

1 **Running Title:** *Drosophila* descending neuron function

2

3 **Title**

4 Optogenetic dissection of descending behavioral control in *Drosophila*.

5

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24 **Abstract**

25 In most animals, the brain makes behavioral decisions that are transmitted by descending  
26 neurons to the nerve cord circuitry that produces behaviors. In insects, only a few  
27 descending neurons have been associated with specific behaviors. To explore how these  
28 neurons control an insect's movements, we developed a novel method to systematically  
29 assay the behavioral effects of activating individual neurons on freely behaving terrestrial  
30 *D. melanogaster*. We calculated a two-dimensional representation of the entire behavior  
31 space explored by these flies and associated descending neurons with specific behaviors  
32 by identifying regions of this space that were visited with increased frequency during  
33 optogenetic activation. Applying this approach across a population of descending  
34 neurons, we found, that (1) activation of most of the descending neurons drove  
35 stereotyped behaviors, (2) in many cases multiple descending neurons activated similar  
36 behaviors, and (3) optogenetically-activated behaviors were often dependent on the  
37 behavioral state prior to activation.

## 38 **Introduction**

39           As animals navigate a dynamic environment, their survival depends on their  
40 ability to execute specific motor programs and to adjust motor output in response to  
41 external stimuli. While the brain performs computations essential for behavior, the motor  
42 circuits that directly control behavior are located close to the muscles that they control in  
43 the vertebrate spinal cord and insect ventral nerve cord. Information to drive motor  
44 patterns must therefore be transmitted from the brain to the nerve cord to direct behavior.  
45 Since there are many fewer descending neurons than neurons in the central brain,  
46 descending neurons generate an information processing bottleneck, which may generate a  
47 fundamental problem in information coding.

48           In flies, descending commands from the brain to the ventral nerve cord are  
49 transmitted through an estimated 250-550 pairs of descending neurons that arborize in 20  
50 highly-conserved clusters in the brain involved in sensory processing and motor behavior  
51 (Gronenberg & Strausfeld, 1990; Hsu & Bhandawat, 2016). Each descending neuron  
52 extends a single axon through the neck connective to the ventral nerve cord, where they  
53 synapse onto interneurons associated with leg, neck, and wing motor circuitry (Namiki et  
54 al, 2017).

55           Little is known about how so few neurons—approximately 0.5% of all neurons in  
56 the fly (Alivisatos et al., 2012)—encode signals from the brain to control the full range of  
57 movements performed by a freely moving fly. Several potential models have been  
58 suggested. One possibility is that, as with vertebrates, many stereotypical insect  
59 behaviors, such as walking, flying, or “singing” can be decomposed into individual motor  
60 modules controlled by central pattern generators located in the ventral nerve cord.

61 Several recent findings in *Drosophila melanogaster*, together with earlier  
62 electrophysiological studies in larger insects, support this idea. Activation of some  
63 individually-identifiable descending neurons triggers specific motor outputs, such as  
64 courtship song (von Philipsborn et al., 2011a), backwards walking (Bidaye, Machacek,  
65 Wu, & Dickson, 2014), or escape behavior (King & Wyman, 1980). However, some  
66 descending neurons modify motor programs, rather than trigger them. For example,  
67 cricket walking initiation, speed, and turning appear to depend on separately encoded  
68 descending commands (Böhm & Schildberger, 1992; Gras & Kohstall, 1998).  
69 Alternatively, motor activity may result from the summed activity of multiple descending  
70 neurons (Heinrich, 2002). For example, a cluster of descending neurons linking fly visual  
71 centers in the brain to the flight apparatus in the ventral nerve cord (Strausfeld &  
72 Gronenberg, 1990; Namiki et al, 2017) supports the idea that at least some descending  
73 neurons may function this way.

74 Descending neuron function may also be gated by behavioral state. For example,  
75 a single descending neuron may cause a different or modified behavior if the animal is  
76 walking versus flying. For example, DN sensory responses have been shown to be  
77 modified by locomotor state (Staudacher & Schildberger, 1998). This kind of gating has  
78 been observed in other contexts, such as the effect of the neuromodulator pyrokinin on  
79 the oscillatory mechanisms underlying the crustacean gastric mill central pattern  
80 generator (Marder & Goaillard, 2006). However, it has not previously been possible to  
81 undertake a systematic analysis of the context dependency across the DN population.

82 Systematic dissection of descending motor control is challenging for two reasons.  
83 First, it has been difficult to precisely manipulate a large number of descending neurons

84 individually in freely behaving animals. Second, we have not had a high-throughput,  
85 unbiased behavioral phenotyping pipeline capable of objectively categorizing all of an  
86 individual's movements. Historically, insect descending neuron anatomy, connectivity  
87 and function have been described by backfilling neurons with dye and recording from  
88 individual neurons in locusts, grasshoppers, and cockroaches (for a review see  
89 (Strausfeld, Bassemir, & Singh, 1984)), with more recent studies performing similar  
90 experiments in flies (Hsu & Bhandaway, 2016). While this approach has allowed  
91 researchers to describe the anatomy and electrophysiological responses of individual  
92 neurons, it is inherently low throughput and biased towards larger or otherwise more  
93 accessible neurons. Additionally, because experiments are typically carried out on  
94 immobile preparations, only in rare cases have investigators been able to link individual  
95 neurons to behavior (e.g. (E. Staudacher & Schildberger, 1998)). While recent technical  
96 and genetic advances in the model fly *Drosophila melanogaster* have improved our  
97 ability to access and manipulate individual descending neurons, to date only a handful of  
98 *Drosophila* descending neurons have been linked to specific motor outputs (e.g. (Bidaye  
99 et al., 2014; von Philipsborn et al., 2011b; von Reyn et al., 2014)).

100 To assess how descending neurons control motor behaviors on a systems scale, it  
101 will be necessary to move beyond isolated examples and to describe the behavioral  
102 functions of large numbers of descending neurons. Our goal was to identify all of the  
103 behavioral phenotypes observable in one particular setting, freely behaving flies moving  
104 within a two-dimensional arena, for many descending neurons, without any *a priori*  
105 expectation about the neurons' effects on behavior. Namiki et al (Namiki et al, 2017)  
106 created a collection of transgenic *Drosophila* strains that target descending neurons using

107 the split-GAL4 intersectional system (Pfeiffer et al., 2010) in a cell-type specific manner.  
108 We screened 130 of the sparsest lines in this collection, targeting approximately 160  
109 neurons that are divisible into 58 distinct anatomical cell-types. 40 of these cell types  
110 consist of a single pair of bilaterally symmetric descending neurons, while the remaining  
111 18 categories target populations of 3 to 15 descending neurons with similar  
112 neuroanatomy. We used this split-GAL4 collection to drive the expression of the red-  
113 shifted channelrhodopsin *CsChrimson* (Klapoetke et al., 2014a) in specified subsets of  
114 descending neurons, allowing us to photo-activate these neurons in a temporally precise  
115 fashion. We combined these genetic reagents with a recently described method for  
116 objective, quantitative analysis of behavior (Berman, Choi, Bialek, & Shaevitz, 2014) to  
117 comprehensively identify the behaviors associated with the activation of specific neurons  
118 in an unbiased fashion. Unlike supervised machine learning approaches for classifying  
119 behavior, this approach does not rely on a human-trained classifier to decide which  
120 behaviors are of interest. Instead it captures a wide range of movements by converting  
121 high-dimensional postural dynamics into a two-dimensional map using dimensionality  
122 reduction techniques (Berman et al., 2014). Using this method, we associated 80% of the  
123 descending neurons in our collection with specific behaviors.

124 We have generated a behavioral dataset from freely walking animals that  
125 comprehensively describes the activation phenotypes of roughly one third to one half of  
126 the total number of fly descending neurons. The size of this dataset has allowed us to  
127 move beyond individual examples to extract general features of descending neuron  
128 function, and therefore to consider how these neurons might encode information to  
129 modulate behaviors. We find that, with a few exceptions, descending neuron control of

130 behavior appears to be largely modular. In addition, we find many cases in which  
131 descending neuron function is context dependent, even within the confines of a single fly  
132 confined to a two-dimensional substrate.

133

## 134 **Results**

### 135 **Establishing a framework for large scale analysis of descending neuron activation** 136 **phenotypes**

137 Mapping fly behavior using postural dynamics requires high temporal and spatial  
138 resolution video data from a large number of animals. Accordingly, we built a red light  
139 activation apparatus that consisted of an array of 12 USB cameras that allowed us to film  
140 12 flies in separate chambers simultaneously at high resolution (Figure 1A). We crossed  
141 each split-GAL4 line to a UAS-CsChrimson line, and we filmed six experimental  
142 progeny that had been fed retinal, a co-factor necessary for neuronal activation via  
143 channelrhodopsin, and six genetically identical control flies whose food had not been  
144 supplemented with retinal. The flies were backlit using custom light tables, each  
145 consisting of an array of infrared and red LEDs covered by a diffuser. Each chamber was  
146 a 3 cm “fly bubble” (Klibaite (2017)), which had sloping sides coated with silicone. This  
147 encouraged the flies to remain on the flat floor of the chamber, where they could roam  
148 freely and would remain in the focal plane of the camera (Berman et al., 2014). For each  
149 split-GAL4 line, we recorded 30 trials consisting of a 15-second pulse of red light  
150 followed by a 45 second recovery interval (Figure 1A).

151 If the descending neuron(s) labeled by a particular split-GAL4 line are involved in  
152 triggering, maintaining, or modulating a particular behavior, then activating these

153 neurons with *CsChrimson* may be sufficient to activate that behavior. To identify  
154 behavioral phenotypes in an unbiased manner, we utilized the behavior mapping methods  
155 described in Berman et al. (2014). First, we generated a comprehensive “behavior space”  
156 of stereotyped actions that single flies could produce in our assay. We collected a dataset  
157 of approximately 700 million images, which included behaviors recorded from activation  
158 of descending interneuron split-GAL4 lines, previously characterized sparse GAL4  
159 drivers (*fruitless-GAL4* and *pIP10*) that trigger courtship-related behaviors (Stockinger,  
160 Kvitsiani, Rotkopf, Tirián, & Dickson, 2005; von Philipsborn et al., 2011b), and  
161 interneuron drivers targeting the flight neuropil. The additional lines that are not part of  
162 the descending neuron screen were included to sample fly behaviors as widely as  
163 possible, allowing for higher resolution mapping within the space of behaviors. We  
164 computed the behavior space by (1) aligning video images (Figure 1A), (2) decomposing  
165 the pixel value dynamics (which correspond to the fly’s posture changes) into a low-  
166 dimensional basis set using principal component analysis (Figure S1), (3) projecting the  
167 original pixel values onto this basis set and transforming those values using a spectral  
168 wavelet function to produce a time series that was (4) embedded into a two-dimensional  
169 “behavior space” (Figure 1B) using *t*-distributed Stochastic Neighbor Embedding (*t*-  
170 SNE) (van der Maaten & Hinton, 2008).

171 Each position in the behavior space corresponds to a unique set of postural  
172 dynamics. Nearby points represent similar motions, i.e. those involving related body  
173 parts executing similar temporal patterns. By observing the video data underlying sub-  
174 regions of the behavior space (Figure S2 and movies S1-S5), we generated a human-  
175 curated version of the behavior space to aid interpretation (Figure 1C). In this behavior



176 space, anterior directed movements such as eye/antennal grooming and proboscis  
177 extension are located at the top (supplemental movie S1). Anterior-directed foreleg  
178 movements are on the upper left side (movie S2). Extremely slow or still postures are on  
179 the upper right side (movie S3), and complex wing and abdomen movements such as  
180 body and abdomen grooming, abdomen bending and wing extension are in the center  
181 (movie S4). Locomotion, ranging from slow (left) to fast (right) is at the bottom (movie  
182 S5).

183 Red peaks, or density maxima, represent the fly behaviors observed most  
184 frequently in our data set. These tend to be repetitive, stereotyped behaviors, such as  
185 walking or grooming, that our analysis methodology is most sensitive at detecting. By  
186 definition, we could not detect behaviors occurring over time-scales faster than 50 Hz, the  
187 Nyquist frequency of our system. Approximately 93% of all video image data points  
188 could be embedded in this space, irrespective of whether the red light was on or off  
189 (Figure S3), indicating that the majority of red light activated behaviors are well  
190 represented in the behavior space. Un-embeddable imaging errors, such as the fly  
191 wandering partially out of frame, are randomly distributed within the dataset.

192

### 193 **Entropy of behavior space density provides a quantitative and sensitive measure of** 194 **optogenetic activation phenotypes**

195 Having established a behavior space representing the full repertoire of fly  
196 behaviors that could be captured with our apparatus, we next examined which parts of  
197 this space were occupied when individual or subsets of descending neurons were  
198 optogenetically activated by CsChrimson. We focused on 130 split-GAL4 lines that

199 targeted descending neurons with little, or no, extraneous expression in other neurons.  
200 We first considered the timing and duration of red light triggered behaviors. If  
201 descending neuron activation triggered a particular behavior represented in the behavior  
202 space, then we expect that the density of that line in the behavior space should shift into  
203 the region that represents that behavior during periods of red light activation. For  
204 example, upon red light activation, retinal-fed flies expressing *CsChrimson* in a  
205 descending neuron line targeting DNg07 and DNg08 (SS02635) groomed their heads  
206 (supplemental movie S6). We identified regions in the behavior space that experienced a  
207 statistically significant shift in density for experimental flies during the first three seconds  
208 of red light compared to a window at the end of the recovery period when the red light  
209 was off (Figure 2B, C, Figure S4). This same region in the behavior space did not  
210 undergo a significant shift in the control flies (Wilcoxon rank-sum test  $p < 0.05$  using the  
211 Dunn–Šidák correction for multiple hypotheses (Šidák, 1967)). Likewise, when  
212 considering densities over the whole behavior space in three second sliding windows, the  
213 experimental, but not the control, flies shift into the head grooming region (arrowheads,  
214 Figure 2C).

215         The shift in behavior in the experimental animals, and the timing of this shift  
216 relative to red light activation, can be detected as a reduction in the entropy of the  
217 behavior space density during this epoch (Figure 2A). Entropy measures the degree of  
218 disorder inherent in the distribution of the flies in the behavior space. When the red light  
219 was off, flies exhibited a range of different behaviors, and the probability that they  
220 performed any one behavior was low. This results in a low probability density  
221 distributed throughout the behavior space and correspondingly high entropy (Figure 2A).

222 Upon red light activation, the experimental fly line engaged in red light triggered  
223 behaviors at the expense of other natural behaviors. This increased the probability that  
224 they occupied a small region within the behavior space, generating a drop in entropy  
225 whose timing and duration mirrored that of the red light triggered behaviors (Figure 2A  
226 movie S7). We can therefore use entropy as a proxy for the duration and onset of red light  
227 triggered movements in the behavior space without needing to know, *a priori*, which  
228 behaviors are activated (i.e. which part of the behavior space to examine).

229         The region density and entropy are quantitative measurements sensitive to small  
230 changes in behavior map distribution. We therefore used these values to identify subtle  
231 phenotypes that could not be easily identified by manual inspection of the movies. For  
232 example, the rapid activation of descending neuron DN<sub>g</sub>25 induced a transient rapid  
233 running phenotype (Figure 2E & 2F) that was identified by the transient drop in entropy  
234 in the behavior space (Figure 2D) and the transient increase in density in the fast  
235 locomotion region of the space (Figure 2E & 2F, supplemental movie S8).

236

### 237 **Comprehensive characterization of descending neuron split-Gal4 line activation** 238 **phenotypes**

239         To characterize the time-course and likelihood of optogenetically-induced  
240 phenotypes across the entire collection of descending neuron lines, we examined the  
241 entropy time course of each line (Figure 3). We found that most lines displayed the  
242 largest entropy drop immediately after red light activation (Figure 3A). For roughly a  
243 third of the lines, this entropy drop persisted throughout the entire red light activation  
244 window (Figure 3B). For most of the rest of the lines, however, the entropy drop was

245 transient and diminished after several seconds (Figure 3B). For a minority of lines, the  
246 entropy minimum occurred near the middle or end of the activation window (Figure 3B).  
247 We reviewed the raw video data for these lines and found that most of these flies  
248 performed some action upon red light activation, followed by a freeze. This explained  
249 why the entropy was lower in the later part of the activation window, because consistent  
250 stillness is a low entropy state (see Figure S5 for a line by line description of  
251 phenotypes). We therefore performed our system-wide analysis using the first 3 seconds  
252 of the red light activation period, because this time period captured the majority of  
253 CsChrimson activated behaviors.

254 In our initial analysis, we looked for behaviors produced when our descending  
255 neuron lines were activated using a comparatively low level of red light, (5 mW/cm<sup>2</sup>).  
256 Under these conditions, 91 of the 130 lines (69%) displayed a statistically significant  
257 increase in density of some area of the behavior space. We then re-tested most of the 41  
258 lines that did not produce a significant density increase by driving CsChrimson at higher  
259 levels by growing the flies on food containing an increased retinal concentration and  
260 exposing flies to higher intensity red light (9 mW/cm<sup>2</sup>). Under these conditions, 80% of  
261 the lines that had previously displayed no phenotype produced a statistically significant  
262 increase in density in the behavior space.

263 Pooling the data from the low and high activation protocols, we detected  
264 statistically significant increases in the behavior space in 119 of the 130 (90%)  
265 descending neuron lines (Figure 4A). In 86 cases, we observed an increased density in  
266 only a single statistically significant region in the behavior space. However some lines  
267 generated density increases in multiple non-contiguous regions of the behavior space

268 (Figure 4A, examples shown in Figure 4B-D, supplemental movies S9-S11, supplemental  
269 figure S5). In many cases, these multi-region lines reflect multiple behaviors performed  
270 approximately simultaneously by the flies. For example, a line targeting DNp10 induced  
271 anterior reaching movements and wing flicking with similar timing (Figure 4D, red and  
272 blue regions respectively). However, in other cases, multiple activated regions reflect a  
273 stereotyped sequence of behaviors. For example, the DNp09 line shown in Figure 4B  
274 repeatedly ran and then paused throughout the entire 15 second activation period. The  
275 increased density in the run region of the behavior space (Figure 4B, red) appeared before  
276 the increased density in the paused region (Figure 4B, blue), reflecting the sequential  
277 timing of the two behaviors. However, the flies rapidly became asynchronous as they  
278 repeated this series of behaviors, so this behavioral series is detected as density increases  
279 in the running and still regions throughout the red light activation window. A line  
280 targeting descending neuron DNb01 displayed a simple behavior series; flies produced an  
281 anteriorly directed twitch of the front legs when the red light was turned on (Figure 4C,  
282 red region), then froze for the majority of the red light activation period (Figure 4C, black  
283 region), and then twitched when the light was turned off (Figure 4C, blue region). Thus,  
284 examining the timing of density shifts illuminates the more complicated behavior series  
285 produced by red light activation. This level of analysis is provided for all lines in  
286 supplemental Figure S5.

287

### 288 **Behavioral result of descending neuron activation is often context dependent**

289 Why does activation of some descending neurons result in multiple, distinct  
290 behavioral outputs? One possibility is that the behavioral output of some descending

291 neurons depends on the behavioral context of the fly when the descending neuron is  
292 activated. To address this possibility, we calculated the mutual information between the  
293 density distribution of the experimental flies in the behavior space at 1.5 to 0.5 seconds  
294 before the red light was turned on versus the first second after red light activation. Mutual  
295 information is a non-linear measure of the degree of dependence between two variables  
296 and is typically measured in units of bits (Cover & Thomas, 2005). The higher the mutual  
297 information, the more the first variable, here the behavior of flies immediately prior to  
298 red light activation as measured by their distribution in the behavior space at  $t = -1.5$  to -  
299 0.5 seconds, informs the value of the second variable, the region of the behavior space  
300 occupied in the first second of red light activation.

301 We found that, in all cases, experimental animals displayed non-zero mutual  
302 information between the pre- and post-stimulation behaviors (Figure 5A). In addition, for  
303 most lines, more information was available in the experimental flies than in the controls  
304 (Figure 5B). This means that even in those cases where red light activation produced only  
305 one significant region in the behavior space, the fly's activity prior to red light activation  
306 influenced whether or not it performed the behavior. However, lines with multiple red  
307 light activated regions in the behavior space were also those with a relatively high level  
308 of mutual information (Figure 5A-B). Thus, a given fly's behavior before red light  
309 activation was highly informative of which behavior that fly would perform after red  
310 light activation, as indicated by the different significantly activated regions in the  
311 behavior space. Figure 5C displays this phenomenon for one of the lines with the highest  
312 mutual information, SS02542 (asterisk in figure 5A-B, also shown in Figure 4C). Here,  
313 if the flies were performing an action in the wind/abdomen movement regions of the

314 behavior space prior to the stimulation, they were likely to perform an anterior movement  
315 (region 1) immediately following stimulation. Similarly, flies performing anterior  
316 grooming were likely to transition to the small anterior twitch region (region 2), and flies  
317 that were initially still tended to remain still post-stimulation (region 3).

### 318 **Individual descending neurons produce mainly stereotyped, modular behaviors**

319 So far, we have analyzed split-GAL4 lines as if they were a proxy for individual  
320 descending neurons or anatomical classes of descending neurons. However, these lines  
321 vary in both their strength of expression and in the number and identity of additional cells  
322 labeled. To estimate phenotypes for individual descending neurons, we therefore  
323 averaged the behavior space densities of multiple lines for those cases where we had  
324 multiple lines targeting the same descending neuron (Figure 6). Using this method, and  
325 combining it with those descending neurons for which we had only a single  
326 representative split-GAL4 line, we estimated phenotypes for 47 of the 58 descending  
327 neuron cell types. We have also included six lines and line averages that target two  
328 different types of descending neurons cleanly, but for which we have no lines that target  
329 each type individually. Twenty-six descending neurons drove locomotion phenotypes and  
330 ten drove anterior directed foreleg movements. We also identified six new descending  
331 neurons that triggered wing and abdomen movements (plus the previously published  
332 pIP10 (von Philipsborn et al., 2011b)), two that drove anterior grooming, one that drove  
333 abdomen stroking, and four that drove still or slow behaviors.

334 In general, we found that activation of each type of descending neuron drove  
335 behaviors that mapped to a relatively small region of the behavior space. For example,  
336 some descending neurons drove slow locomotion, whereas others drove fast locomotion.

337 Only a few, such as DNa01, DNa02 and DNp26, seemed to produce a global increase in  
338 locomotor activity. Likewise, we found descending neurons that produced different types  
339 of grooming, such as head grooming (DNg07 & DNg08, and DNg12) or abdomen  
340 grooming (DNp29), different types of anterior reaching movements (DNg10 versus  
341 DNg13) and different types of slow movements (e.g. DNd02 versus DNp02).

342

### 343 **Discussion**

344 Using optogenetic activation and automated behavioral quantification, we  
345 assigned behavioral phenotypes to 80% of the descending neurons cell types in our  
346 collection of lines, or one third to one half of the estimated total number of descending  
347 neurons present in the fly. Using a dataset of this scope, it is possible for the first time to  
348 move beyond isolated examples to consider systems-level trends in how descending  
349 neurons control behaviors.

350 For several reasons, this is unlikely to be a comprehensive categorization of the  
351 activation effects of these descending neurons. First, behaviors performed more quickly  
352 than the Nyquist frequency of 50Hz for our movies could not be detected. Second, we  
353 assayed only behaviors that can be activated when flies are standing and walking.  
354 Descending neurons controlling behaviors gated by flight, for example, would not be  
355 detected. Third, we assayed only males, so any female-specific behaviors may not be  
356 identified. Finally, we assayed solitary flies, so any behaviors gated by social  
357 interactions, for example courtship, may not have been detected.

358 There are several, non-mutually exclusive ways a limited number of seemingly  
359 highly modular descending neurons could encode the wide range of behaviors undertaken



360 by freely moving animals. First, descending neurons could be more important for  
361 triggering and maintaining behaviors than for controlling individual details of a given  
362 motor program (Heinrich, 2002). Many motor programs, particularly those controlling  
363 repetitive, rhythmic actions such as walking or stridulation, can function in the absence of  
364 descending control ((Bentley, 1977; Kien, 1983), for a review on walking circuits see  
365 (Ritzmann & Bü Schges, 2007)). For example, Hedwig (1994) identified two pairs of  
366 descending neurons that control stridulation in grasshoppers. In this system, tonic  
367 activation of the descending neurons was sufficient to induce and modulate the activity of  
368 the stridulation central pattern generator in the thorax, indicating that the descending  
369 neurons play only a limited role in patterning stridulation. Several of our lines, including  
370 the DN<sub>g</sub>07 & DN<sub>g</sub>08 head grooming line (Figure 2A & 2B), appear to reflect a similar  
371 phenomenon, driving a repeated stereotyped behavior during the entire CsChrimson  
372 activation window.

373         Second, behaviors might be controlled not by single descending neurons acting as  
374 command neurons, but by combinations of descending neurons acting in concert  
375 (Heinrich, 2002). Neuroanatomy suggests this possibility; roughly a third of described  
376 descending neurons appear to have unique projection patterns in *Drosophila* and  
377 *Calliphora*, while the rest share common input and/or output regions in the brain and  
378 ventral nerve cord with other descending neurons (Gronenberg & Strausfeld, 1990; Milde  
379 & Strausfeld, 1990; Namiki et al, 2017). In addition, several examples illustrate that  
380 multiple descending neurons control the same specific behaviors (Griss & Rowell, 1986;  
381 Gronenberg & Strausfeld, 1990; Hensler, 1992; Kanzaki, Ikeda, & Shibuya, 1994; Kien,  
382 1983, 1990; Milde & Strausfeld, 1990; Rowell & Reichert, 1986; E. M. Staudacher,

383 2001). In this case, Larimer et al (1988) suggest that sufficiently strong stimulation of one  
384 neuron in a command cohort or module is sufficient to recruit the activity of the other  
385 descending neurons, triggering the behavior. Our data do not allow us to definitively  
386 address this question. However, the large number of descending neurons that drive  
387 similar patterns of fast locomotion, slow locomotion and anterior reaching suggest that,  
388 for these motor circuits at least, this is a possibility. Alternatively, it is possible that many  
389 of these descending neurons modulate distinct aspects of these motor programs.

390 Third, another way to generate behavioral complexity is through coding different  
391 behaviors via combinations of descending neurons. We examined a few lines that target  
392 multiple descending neurons. We compared behaviors produced by these “multi-hit”  
393 split-GAL4 lines with lines that targeted the individual neurons and found only weak  
394 evidence for the emergence of new behaviors when descending neurons were triggered in  
395 combination. For example, both DNa05 and DNd02 produce slightly different  
396 phenotypes when activated in combination with DNa07 and DNd03, respectively, as  
397 compared to when lines targeting these neurons are activated alone (see Figure S5).  
398 However, our collection contains, by design, few lines driving expression in  
399 combinations of descending neuron types. Therefore, further exploration of this idea will  
400 require the generation and characterization of additional lines.

401 Finally, descending neurons could be re-used in multiple behavioral contexts.  
402 While there are to date no published examples of a single descending neuron triggering  
403 different context-dependent behaviors, there are multiple cases in which descending  
404 neurons exhibit different physiological responses depending on the state of the animal  
405 (e.g. walking, flying, courting, etc.) (Böhm & Schildberger, 1992; B Hedwig, 2000; R.

406 M. Olberg, 1983; R. Olberg & Willis, 1990; E. M. Staudacher, 2001; E. Staudacher &  
407 Schildberger, 1998; Strausfeld & Bassemir, 1985; Zorović & Hedwig, 2011). Our results  
408 strongly support a role for context dependency for two reasons. First, the high level of  
409 mutual information between behaviors immediately before and after red light activation  
410 seen in lines that have multiple red light activated regions indicates that even within the  
411 relatively confined system of our assay, the behavior of the fly immediately before  
412 descending neuron activation biases the behavioral output in many cases. Second, our  
413 observation that substrate specific behaviors, such as foreleg tapping, reaching, and  
414 locomotion are strongly represented in our dataset, while flight and courtship behaviors  
415 are less prevalent suggests that descending neuron outputs may be context dependent. By  
416 forcing the flies to remain on a two dimensional substrate in isolation, we may have  
417 observed predominantly indirect results of behaviors that would normally take place in a  
418 different context. For example, when we activated a line expressing in DNp01, the giant  
419 fiber, a neuron known to elicit a rapid escape response initiated by a jump when  
420 optogenetically activated (Lima & Miesenbock, 2005) we detected the flies running after  
421 returning back to the ground because the jump was too fast (~30 ms) to be detected in our  
422 assay. It is also possible that some of these descending neurons are never naturally  
423 activated in the two-dimensional context of walking and that proprioceptive feedback  
424 may have generated abnormal behaviors in our assay.

425 Our objective, quantitative assessment of a descending neuron activation screen  
426 provides a foundation for understanding descending neuron functions more broadly.  
427 Using similar analytical approaches to study the results of descending neuron activation  
428 and inactivation in other behavioral settings in the future will broaden our understanding

429 of how descending neurons direct motor patterns in specific behavioral contexts and  
430 reveal how the fly's rich behavioral repertoire can be encoded with only a few hundred  
431 neurons.

432

### 433 **Materials and Methods**

#### 434 *Fly stocks and fly handling*

435 The descending neuron split-GAL4 driver collection is described in Namiki et al  
436 (Namiki et al 2017). Male flies were crossed to virgin females carrying *20xUAS-*  
437 *CsChrimson-mVenus* (Klapoetke et al., 2014b) integrated into the *attP18* landing site  
438 (Markstein, Pitsouli, Villalta, Celniker, & Perrimon, 2008) and transferred to Dickson lab  
439 power food (1L water, 10g agar, 80g Brewer's yeast, 20g yeast extract, 20g peptone, 30g  
440 sucrose, 60g dextrose, 0.5g MgSO<sub>4</sub>\*6H<sub>2</sub>O, 0.5g CaCl<sub>2</sub>\*2H<sub>2</sub>O, 6mL propionic acid and  
441 7mL 15% Nipagin). For the initial screen, experimental animals were raised on power  
442 food supplemented with 0.2 mM retinal. This concentration was increased to 0.4 mM for  
443 animals that were re-assayed at a higher light intensity. All flies (except parental stocks)  
444 were handled under 453 nm blue LEDs and reared in dark blue acrylic boxes (acrylic  
445 available from McMaster-Carr, # 8505K84) at 22°C on a 12 hour lights on:12 hour lights  
446 off day:night cycle. Individual male flies were collected upon eclosion and housed singly  
447 in 2mL wells in a 96 well "condo," with power food (with or without retinal) deposited in  
448 the bottom of each well, which was sealed at the top with an airpore sheet (Qiagen  
449 #195761). Flies were imaged at age 7-12 days, within 4 hours of lights on.

450

451

452 *Data Collection*

453 Single flies were loaded into individual trays made from 4.5 mm clear acrylic  
454 topped with a fly “bubble” 3 cm in diameter and 4 mm at its tallest point, which was  
455 vacuum molded from clear 0.020” PETG thermoform plastic (WidgetWorks, available on  
456 Amazon) (Klibaite (2017)). PETG was placed in a frame, heated in a Oster Convection  
457 Bake pizza oven set at 350°F until the plastic started to deform (about 20 seconds), then  
458 placed on a vacuum manifold. To further encourage the flies to remain on the 2  
459 dimensional acrylic surface, the bubbles were coated with Sigmacote siliconizing reagent  
460 one day prior to imaging and lightly wiped with ethanol to remove the excess silicone.

461 For each descending neuron split-GAL4 line six retinal-fed experimental animals  
462 and six non-retinal-fed control animals were imaged simultaneously. For imaging, flies  
463 were placed in individual fly bubbles atop custom light tables (3 identical light tables,  
464 each imaging 4 flies). These tables consisted of a custom light board topped with a 0.75”  
465 3D printed white plastic standoff that was lined with infrared reflective tape, and which  
466 was capped with a diffuser made from 0.125” white plexiglass acrylic (available from  
467 eplastics.com, # ACRY24470.125PM24X48), which had 50% light transmittance. The  
468 light board itself consisted of an array of 256 IR LEDs (Osram Opto SFH 4050-Z, 850  
469 nm wavelength) arrayed in a 16 x16 pattern, spaced 7.14 mm apart, and 64 red LEDs  
470 (Philips Lumileds, LXM2-PD01-0050, 627 nm) arranged in an 8x8 pattern, spaced 14.28  
471 mm apart. IR and red LED intensity was controlled separately by 0-2.5V control  
472 voltages, yielding 0-100mA for the IR LEDs and 0-400 mA for the red LEDs. We set the  
473 IR LEDs to 1V, which provided even illumination without overheating the flies. We used  
474 0.2V (4.5 mW/cm<sup>2</sup>) red light in the initial screen and 1.0V (9 mW/cm<sup>2</sup>) red light when

475 re-screening a subset of lines. All three light tables were connected to a 68-Pin  
476 unshielded I/O connector block (National Instruments, CB-68LP), then to an M Series  
477 multifunction DAQ board (National Instruments, NI USB-6281), so that all tables could  
478 be run simultaneously from a single computer. Each light table was topped with a 10”  
479 square frame constructed from off the shelf parts from Thorlabs, which supported four  
480 1.3 MP grayscale USB cameras (Point Grey FL3-U3-13Y3M-C, 1 camera per fly) on  
481 optical rails, whose X/Y/Z coordinates could be adjusted relative to the fly bubble. Each  
482 camera was fitted with an HR F2/35mm lens from Thorlabs and an 800 nm longpass  
483 filter (Thorlabs, FEL0800). Each set of four cameras was connected to a separate Dell  
484 Precision T3600 Tower Workstation. Each was fitted with two 100 GB internal solid  
485 state drives, so that 2 cameras wrote to each SSD.

486       Cameras were programmed using NI-MAX and custom software written in  
487 Labview (National Instruments). Data acquisition and the LED light tables were  
488 controlled by custom software written in Labview. In brief, a master computer ran a  
489 single program that (1) turned the red and infrared (Berman et al., 2014) LEDs on for all  
490 three light tables, the former running a program of 15 seconds, then off for 45 seconds,  
491 for 30 cycles; (2) started all 12 cameras recording; (3) recorded the position of the fly’s  
492 centroid for each frame for each camera; and (4) grabbed the frame number for each  
493 camera over the network every 2-3 frames, and wrote the frame number and red LED  
494 status from the light tables to a single text file. All movies were recorded as  
495 uncompressed avi files at 100 frames per second. Each camera was set to 1024 x 1024  
496 pixel resolution that encompassed the entire 3 cm arena. However, flies were tracked

497 using a blob detector and only a 150x150 pixel box centered on each fly was saved and  
498 used for analysis.

499

### 500 *Behavior Space Generation*

501 Our approach for generating a behavior space largely follows the methodology  
502 originally described in Berman, 2014 (Berman et al., 2014), which describes much of the  
503 procedure in additional detail. We first segmented flies using Canny's method for edge  
504 detection (Canny, 1986) and morphological dilation to find the outline of the fly. All  
505 pixels within the corresponding closed curve were considered part of the fly. We assumed  
506 that all flies had identical morphology but variable sizes. We calculated a rescaling factor  
507 for each fly by segmenting 100 randomly-selected images from a single fly and finding  
508 the pixels belonging to that fly's body (head, thorax, and abdomen) in each of them,  
509 ignoring pixels associated with the wings and legs. Body pixels were assigned via a two-  
510 component Gaussian mixture model, and the average value of the number of pixels was  
511 chosen as the body area. All frames from a single movie were then uniformly re-scaled to  
512 make the number of body pixels in the average image equal to that in a reference image  
513 of a fly. We then rotationally aligned segmented, recalled images by finding the maximal  
514 angular cross-correlation of the magnitudes of the two-dimensional polar Fourier  
515 transforms between the image and a reference image. This reference image was common  
516 to all aligned images. Translational registration was then performed by maximizing the  
517 spatial cross-correlation.

518 Postural decomposition was performed as described in Berman (2014). Images  
519 were Radon-transformed using a 2 degree spacing, and the 9,781 Radon-space pixels that

520 contained the most variance were kept for further analysis (>95% of the total variance).  
521 We then performed principal components analysis (PCA) on these data, keeping the 50  
522 modes capturing the most variance (>90% of the total variance). We projected the  
523 segmented and aligned images onto the found eigenvectors to create a set of time series  
524 that were representative of the postural movements of the fly. To obtain dynamic  
525 information about these time series, we applied a Morlet continuous wavelet transform to  
526 these time series. We transformed each mode separately, using 25 frequency channels  
527 that were dyadically spaced between 1 Hz and 50 Hz, retaining only the amplitudes of the  
528 resulting complex numbers.

529 Low dimensional embedding of these wavelet time series using t-Distributed  
530 Stochastic Neighbor Embedding (t-SNE) (van der Maaten & Hinton, 2008) largely  
531 followed the approach in (Berman et al., 2014) as well. A distance metric between points  
532 in time was calculated via the the Kullback-Leibler divergence (Cover & Thomas, 2005)  
533 between their associated normalized mode-frequency spectra. Because this data set  
534 contains several orders of magnitude more data than can be calculated through brute-  
535 force minimization of the t-SNE cost function, we used the sub-sampling technique  
536 described in (Berman et al., 2014) to identify 600 representative data points from each of  
537 the recording sessions. From here, points were randomly assigned subsequent groupings  
538 such that each of these groups contained 36,000 data points. The same sub-sampling  
539 process was performed amongst these data points, but now keeping twice as many data  
540 points as in the previous iteration. This process was repeated until a data set of 36,000  
541 points was obtained. We minimized t-SNE for this data set to create a low-dimensional  
542 embedding. We used the re-embedding procedure described in (Berman et al., 2014) to



543 include data from outside the 36,000-point training set into the embedding, resulting in  
544 the overall density seen in Figure 1.

545

#### 546 *Statistical Analysis*

547 Our main goal for the statistical analysis of the behavior space data was to isolate  
548 regions of the map that were significantly affected by optogenetic stimulation. Here, we  
549 assessed significance by (1) comparing the flies' behavior when the LED was on versus  
550 when the LED was off, and (2) requiring that the effect of the LED stimulation be larger  
551 in the experimental flies than in the control flies. Specifically, we compared the flies'  
552 behavior during the first three seconds of stimulation ( $t=0$ s to  $t=3$ s, where the LED turns  
553 on at  $t=0$ ) to their behavior between stimulation ( $t=30$ s to  $t=45$ s). To statistically assess  
554 whether a particular region of the behavior space was significantly affected by the  
555 stimulus, we first defined  $\rho_{i,n}^{on}(x,y)$  to be the average behavior space density for fly  $i$   
556 during the  $n$ th cycle at location  $(x,y)$  during the first 3 seconds of excitation and  
557  $\rho_{i,n}^{off}(x,y)$  to be the same, but during the 15s window furthest from the stimulation. We  
558 then tested whether  $\rho_{i,n}^{on}(x,y)$  was significantly different from  $\rho_{i,n}^{off}(x,y)$  through a  
559 Wilcoxon rank sum test with Šidák corrections ( $p < .05$  after corrections) (Šidák, 1967).  
560 To calculate the number of corrections, we conservatively assumed that the number of  
561 measurements was equal to  $2^H$ , where  $H$  was the entropy of the mean density of the  
562 behavior space. This is likely an over-estimate of the number of comparisons, but it  
563 provides an upper-bound for the number of distinctions that could be made.

564 To compare the effect of the optogenetic stimulus on the experimental flies to that  
565 of the effect on the control flies, we computed the quantity  $\chi_{i,n}(x,y) = \rho_{i,n}^{on}(x,y) -$

566  $\frac{1}{2}(\rho_{i,n-1}^{off}(x,y) + \rho_{i,n}^{off}(x,y))$ , which was the behavior space density during light  
567 stimulation compared to the average of the two preceding time periods with no light  
568 stimulation. We thus assessed statistical significance by using a Wilcoxon rank sum test  
569 with Šidák corrections ( $p < .05$  after corrections) to compare  
570  $\{\chi_{i,n}(x,y)\}_{i \in experimental\ flies}$  with  $\{\chi_{i,n}(x,y)\}_{i \in control\ flies}$ . For a point,  $(x,y)$ , in the  
571 behavior space to be considered significantly affected by the stimulus, we required that  
572 both of these tests—within experimental flies test and experimental versus control flies  
573 test—yielded a significant result.

574 Behavior activation maps for individual descending interneurons (Figure 6) were  
575 calculated by averaging together the maps of significantly significant activations  
576 ( $E[\chi_{i,n}(x,y)]_{i,n} > 0$ ) from each of the lines exciting that neuron.

577 Stimulation-response entropy curves (Figure 2A & D) were generated by first  
578 aligning each time point to its associated phase within the 60 second LED on-off cycle.  
579 For each phase within the cycle, we found all embedding points from all relevant trials  
580 that were detected within  $\pm 200$  ms (using periodic boundary conditions). We then  
581 generated a histogram of these points, normalized and convolved the resulting values  
582 with a symmetric two-dimensional Gaussian of width  $\sigma = 2$ , to generate a probability  
583 density function,  $p_t(x,y)$ . From this, the entropy curve value at phase  $t$  was given by  
584  $H(t) = \int dx dy p_t(x,y) \log p_t(x,y)$ . We then pooled data from all individuals of a  
585 specific type together (i.e. all control flies from a given line or all experimental flies from  
586 a given line) to calculate these curves.

587 Mutual information between pre-stimulus behavior space densities and post-  
588 stimulus regions was computed by numerically integrating the integral:

589  $MI(\rho_{pre}; R_{post}) = \sum_{k=0}^m \int d\vec{x} p_{pre}(\vec{x}|R_k) \log_2 \frac{p_{pre}(\vec{x}|R_k)}{\sum_{\ell=0}^m p_{pre}(\vec{x}|R_\ell)p(R_\ell)}$ , where  $p_{pre}(\vec{x}|R_k)$  is  
590 the conditional probability of observing the fly's behavior to be at location  $\vec{x}$  between 1.5  
591 and 0.5 seconds before the stimulus onset and  $p(R_k)$  is the probability that the fly  
592 transitions to region  $R_k$  following the stimulus onset. Finite data-size corrections were  
593 performed by drawing subsets of the data with replacement and extrapolating to an  
594 infinite number of trials, and error bars were generated by extrapolating the calculated  
595 variance in a similar manner (Bialek, 2012). The region of transition for each trial was  
596 assigned by finding the mode of the behavior space distribution during the first second  
597 subsequent to the onset of the stimulus. If the location of the mode of the distribution for  
598 that trial was within or closer than 5-pixels to the edge of a region, it was assigned to that  
599 region, unless another region was closer. Trials not assigned to any of the regions were  
600 given a “zero” label, as reflected in the previous equation.

601 To provide a sense of scale, if there are  $N$  significantly activated regions, the  
602 maximum possible mutual information one could potentially measure between the prior  
603 distribution and the activated region would be  $\log_2(N)$  bits. Note, however, that we  
604 assigned an additional state corresponding to the fly performing a behavior outside of the  
605 significantly activated regions subsequent to the light turning on, thus making the  
606 maximal possible mutual information  $\log_2(N + 1)$ . This additional “zero” state is  
607 necessary to account for the possibility that the significant regions might be exhibited  
608 only in a context-dependent manner, leading to no significant phenotype when the fly is  
609 performing some behaviors at the onset of red light stimulation and leading to a  
610 phenotype if other actions are being exhibited.

611

612

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620

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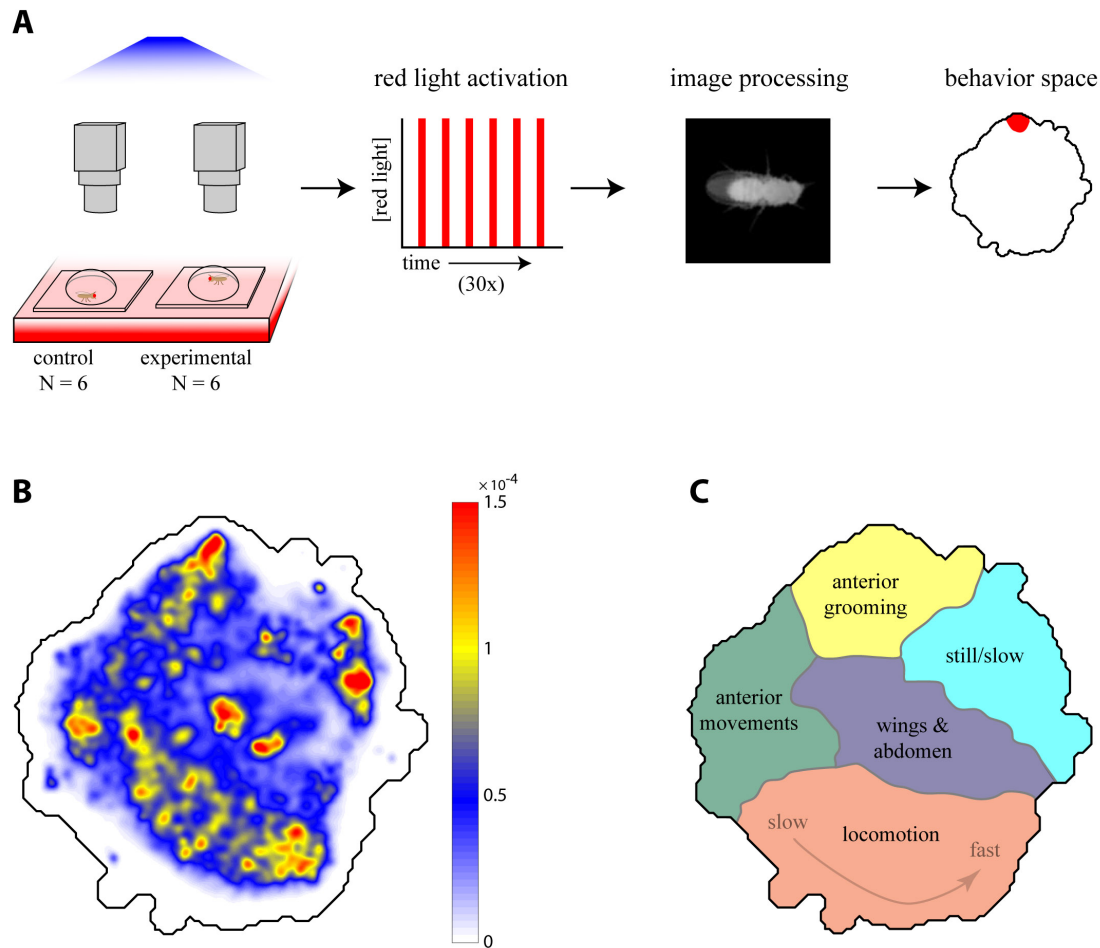
758

759

760 **Figures and Figure Legends**

761

762 Figure 1.



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764

765 Figure 1. Descending neuron phenotyping pipeline and behavior space. (A) The red light

766 activation rig. Six no retinal control flies and six retinal fed experimental flies were

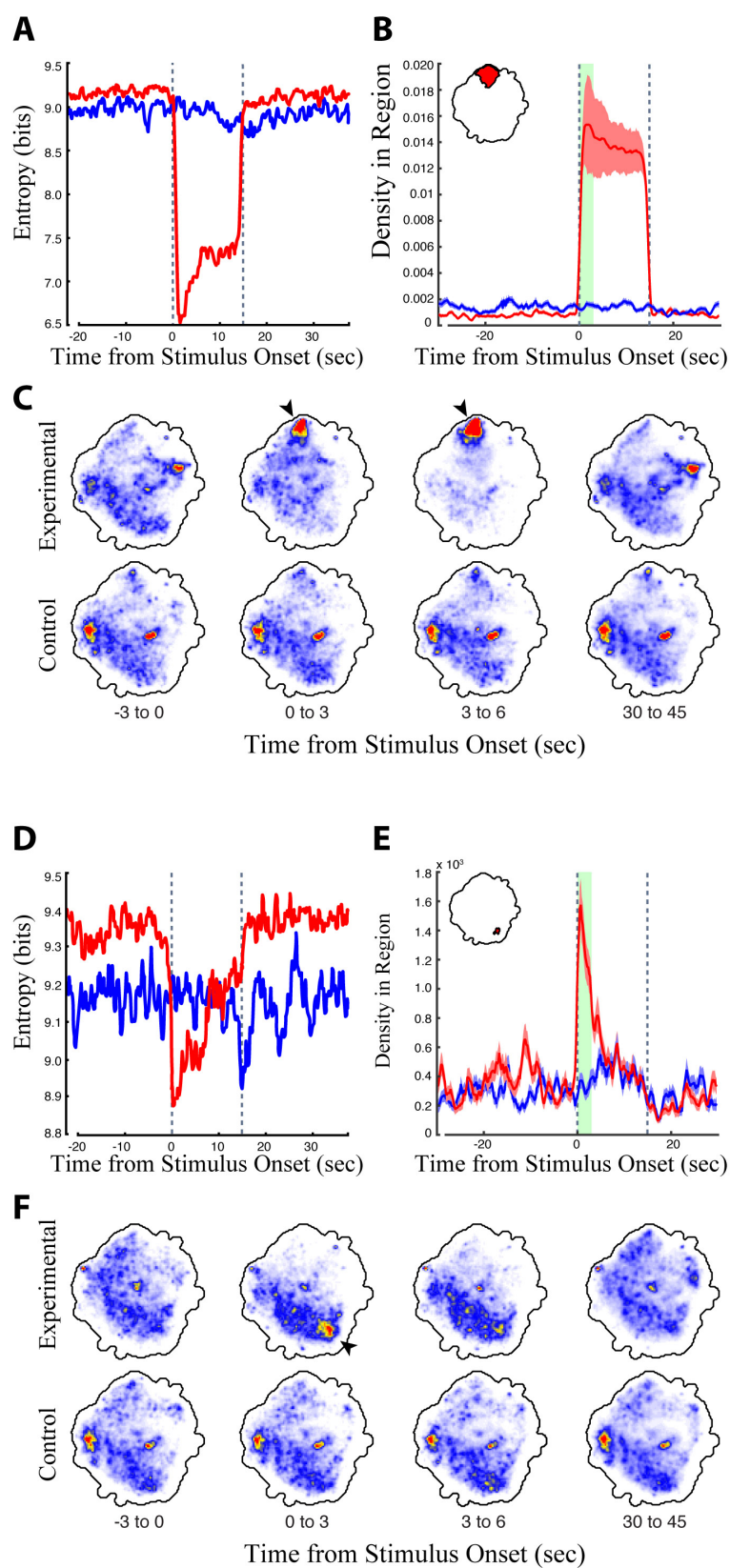
767 mounted in parallel in individual 3 cm diameter plexiglass bubbles on top of three custom

768 light boards with constant 850 nm infrared light and variable 617 nm red light. The red

769 LEDs were repeatedly turned off and on for 45 sec and 15 sec, respectively. Each fly was

770 filmed at 150x150 pixel, 100 fps resolution by a single camera. Video data was then  
771 aligned and processed, and the line was characterized for its occupancy in the descending  
772 neuron behavior space with respect to red light activation and controls. (B) A 2D  
773 representation of behaviors in the descending neuron video dataset was generated by  
774 applying a probability density function to all the embedded data points (scale bar), which  
775 was then convolved with a Gaussian ( $\sigma = 1.5$ ) (C) Localization of various behaviors  
776 within the descending neuron behavior space seen in (B), based on human curation of  
777 watershedded regions in the space (Supplemental Figure 2, Supplemental movies S1-S6).  
778

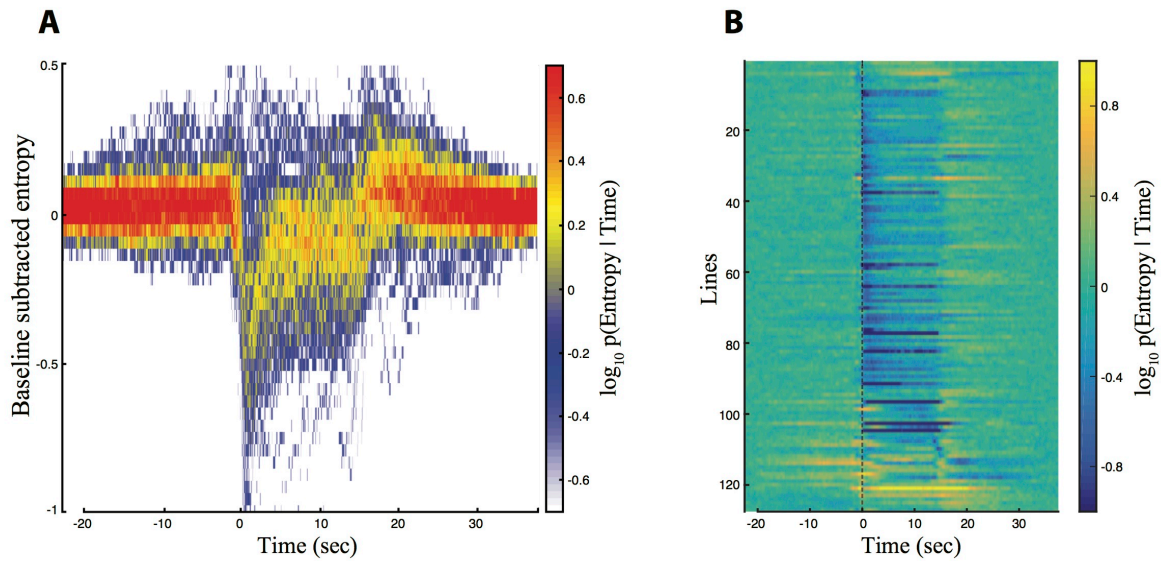
779 Figure 2.



780

781 Figure 2. Analysis of the head grooming DNg07 & DNg08 line (SS02635) and the  
782 transient fast-running DNg25 line (SS01602). (A, D) Digital entropy of the distribution of  
783 the retinal fed experimental flies (red) and non-retinal fed control flies (blue) in the  
784 behavior space relative to the timing of red light stimulus onset (red light turned on at  $t =$   
785 0 sec, the time of red light activation is indicated by dashed lines). Experimental flies  
786 experience a decrease in entropy when they perform a specific set of behaviors, because  
787 they shift from the full range of normal fly behaviors to a subset of red light-activated  
788 behaviors. (B, E) Average density +/- the standard deviation in the head grooming map  
789 region indicated in red (inset, upper right) in experimental flies (red) and controls (blue)  
790 relative to red light activation. The head grooming region was calculated as the region in  
791 the map that experienced a statistically significant shift in density in experimental flies  
792 but not controls when comparing the first 3 seconds (green bar) of the activation period to  
793 the last 15 seconds of the recovery period (Wilcoxon rank sum test,  $p < 0.05$ , using the  
794 Dunn-Šidák correction for multiple hypotheses). (C, F) Average density in the map over  
795 a series of 3 second windows (calculated from 6 animals, 30 trials each). Red and blue  
796 indicate regions of high and low density, respectively.  
797

798 Figure 3.



799

800 Figure 3. Entropy for all of of descending neuron split-GAL4 lines. Trials were aligned  
801 such that the onset of red light activation occurred at  $t = 0$  sec (A, B). The red light was  
802 turned off at  $t = 15$  sec. (A) Baseline subtracted entropy (Y-axis) versus time (X-axis) for  
803 all experimental animals and all trials. Colors indicate a probability distribution  
804 describing the entropy of experimental animals at a given time in the trial. (B) Entropy  
805 levels of experimental animals over the course of the aligned trials (X-axis) shown by  
806 line (Y-axis). Warm and blue colors indicate high and low entropy, respectively.

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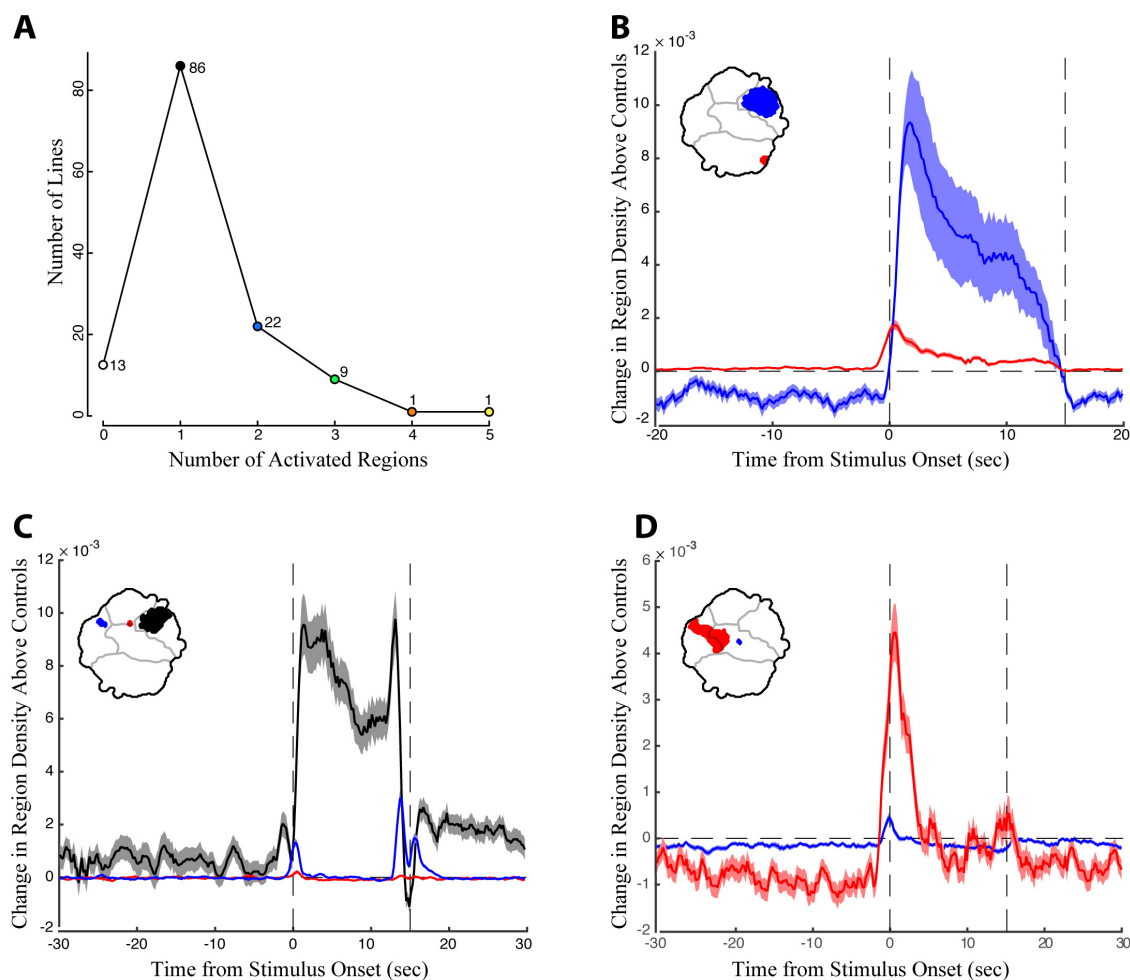
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814 Figure 4

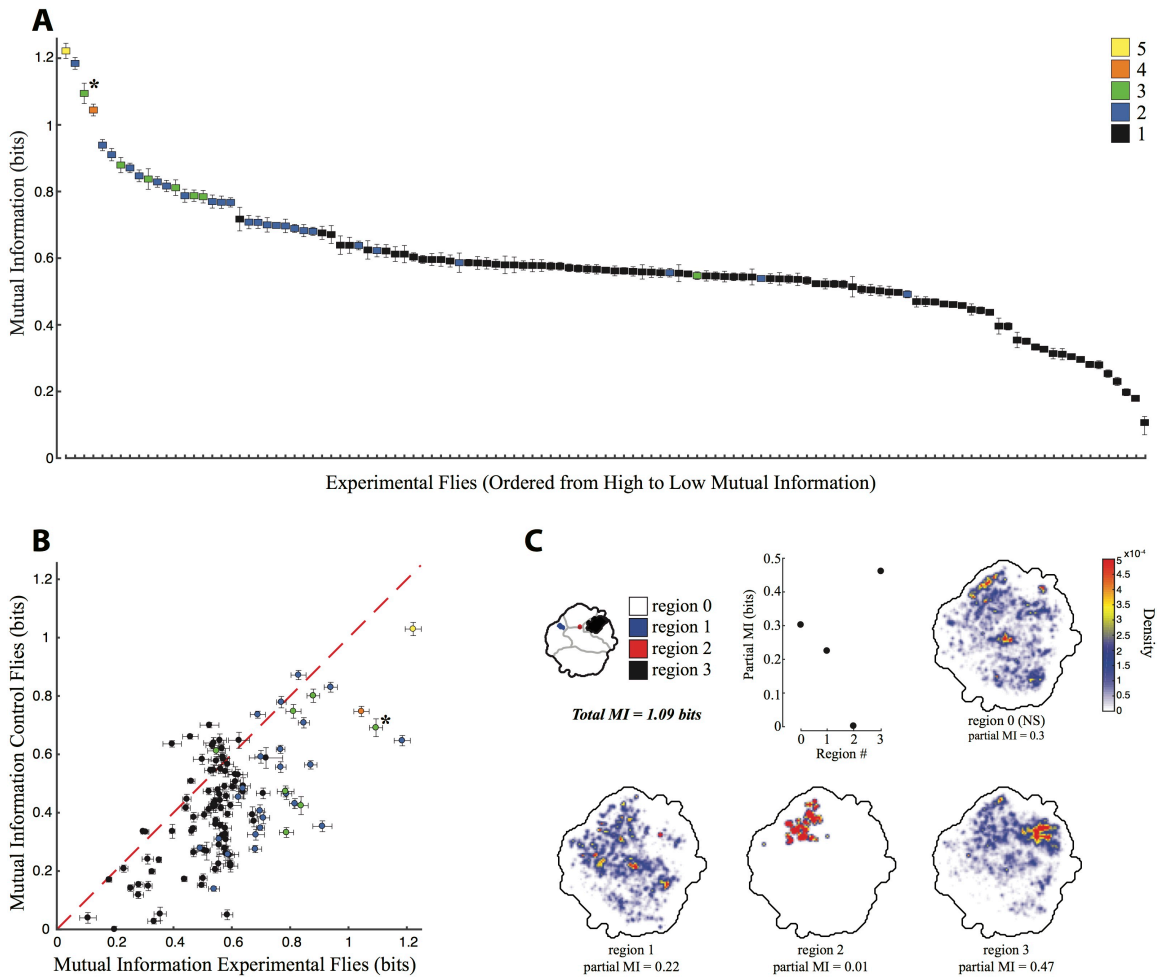


815

816 Figure 4. The timing of density shifts in descending neuron lines that occupy multiple  
817 behavior space regions. (A) Most lines (86) increase density in only one region of the  
818 behavior space upon red light activation. However, some lines occupy multiple regions in  
819 the behavior space (B-D) Examples of lines that occupy multiple discontinuous regions  
820 upon red light activation. Time is indicated on the X-axis, with the red light turned on at  
821  $T = 0$  sec and off at 15 sec. Change in density in the color coded regions in the  
822 experimental animals above the controls is indicated on the Y-axis. (B) Line SS01540  
823 targeting descending neuron DNp09; (C) SS02542 targeting descending neuron DNb01;  
824 (D) SS01049 targeting descending neuron DNp10.



825 Figure 5.



826

827 Figure 5. Mutual information between behaviors performed before and during red-light

828 activation. (A) Mutual information for experimental flies, calculated using the density of

829 the flies in the behavior space at  $t = -1.5$  to  $-0.5$  seconds prior to red light activation

830 versus density at  $t = 0$  to  $1.0$  seconds after red light activation. Y-axis indicates the mutual

831 information, X-axis is all the lines in order of most to least mutual information, error bars

832 indicate the standard deviation. Lines are color coded by the number of significant

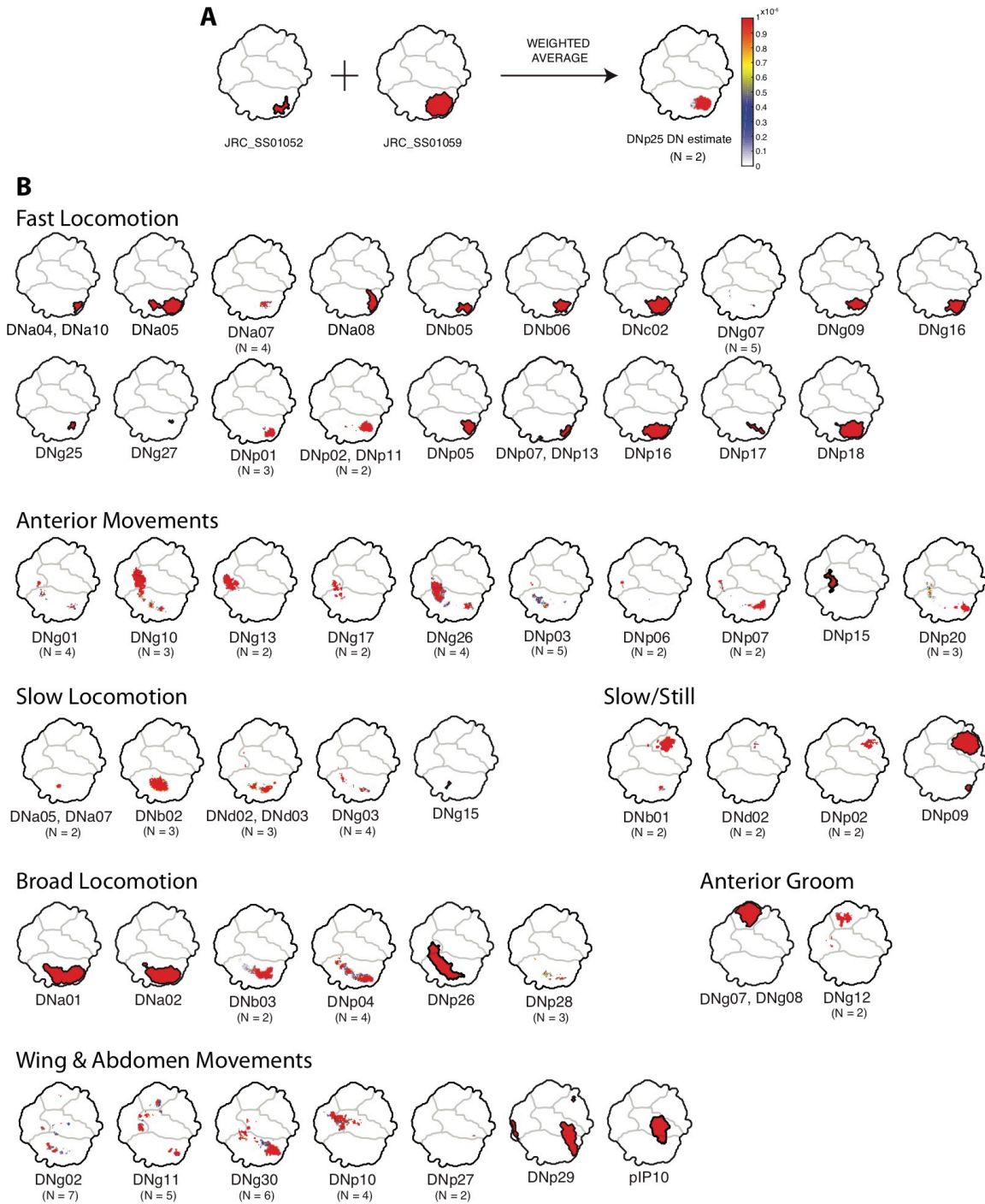
833 regions produced by red light activation, the key is indicated in the upper right, line

834 SS02542 is indicated by an asterisk. (B) Mutual information of experimental flies (X-

835 axis) plotted against that of non-retinal fed control flies (Y-axis), with the same color

836 coding as (A). (C) Partial mutual information for SS02542, showing the density at  $t = -$   
837 1.5 to 0.5 that has mutual information with the different regions of the behavior space  
838 after red light activation indicated in the key in the upper left portion of the figure.

839 Figure 6.



840

841 Figure 6. Averages of representative lines for individual descending neurons. Descending

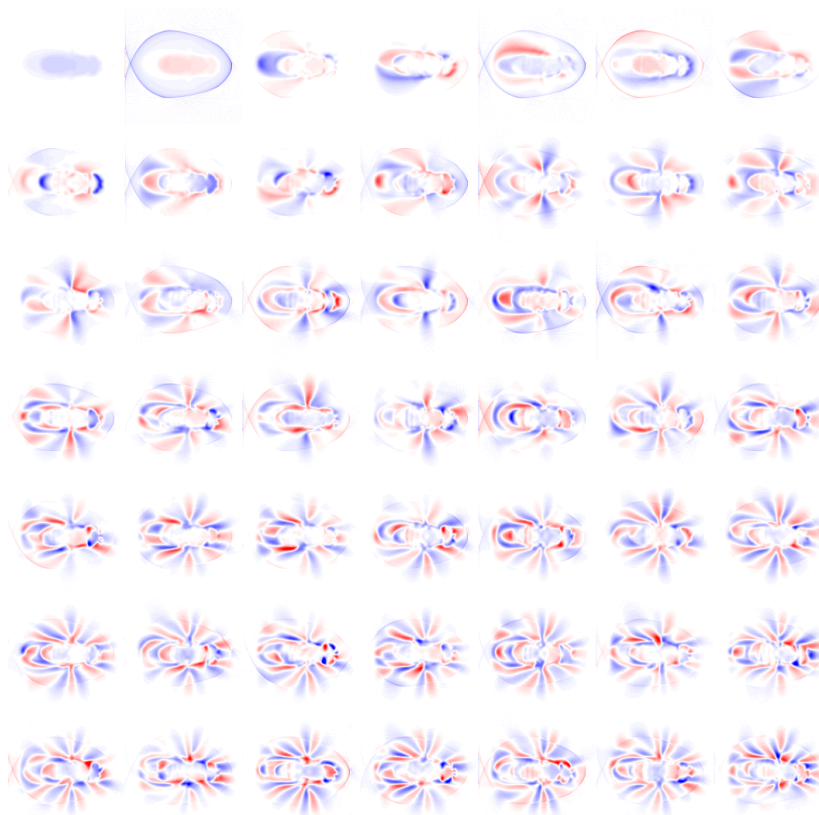
842 neurons are organized according to the region of the behavior space that they activate

843 (Figure 1). (A) Example illustrating the averaging of two lines to produce an estimated

844 phenotype. Colors indicate the degree to which particular regions are represented in the  
845 average. Red regions are highly represented, blue less so, and white not at all. (B)  
846 Phenotypes for 53 of the 58 descending neurons in the collection, plus pIP10 (von  
847 Philipsborn et al., 2011b). Some descending neurons are represented by a single clean  
848 line, others by averaging multiple clean lines. For averages, the number of lines are  
849 indicated under the descending neuron name.  
850

## 851 **Supplementary Information**

852 Figure S1.



853

854 Figure S1. Postural eigenmodes used to build the descending neuron behavior space

855 (Figure 1B).

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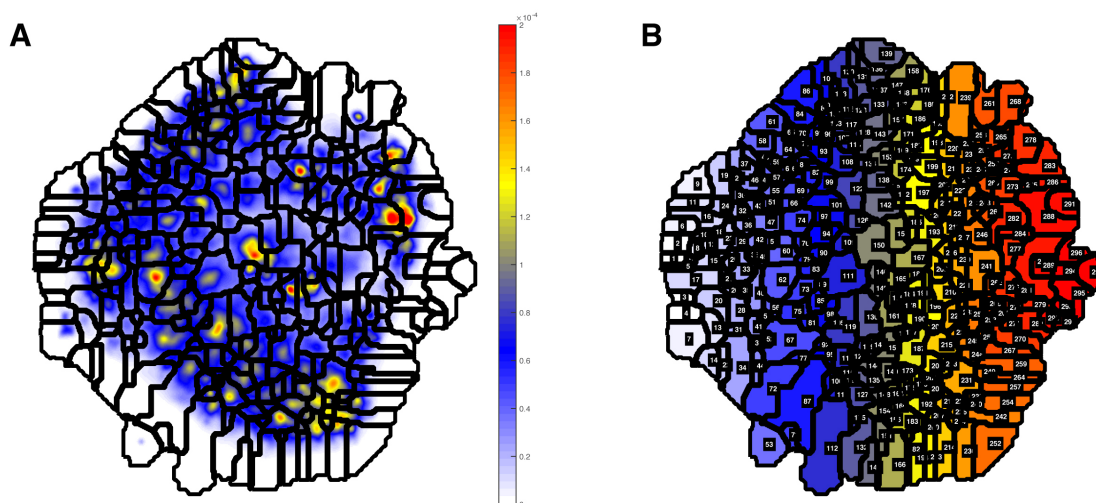
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863 Figure S2.

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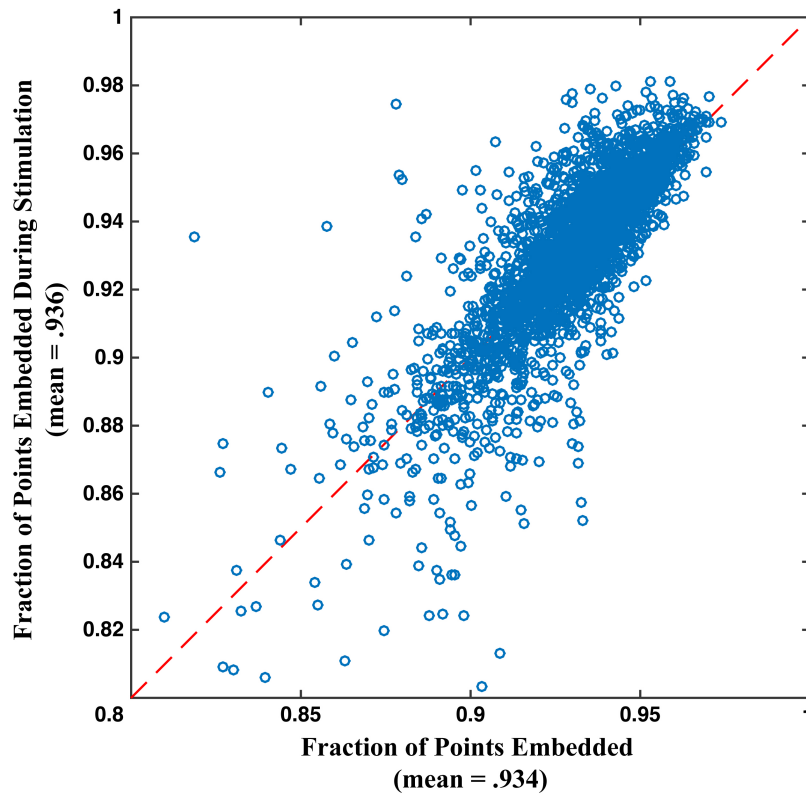
865

866 Figure S2. Watershedded regions in the descending neuron behavior space. (A) A  
867 watershedding algorithm was used on the behavior space in Figure 1B to identify local  
868 density maxima, which represent stereotyped behaviors. (B) Numbered watershedded  
869 regions. These correspond to videos underlying all the watershedded regions (Movies  
870 S1...S6) which were examined to create the human curated version of the behavior space  
871 in Figure 1C.

872

873 Figure

S3.



874

875 Figure S3. Fraction of video data points for each movie embedded in the behavior space,

876 during the red light stimulus window (Y axis) and during the recovery period (X axis).

877 For most movies, between 90 – 95% of frames embedded, with very little difference

878 between when the red light is on and when the red light is off, indicating that

879 CsChrimson and red light induced behaviors are well represented in the behavior space.

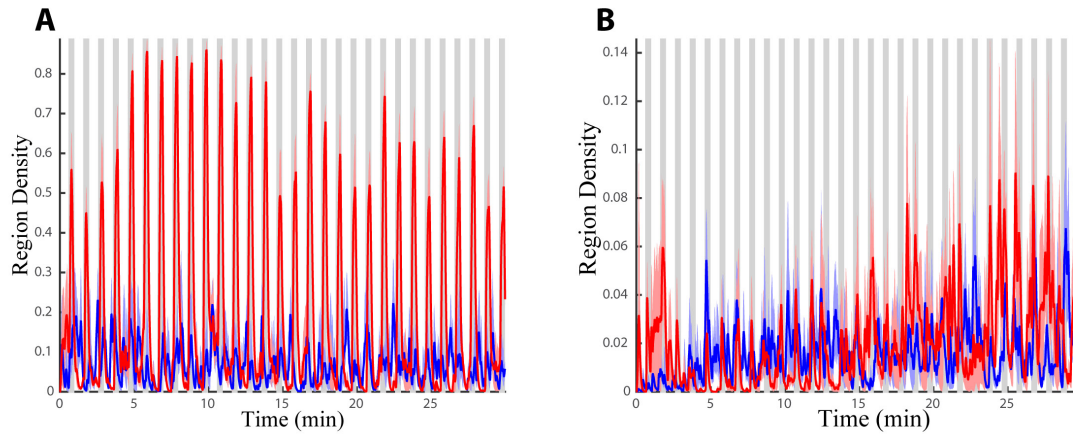
880 Failure to embed is usually indicative of imaging flaws, for example when part of the fly

881 is out of the field of view of the camera.

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883

884 Figure S4.



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886 Figure S4. Density in experimental (red) and control (blue) animals in the regions defined  
887 in Figure 2A and 2D (A and B, this figure, respectively). Densities shown in Fig. 2B and  
888 2E were averaged for six animals over 30 minutes. Gray bars indicate periods of red light  
889 activation.

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902 Figure S5. Analysis of all the descending neuron split-GAL4 lines. The line by line

903 analysis shown here follows the model of the selected examples described in Figure 2 of

904 the main text.

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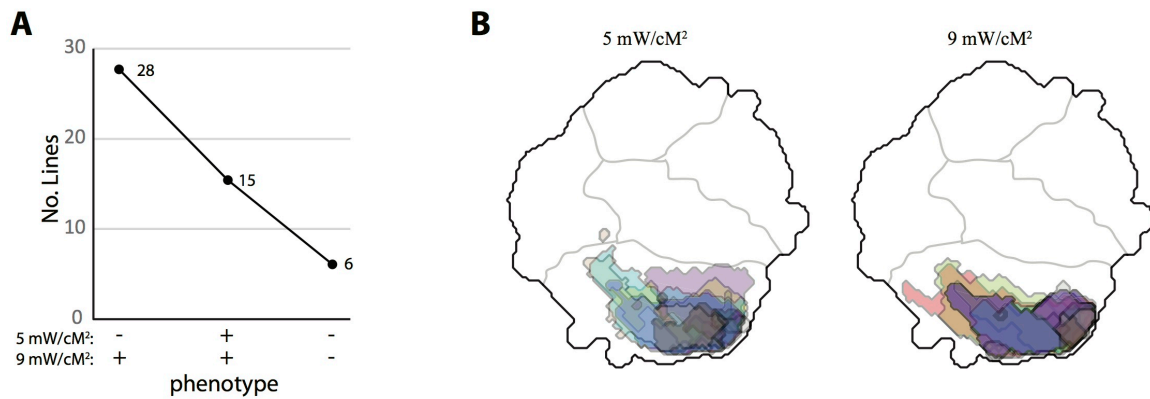
906 *Available at:* [http://www.biology.emory.edu/Berman/files/FigureS5\\_Cande\\_et\\_al.pdf](http://www.biology.emory.edu/Berman/files/FigureS5_Cande_et_al.pdf)

907 (Large file size)

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910



911

912 Figure S6. Choosing the red light intensity levels. (A) While most lines were screened at  
913 5 mW/cm<sup>2</sup>, 49 lines that had no or only a weak phenotype at this light level were  
914 repeated at 9 mW/cm<sup>2</sup> and a higher retinal concentration. 43 of these lines acquired a  
915 phenotype or a strengthened statistical significance of the previously observed phenotype  
916 under these conditions. No lines lost their phenotype at the higher intensity. (B) 10  
917 transient locomotion lines, overlaid in the same behavior space in different colors, were  
918 repeated at the lower and higher light intensities and retinal concentrations. The  
919 boundaries of the significant regions in the behavior space shift slightly between  
920 treatments, but overall the phenotypes remain largely unchanged.

921