way as the wing. All body parts were imaged using a Zeiss 710 confocal

485   microscope using 10x and 20x air objectives. Native GFP fluorescence was imaged using an

486   excitation wavelength of 488 nm, whereas autofluorescence from cuticle was imaged using 568

487   nm. Body parts from at least three flies were imaged from separate crosses on different days. In

488   some cases, the body parts were imaged at 20x and then stitched using a FIJI plugin (Preibisch,

489   Saalfeld, and Tomancak 2009).

490

491   The different sensory neuron types on each body part were classified based on previous

492   descriptions (Ghysen 1980; Cole and Palka 1982; Dickinson and Palka 1987; Murphey et al.

19

493   1989; Smith and Shepherd 1998). The numbers of campaniform sensilla and mechanosensory

494   bristle neurons on the wings were previously counted (Cole and Palka 1982; Dickinson and

495   Palka 1987; Hartenstein and Posakony 1989). Proximal campaniform sensilla described in this

496   work include ANWP, Teg, d.Rad.A, d.Rad.B, d.Rad.C, d.Rad.D, d.Rad.E, d.HCV, v.Rad.A,

497   v.Rad.B, v.Rad.C, v.HCV, and vL.III. Distal campaniform sensilla described in this work include

498   GSR, p.TSM, d.TSM, L3-V, ACV, L3-1, L3-2, and L3-3. We classified neurons on the wings as

499   bristle mechanosensory rather than chemosensory given that their dendrites appear to

500   terminate at the base of the bristle rather than projecting to the bristle tip.

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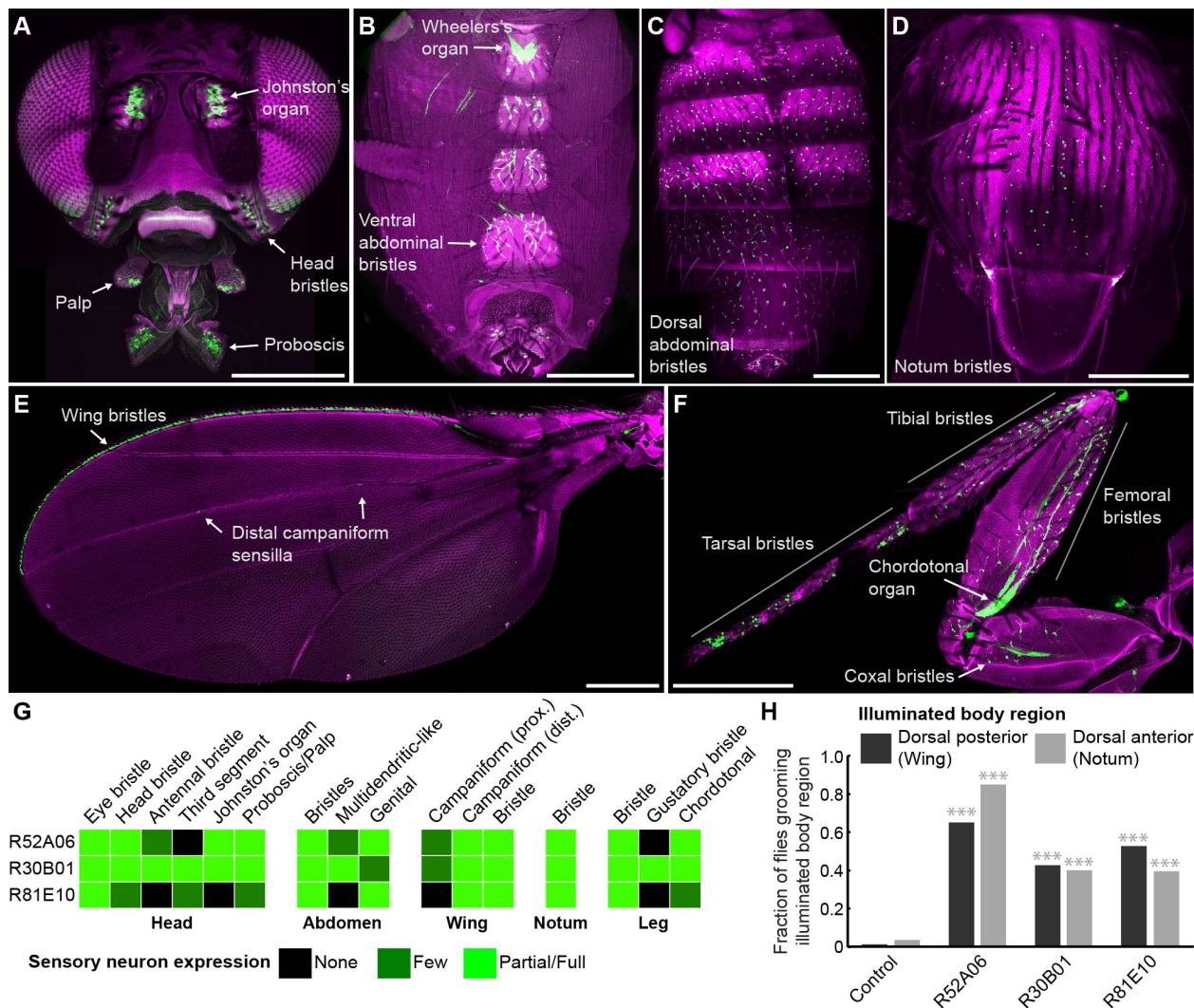
Figure 1. GAL4 lines expressing in sensory neurons whose activation elicits grooming.

(**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

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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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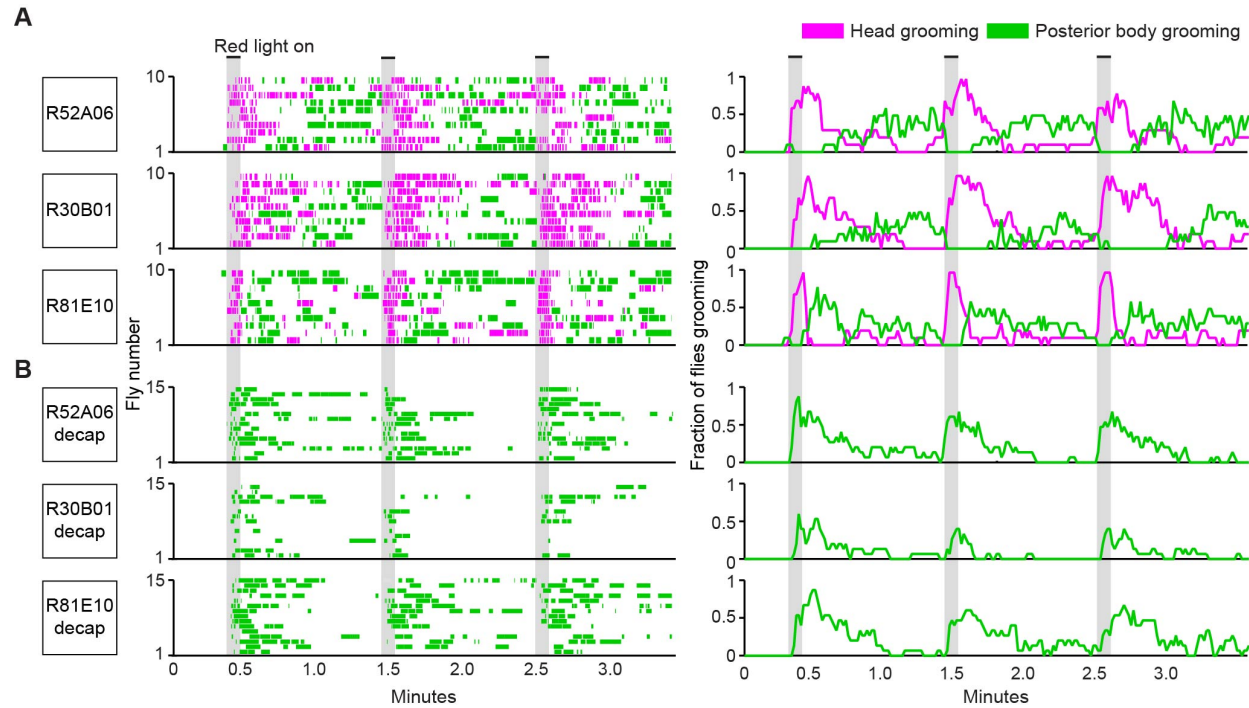
Figure 2. Simultaneous optogenetic activation of body sensory neurons elicits sequential grooming. (**A**-**B**) Head (magenta) or posterior body grooming movements (green) elicited with red light-illumination of R52A06-, R30B01-, and R81E10-GAL4 flies expressing CsChrimson. The movements are mutually 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**).
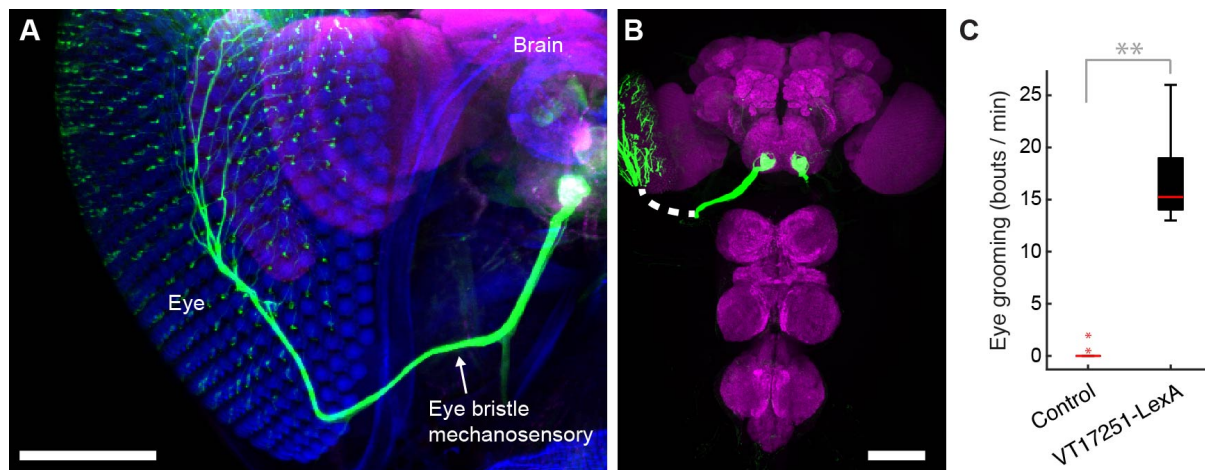
Figure 3. Interommatidial bristle mechanosensory neurons elicit eye grooming. (**A-B**) The expression pattern of VT17251-LexA in eye bristle mechanosensory neurons. The neurons were stained with anti-GFP (green) and the brain neuropile is stained with anti-Bruchpilot (magenta). Both images are maximum 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).

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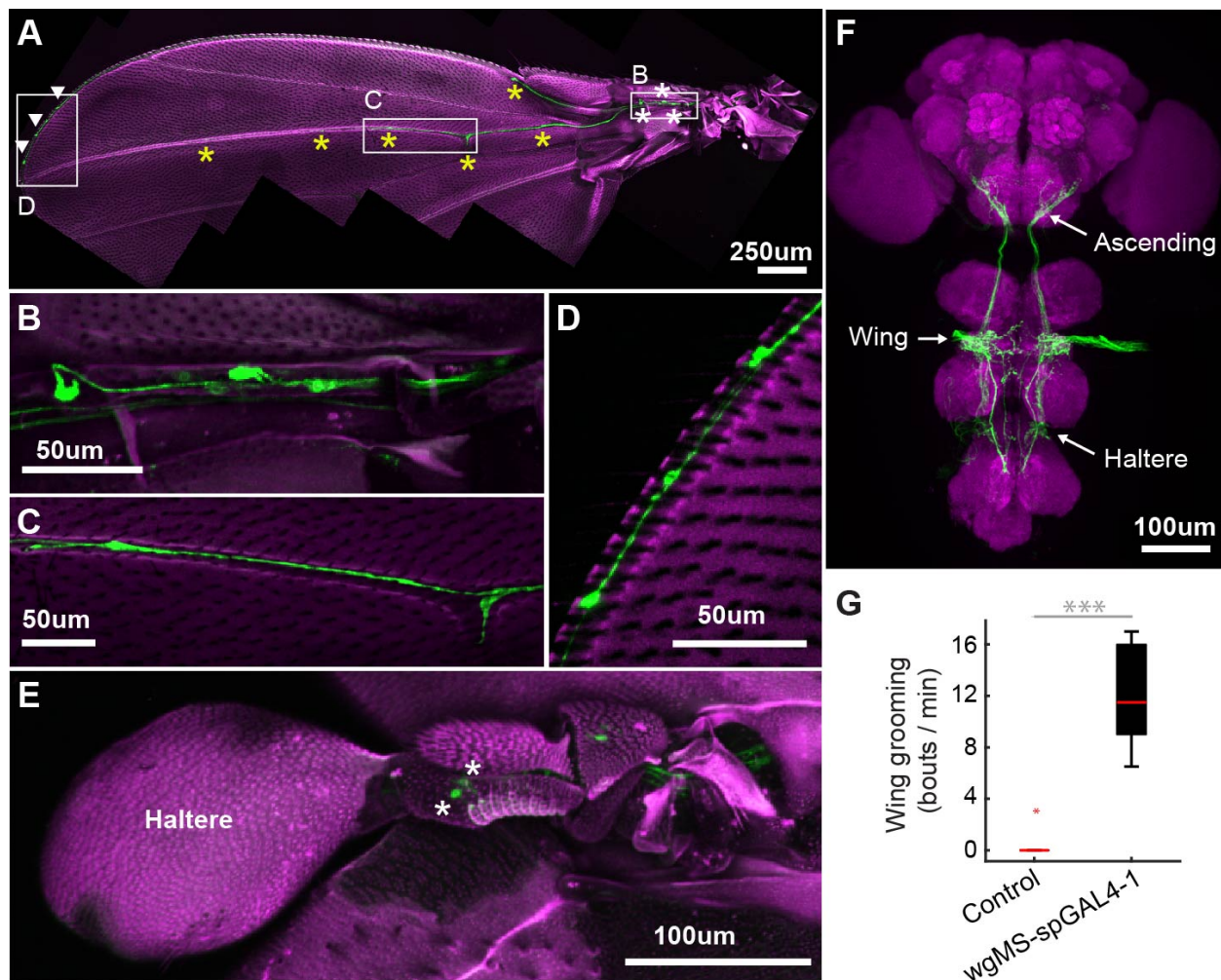
Figure 4. spGAL4 driver that expresses in wing and haltere sensory neurons whose activation elicits wing grooming. (**A-E**) The expression pattern of R30B01-AD ∩ R31H10-DBD in sensory neurons of the wings and halteres. Native GFP fluorescence is shown in green and autofluorescence from the cuticle is 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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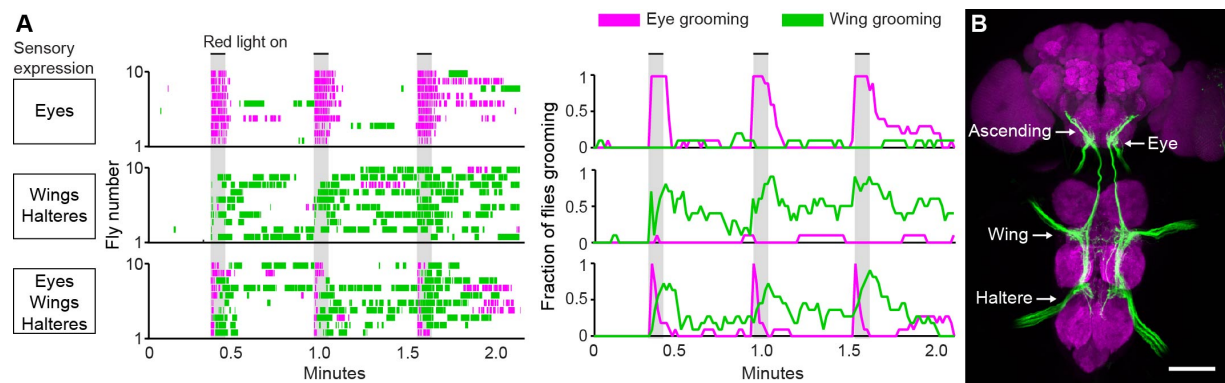
Figure 5. Simultaneous excitation of eye and wing/haltere sensory neurons produces sequential grooming. (**A**) Ethograms (left) and histograms (right) showing eye grooming (magenta) or wing grooming (green) elicited with red light-activated CsChrimson expressed in different transgenic lines. The lines 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.

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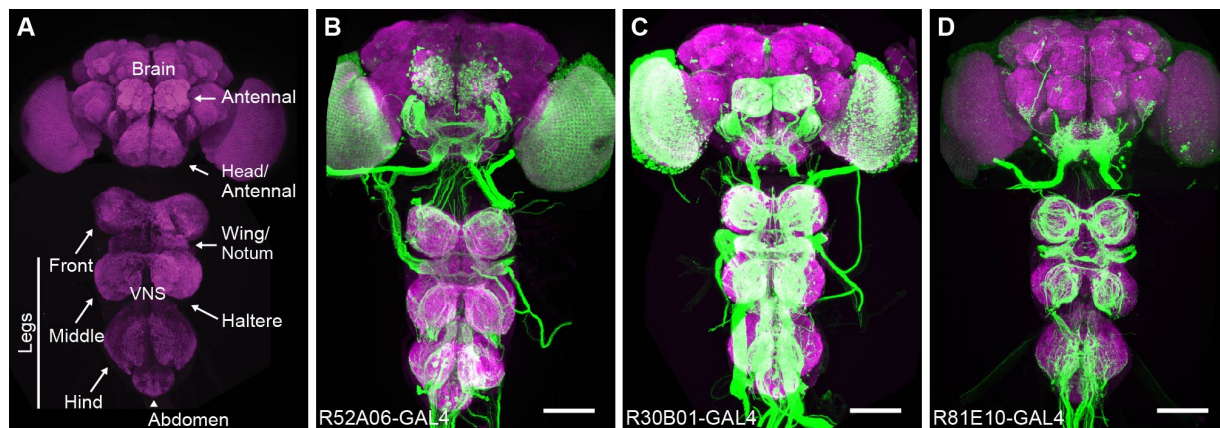
Figure 1 – figure supplement 1. Anatomy of sensory GAL4 lines. (**A**) Sensory neurons project their axons (afferents) to specific regions of the *central nervous system* (CNS) depending on which body part they are from. The neuropile of the CNS visualized with anti-Bruchpilot (magenta). A confocal image 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**).

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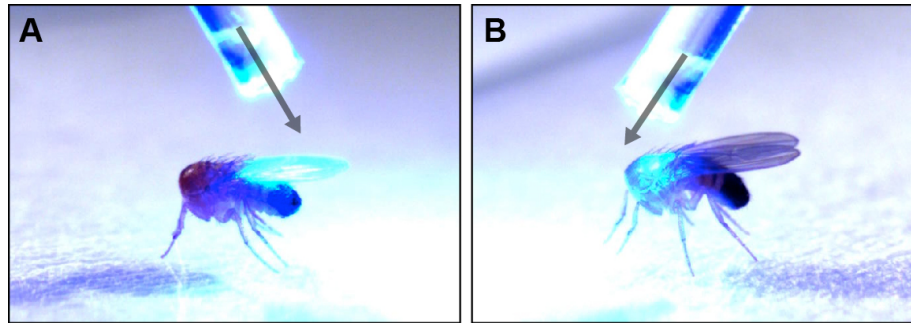
810 **Figure 1 – figure supplement 2. Optogenetic illumination of sensory neurons on different**

811 **body regions.** (**A-B**) A fiber optic probe that was connected to a blue light LED was used to

812 direct light to specific body regions. Images show illumination of the posterior (**A**) or anterior (**B**)

813 dorsal surfaces of decapitated flies. See **Video 1** and **Video 2** for representative examples.

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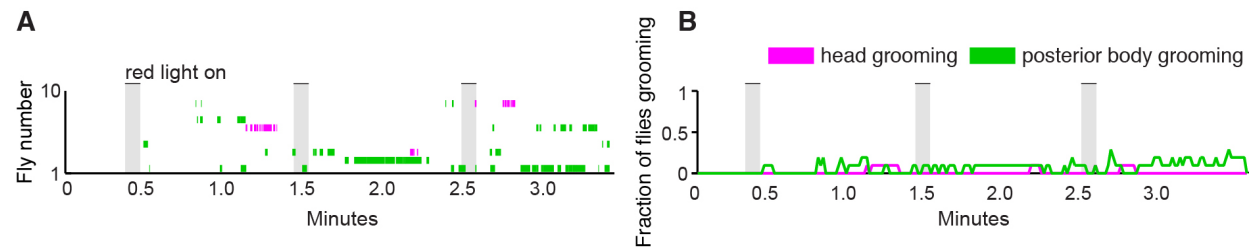
Figure 2 – figure supplement 1. Illumination of control flies does not elicit grooming. (**A**) Ethograms showing head (magenta) or posterior body grooming (green) with red light-illumination of control flies. Ethograms of individual flies are stacked on top of each other. (**B**) Histogram shows the 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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Figure 2 – figure supplement 2. Simultaneous optogenetic activation of body sensory neurons elicits a grooming sequence. (**A**) Ethograms showing different grooming movements elicited with red light-illumination of R52A06-, R30B01-, or R81E10-GAL4 flies expressing CsChrimson. Ethograms of ten individual flies are stacked for each GAL4 line. Gray bars indicate five-second presentations of a red light stimulus. Colors indicating the grooming movements are shown above the ethograms. The same ethograms with binned head and posterior grooming movements are shown in **Figure 2A**. (**B**) Grids show the fraction of flies performing a specific grooming movement as thei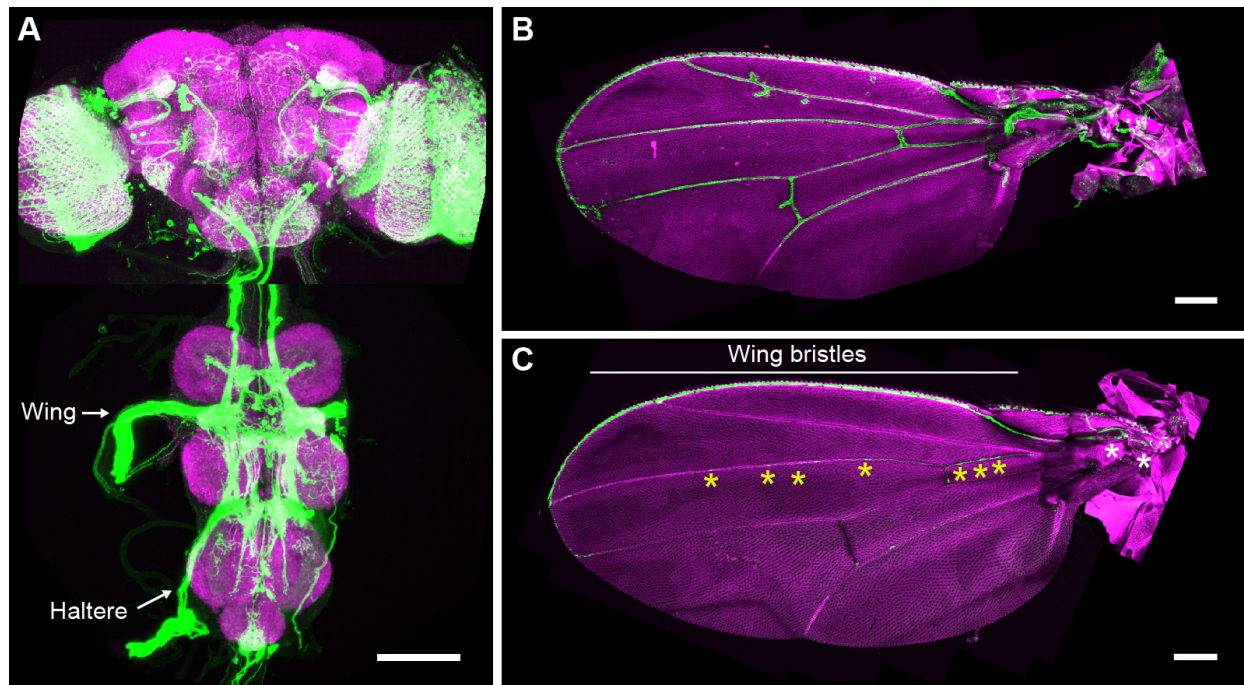r first, second, third, or fourth novel movement from the onset of the red light stimulation until the beginning of the next red light stimulus. The fraction of flies performing notum grooming is low because most flies did not perform that grooming movement. Notum grooming was similarly rare with dust induced grooming (Seeds et al. 2014).

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855 **Figure 4 – figure supplement 1. GAL4 lines that express in wing sensory neurons.** CNS

856 (**A**) and wing (**B**) expression patterns of R31H10-GAL4 that targets neurons whose activation

857 elicits wing grooming. Maximum intensity projections are shown. (**A**) The CNS expression is

858 visualized by co-staining with anti-GFP (green) and anti-Bruchpilot (magenta). Arrows indicate

859 the CNS entry points of afferents from the wings and halteres. Scale bar, 100 µm. (**B**) Wing

860 expression pattern visualized using the native GFP fluorescence in green and autofluorescence

861 from the cuticle in magenta. Scale bar, 250 µm. (**C**) Wing expression pattern of R30B01-GAL4.

862 The different symbols indicate the sensory neuron types on the wing as proximal campaniform

863 sensilla (white asterisks), distal campaniform sensilla (yellow asterisks), or bristle

864 mechanosensory (white line). Scale bar, 250 µm.