

1 **Sun navigation requires compass neurons in *Drosophila***

2

3 **One sentence summary:** Silencing the compass neurons in the central complex of
4 *Drosophila* eliminates sun navigation but leaves phototaxis intact.

5

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15

16 **Abstract:**

17 To follow a straight course, animals must maintain a constant heading relative to a
18 fixed, distant landmark, a strategy termed menotaxis. In experiments using a flight
19 simulator, we found that *Drosophila* adopt arbitrary headings with respect to a
20 simulated sun, and individuals remember their heading preference between successive
21 flights—even over gaps lasting several hours. Imaging experiments revealed that a class
22 of neurons within the central complex, which have been previously shown to act as an
23 internal compass, track the azimuthal motion of a sun stimulus. When these neurons
24 are silenced, flies no longer adopt and maintain arbitrary headings, but instead exhibit
25 frontal phototaxis. Thus, without the compass system, flies lose the ability to execute
26 menotaxis and revert to a simpler, reflexive behavior.

27

28 Despite their small brains, insects can navigate over long distances – in some cases,
29 thousands of kilometers – by orienting to sensory cues such as visual landmarks (1),
30 skylight polarization (2–9) and the position of the sun (3, 4, 6, 10). Although *Drosophila*
31 are not generally renowned for their navigational abilities, mark-and-recapture
32 experiments in Death Valley revealed that they can fly nearly 15 km across open desert
33 over the course of a single evening (11). To accomplish such feats on available energy
34 reserves (12), flies would have to maintain relatively straight headings and rely on
35 celestial cues to do so (13).

36

37 Celestial cues such as sun position and polarized light are thought to be integrated in
38 the central complex, a set of highly conserved unpaired neuropils in the central brain of
39 arthropods (14). Central complex neurons in locusts (15), dung beetles (4), and monarch
40 butterflies (16) respond to the angle of polarized light and the position of small bright
41 objects mimicking the sun or moon. Extracellular recordings from the central complex
42 in cockroaches revealed neurons that act as head-direction cells in the absence of visual
43 cues or relative to a visual landmark (17). Recently, a group of cells (E-PG neurons) in
44 the *Drosophila* central complex have been shown to function as an internal compass (18–
45 20), similar to head-direction cells in mammals (21). Using the wide array of genetic
46 tools available to measure and manipulate cell function in *Drosophila*, we set out to test
47 whether flies can navigate using the sun and to identify the role of E-PG cells in this
48 behavior.

49

50 We tested the hypothesis that *Drosophila* can use the sun to navigate by placing tethered
51 wild-type female flies in a flight simulator and presenting an ersatz sun stimulus (Fig.
52 1A). The fly was surrounded by an array of LEDs on which we presented either a
53 single 2.3° bright spot on a dark background or a 15°-wide dark vertical stripe on a
54 bright background. Given previous studies on other species (4, 15, 16), we expect that

55 flies react to our small bright spot as they would to the actual sun, and thus we call it a
56 'sun stimulus'. Experiments were conducted in closed loop, such that the difference in
57 stroke amplitude between the fly's two wings determined the angular velocity of the
58 stimulus (12). Flies generally maintained the dark stripe in front of them (Fig. 1C, D), a
59 well-characterized behavior termed stripe fixation (22–24). However, when presented
60 with the sun stimulus, individual flies adopted arbitrary headings, thus exhibiting
61 menotaxis (Fig. 1B, D). We quantified how well flies maintained a heading by
62 calculating vector strength, which is the magnitude of the mean of all instantaneous
63 unit heading vectors for the entire flight. A vector strength of 1 would indicate that a fly
64 held the stimulus at the exact same heading during the entire flight bout. Because we
65 tested each individual with both a stripe and sun stimulus, we could compare the flies'
66 performance under the two conditions. We found no correlation between the mean
67 heading exhibited by individual flies during sun menotaxis and stripe fixation (Fig. 1E),
68 suggesting that heading preference for the sun stimulus is independent of the response
69 to a vertical stripe. To ensure that flies' stabilization of the sun stimulus was not an
70 artifact of our feedback system, we also conducted control closed-loop experiments in
71 which the bright spot was switched off. As expected, the flies exhibited no orientation
72 behavior under this condition, with all vector strength values lower than 0.16 (Fig. 1D).
73 Collectively, these experiments indicate that flies are capable of orienting to a small
74 bright spot and that this behavior is distinct from stripe fixation. *Drosophila* can also
75 perform menotaxis using the axis of linearly polarized light (8, 9, 25). It is not known
76 whether the orientation responses of flies to the sun and polarized light are
77 independent, as they are in dung beetles (4), or linked to create a matched filter of the
78 sky, as they are in locusts (15).

79

80 Given that individual flies adopted arbitrary headings with respect to the sun stimulus,
81 we tested whether they retained a memory of their orientation preference from one

82 flight to the next. We presented flies with the sun stimulus in closed loop, interrupted
83 flight for a defined interval (5 min, 1, 2, or 6 hours), and then again presented the sun
84 stimulus. To provide an independent metric of flight performance, we also presented a
85 stripe under closed loop conditions for 1 min before the first sun bout and after the
86 second. Across inter-flight intervals of 5 minutes, 1 hours, and 2 hours, flies remained
87 loyal to their first heading during the second flight (Fig. 1F). If each fly adopted the
88 identical heading in both flights, the mean heading difference would be zero, whereas if
89 there was no correlation in heading from one flight to the next, the mean absolute value
90 of the heading difference would be 90° , provided that the orientations were uniformly
91 distributed. To test whether the consistency in flight-to-flight orientation could arise
92 from chance, we bootstrapped 10,000 random pairs of mean heading values from the
93 first and second flights and compared the resulting distribution with the mean absolute
94 heading difference of the actual data (Figure 1G). In all cases, the measured mean
95 difference was less than that of the bootstrapped values (5 min: 54.2° vs. 79.2° ; 1 hour:
96 66.6° vs. 77.4° ; 2 hours: 66.8° vs. 84.8° ; 6 hours: 71.0° vs. 75.5°). We calculated
97 probability values directly from the proportion of the 10,000 bootstrapped simulations
98 that resulted in a smaller mean absolute angle difference than the observed data (Fig.
99 1G). With the exception of the 6-hour gap, this probability was quite low (5 min: $p=0.00$;
100 1 hour: $p=0.03$; 2 hours: $p=0.001$; 6 hours, $p=0.084$). Collectively, these results suggest
101 that headings are not selected at random with each subsequent takeoff, but rather that
102 flies remember their headings from previous flights, at least for up to 2 hours. A similar
103 result was found for the orientation responses to linearly polarized light (8). Fully
104 determining the mechanisms by which flies attain their initial heading preference (i.e.
105 genetic vs. developmental vs. learning) require experiments that are beyond the scope
106 of this current study.

107

108 The finding that flies remember their flight heading for at least 2 hours makes
109 ethological sense. *Drosophila* are crepuscular, exhibiting dawn and dusk activity peaks
110 (26). Assuming our laboratory measurements are representative of dispersal events, a
111 memory that allows an individual to fly straight for a few hours would be sufficient to
112 bias a day's migration in one direction. To our knowledge, there is no evidence that
113 *Drosophila* make multi-day, long-distance migrations that would require the ability to
114 maintain a constant course from one day to the next or a time-compensated sun
115 compass. The most parsimonious ecological interpretation of their sun orientation
116 behavior is that it allows flies to disperse opportunistically to new sources of food and
117 oviposition sites within a single day.

118
119 The visual information conveying sun position likely provides inputs to the recently
120 identified neurons constituting the fly's internal compass (18–20). These columnar
121 neurons receive input in the ellipsoid body and send divergent output to the
122 protocerebral bridge and gall, and are hence named E-PG neurons (27). These neurons
123 track azimuthal position of vertical stripes and more complex visual stimuli, and in the
124 absence of visual input can continue to track azimuthal orientation by integrating
125 estimates of angular velocity (18, 20, 28). Given these functional attributes, an obvious
126 question is whether E-PG neurons respond to a sun stimulus and whether they exhibit
127 different responses to other visual stimuli. We used the split-GAL4 line SS00096 (28),
128 which expresses in the E-PG neurons, to drive the genetically encoded calcium indicator
129 GCaMP6f, and measured activity in tethered, flying flies using a 2-photon microscope
130 (Fig. 2A). As described previously, the set of 16 E-PG neurons tile the toroidally shaped
131 ellipsoid body. Notably, a region of activity, or 'bump', rotates around the ellipsoid
132 body corresponding to azimuthal position (18, Movies S1, S2). Instead of recording from
133 the ellipsoid body, we imaged the activity at E-PG terminals in the protocerebral bridge
134 (Fig. 2B) because fluorescence signals were stronger in these more superficial glomeruli.

135 Based on well-established anatomy, we re-mapped the neural activity in the medial 16
136 glomeruli of the protocerebral bridge into the circular reference frame of azimuthal
137 space (Fig. 2C, 27) and computed a neural activity vector average, or bump position, for
138 each image (similar to 28; see Materials and Methods for details).

139

140 As in our flight arena experiments (Fig 1A), flies adopted arbitrary headings with
141 respect to the sun stimulus (Fig. 2G, H), which they maintained over a 5-minute break
142 (Fig 2G). By presenting sun and stripe stimuli to the same fly, we tested whether these
143 two stimulus types are represented differently by the E-PG neurons. Bump position
144 faithfully tracked the position of both the sun and stripe stimuli (Fig. 2D-F). Prior
145 studies found that while the E-PG bump tracks the azimuthal position of a vertical
146 stripe, it does so with an arbitrary azimuthal angular offset (18). We found an identical
147 result with the sun stimulus; the bump rotated with changes in sun position, but with a
148 bump-to-stimulus offset that varied from individual to individual. In addition, the
149 bump-to-stimulus offset did not differ between the first and second sun presentation
150 trials or between the sun and stripe presentation trials (Fig 2J, K). The offset was not
151 correlated with the azimuthal angle at which individual flies tended to hold the sun
152 (Fig. 2I). Together, these imaging results suggest that the representation of the sun and
153 stripe in the E-PG neurons is similar despite the distinct behavioral responses to the
154 stimuli, and that the bump-to-stimulus offset does not encode heading preference.

155

156 We next tested the causal contributions of E-PG neurons to sun navigation and stripe
157 fixation, predicting that the highly variable headings adopted in sun navigation might
158 require the instantaneous positional information provided by E-PG neurons. We took
159 advantage of the sparse expression patterns of three different split-GAL4 lines (Fig. 3A)
160 to selectively drive the inwardly rectifying potassium channel Kir2.1 (29). As a control,
161 we crossed UAS-Kir2.1 to an engineered split-GAL4 line that was genetically identical

162 to the experimental driver lines, but carried empty vectors of the two GAL4 domains in
163 the two insertion sites (30). Driving Kir2.1 in three, separate split-GAL4 lines yielded
164 flies that lost the ability to maintain the sun at arbitrary azimuthal positions, although
165 they could fixate the sun and stripe frontally. To assess the degree to which this effect
166 could have occurred by chance, we employed a bootstrapping approach similar to that
167 used in our time gap experiments. We randomly selected 50 values from our control
168 dataset 10,000 times, in each case calculating the circular variance of the subsampled
169 population. We then determined the proportion of bootstrapped mean variances that
170 had smaller values than the variance of the actual experimental data and concluded that
171 the observed frontal distributions of our experimental groups were highly unlikely to
172 have occurred by chance (SS00096: $p=0.000$; SS00408: $p=0.000$; SS00131: $p=0.004$). Thus,
173 E-PG neuron activity appears necessary for menotaxis, i.e. maintaining the sun in
174 arbitrary non-frontal positions. To our knowledge, this is the first behavioral deficit
175 elicited via experimental manipulation of the compass cell network.

176
177 In the absence of normal E-PG function, flies might directly orient toward the sun
178 because they lack the ability to compare their instantaneous heading to a stored value of
179 their directional preference. Such a loss of function in the compass network might
180 unmask a simpler reflexive behavior – phototaxis – that does not require the elaborate
181 circuitry of the central complex. Consistent with this hypothesis, stripe fixation was not
182 different between control and experimental animals. This interpretation is compatible
183 with a recent model that showed frontal object fixation could result from a simple
184 circuit involving two asymmetric wide-field motion integrators, without the need for
185 the central complex (31).

186
187 Our findings are consistent with an emerging model of a navigational circuit involving
188 the central complex. E-PG cells have an excitatory relationship with another cell class in

189 the central complex (protocerebral bridge-ellipsoid body-noduli or P-EN neurons),
190 creating an angular velocity integrator that allows a fly to maintain its heading in the
191 absence of visual landmarks (19, 20). Furthermore, the E-PG neurons are homologous
192 to the CL1 neurons described in locusts (32), monarchs (16), dung beetles (4), and bees
193 (33) and likely serve similar functions across taxa. In an anatomy-based model of path
194 integration in bees, CL1 neurons are part of a columnar circuit that provides
195 instantaneous heading information to an array of self-excitatory networks that also
196 receive convergent optic flow information, thereby storing a memory of distance
197 traveled in each direction (33). This information is then retrieved as an animal returns
198 home by driving appropriate steering commands in other classes of central complex
199 neurons. The putative memory cells suggested by this model, CPU4 cells, could be
200 homologous to protocerebral bridge-fan shaped body-noduli (P-FN) neurons described
201 for *Drosophila* (27). Furthermore, cells responsive to progressive optic flow are found
202 throughout the central complex of flies, including neuropil in the fan-shaped body
203 containing the P-FN cells (34). The authors of the recent path integration model suggest
204 that the CPU4 network in bees might also function to store the desired heading during
205 sun navigation (33). Although our results do not directly test this model, they are
206 consistent with the role of CL1 neurons in providing heading direction to circuits that
207 generate steering commands towards an arbitrary orientation whose memory is stored
208 in the network of CPU4 (P-FN) neurons.

209
210 Stripe fixation and sun navigation behaviors may represent two different flight modes
211 in *Drosophila*. Stripe fixation is thought to be a short-range behavioral reflex to orient
212 towards near objects (12), which in free flight is quickly terminated by collision
213 avoidance (13) or landing behaviors (35). In contrast, navigation using the sun is likely
214 a component of long-distance dispersal behavior that could be used in conjunction with
215 polarization vision (8, 9) either in a hierarchical (4) or integrative (36) manner.

216 Individuals could differ in where they lie on the continuum of long-range dispersal to
217 local search, which could explain the inter-individual variation we observed in heading
218 fidelity during sun orientation experiments. In general, dispersal is a condition-
219 dependent behavior that is known to vary with hunger or other internal factors (37).

220

221 Given the architectural similarity of the central complex among species (14), the celestial
222 compass we have identified in *Drosophila* is likely one module within a conserved
223 behavioral toolkit (13) allowing orientation and flight over long distances by integrating
224 skylight polarization, the position of the sun or moon, and other visual cues. An
225 independent study has recently found that the E-PG compass neurons are also
226 necessary in walking flies for maintaining arbitrary headings relative to a small bright
227 object (38). The expanding array of genetic tools developed for flies as well as the rapid
228 growth in our understanding of the neural circuitry involved in orientation during
229 walking (18–20) and flight (28) make this a promising system for exploring such
230 essential and highly conserved behaviors.

231

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350

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358 and TLW were involved in early experimental design and analysis. YMG, KJL, IKR and
359 MHD conceived of and conducted experiments. YMG characterized sun compass
360 behavior (Fig. 1), IKR conducted functional imaging experiments (Fig. 2), and YMG and
361 KJL performed genetic silencing experiments (Fig. 3). YMG, KJL, IKR and MHD wrote
362 the paper. All authors contributed in editing the final manuscript. **Competing**
363 **interests:** Authors declare no competing interests. **Data and materials availability:** All
364 data will be made available on Dryad upon publication.

365

366

367 **List of Supplementary Materials**

368 **Supplementary Materials**

369 Materials and Methods

370 Movie S1

371 Movie S2

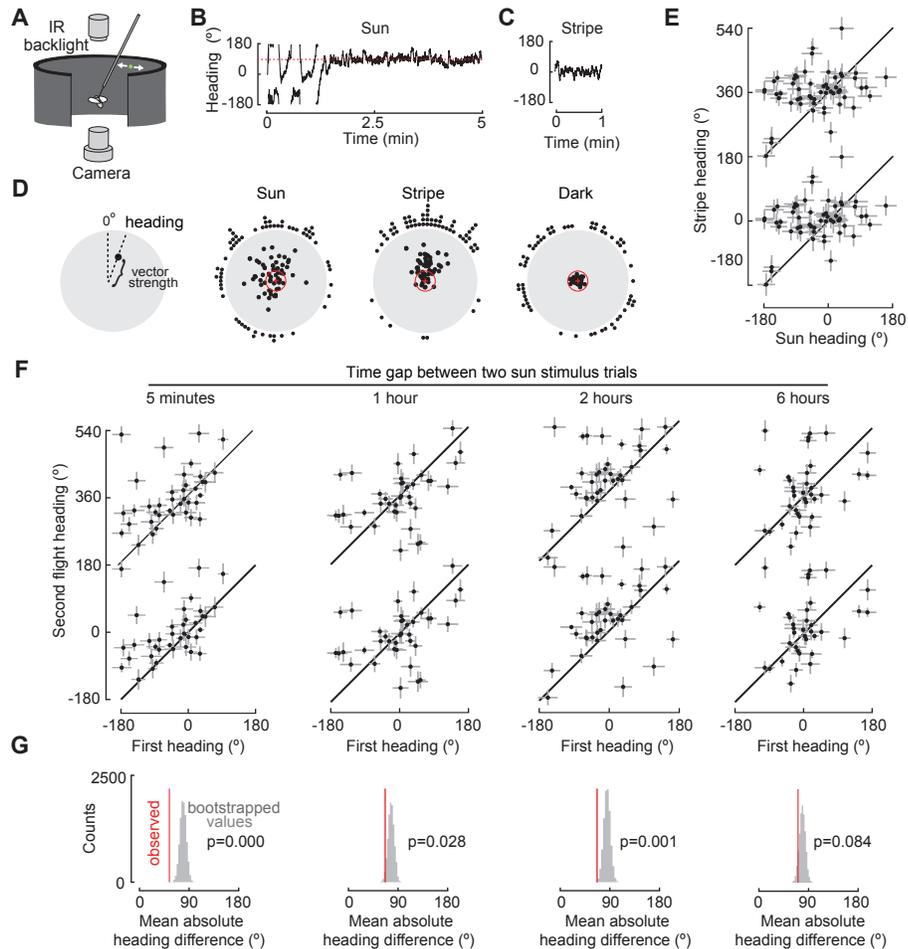


Fig. 1. Flies navigate using a sun stimulus and retain memory of their heading. (A) Tethered fly, backlit with infrared light and surrounded by a cylindrical LED display; a single 1.43° spot simulates the sun. (B) Example trace showing closed-loop behavior. After 88 seconds, the fly stabilized the sun stimulus at a heading of 92° . (C) Heading during a stripe presentation. (D) Polar representation of data. Angular position indicates mean heading; radial distance indicates vector strength. Headings for flies presented with a sun stimulus, a stripe, and in the dark. A histogram of mean heading is plotted around each circle. Dashed red circle, vector strength of 0.2. (E) Sun versus stripe heading. Data are repeated on the vertical axis to indicate their circular nature. Diagonal line indicates identical heading over both trials. Error bars, circular variance times 36 for visibility. (F) Heading in first trial plotted against second trial heading for increasing inter-trial intervals; plotting conventions as in 1E. (G) Distribution of 10,000 bootstrapped heading differences between random pairings of first and second trials from 1F. Red line, mean heading difference of observed data; p-value, proportion of resampled differences that are smaller than the observed mean heading difference.

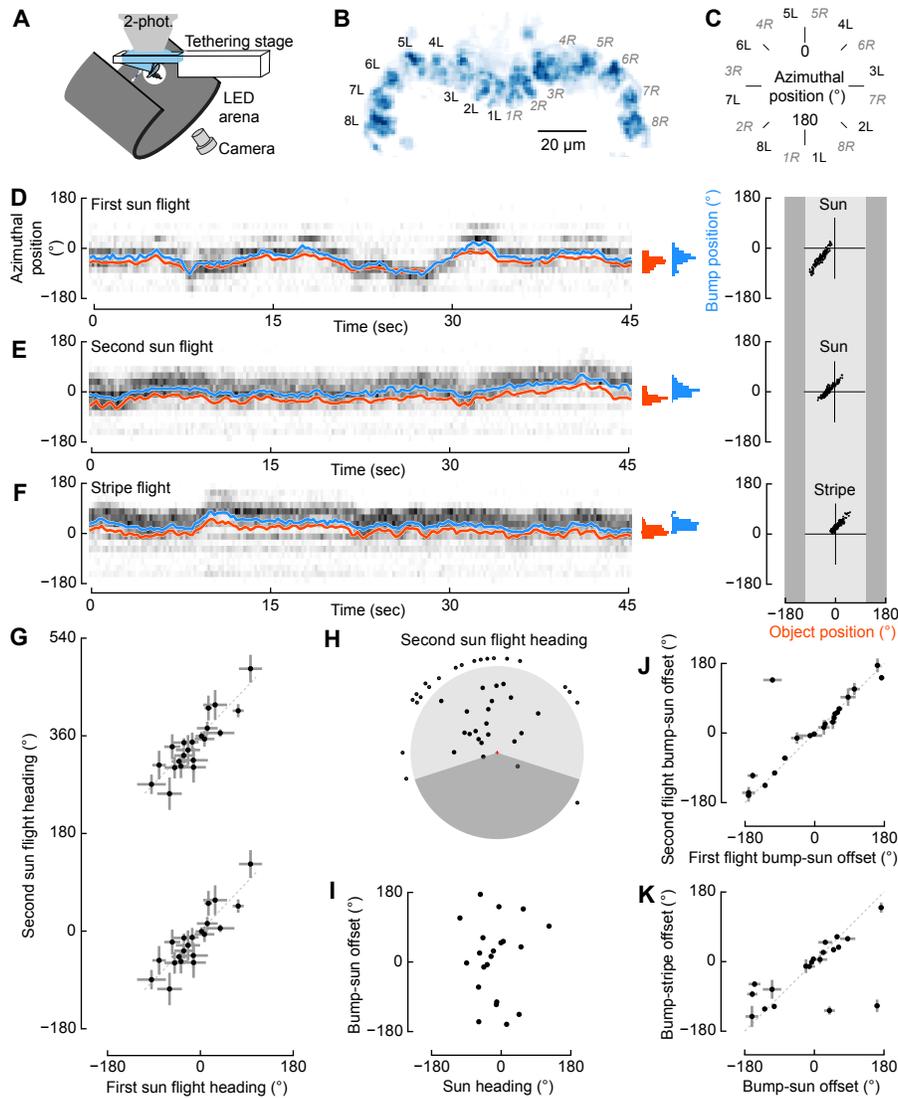


Fig. 2. E-PG neuron activity correlates with both sun and stripe position. (A) Ca²⁺-imaging schematic. (B) Glomeruli assignment in protocerebral bridge based on a standard deviation of GCaMP6f fluorescence in E-PG terminals. (C) Continuous azimuthal representation of glomeruli in B. (D) GCaMP6f fluorescence ($\Delta F/F$), represented as the unwrapped glomerular positions from C, during 45 seconds of a sun presentation (grey). Azimuthal position of E-PG activity bump (blue trace and probability distribution) and sun position (computed as in (25), red) co-vary. Regression of object position on the 216°-wide LED arena (light gray) against bump position for sequence plotted on left. (E) Similar to D, but for second sun presentation and (F) subsequent stripe presentation. (G) Heading during first sun trial plotted against heading in second sun trial with a minimum of 5 min inter-trial interval (N=20; plotted as in Figure 1E). (H) Polar representation of second sun bout headings, similar to Figure 1D. Shaded area not visible by fly. (I) E-PG bump-to-stimulus offset for each second sun flight bout. (J) Regression of the median bump-to-stimulus offset for first sun bout against the offset for the second sun bout. (K) Bump-to-stimulus offset for stripe regressed against the offset for the sun.

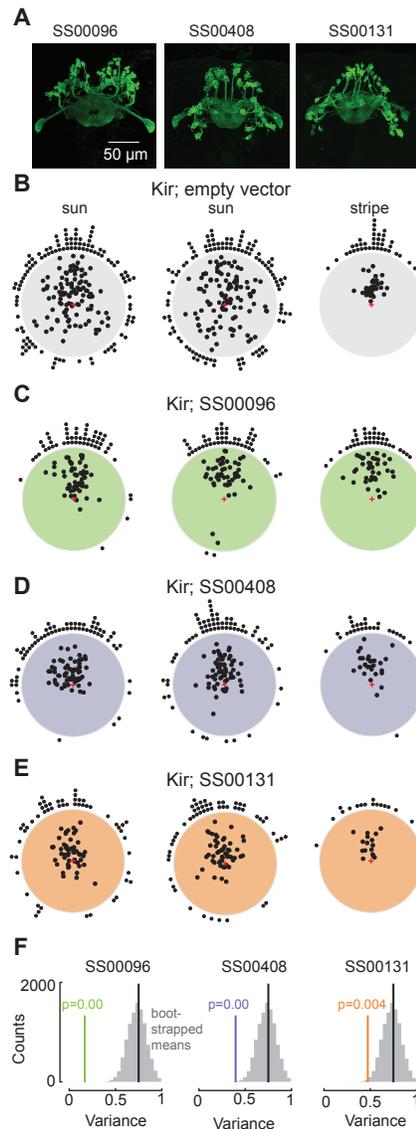


Fig. 3 E-PG neuron activity is necessary for proportional navigation using sun. (A) Fluorescence labeling of GFP expressed in E-PG neurons in three experimental split-Gal4 lines, maximum intensity projections. (B) Sun menotaxis and stripe fixation in genetic controls (Kir; empty vector split-Gal4). (Left) First 5-min trial of sun fixation ($n = 111$ flies). (Center) Second 5-min trial of sun fixation ($N = 108$). (Right) Five minutes of stripe fixation ($N = 38$). (C) Results from the same experimental paradigm for Kir;SS00096 ($N = 54, 49, 28$). (D) Sun and stripe fixation for Kir;SS00408 ($N = 64, 66, 28$). (E) Headings from SS00131;Kir ($N = 54, 54, 16$). (F) Flies with silenced E-PG neurons have smaller variances than the genetic control. Distribution of 10,000 bootstrapped circular variances subsampled from the empty-vector control's second sun trial, shown in gray ($N = 50$ each). Black lines depict the observed heading variance of the entire dataset ($n = 108$). Red lines indicate population heading variance of the second sun trial for each experimental group. P-value indicates the proportion of bootstrapped variances that are smaller than the observed variance for each experimental group.

Supplementary Materials for

Sun Navigation Requires Compass Neurons in *Drosophila*

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This PDF file includes:

Materials and Methods
Captions for Movies S1 to S2

Other Supplementary Materials for this manuscript includes the following:

Movies S1 to S2

372 **Materials and Methods**

373 Experimental animals

374 We conducted all experiments using 2-4 day old female *Drosophila melanogaster*. Our
375 initial analysis of sun orientation behavior (Fig. 1) was conducted using flies from a
376 wild-caught strain ('top banana') collected in Seattle, WA and maintained in the lab
377 since September 2013. We reared flies in incubators on a 12 hour light: 12 hour dark
378 cycle at 25°C on standard cornmeal fly food. For the functional imaging experiments
379 (Fig. 2), we used flies heterozygous for *w⁺;UAS-tdTomato;UAS-GCaMP6f* (39) and the
380 split-Gal4 line SS00096 (28). For silencing experiments (Fig. 3), we crossed a
381 backcrossed version of UAS-Kir2.1 with SS00096, SS00131, and SS00408 (kindly
382 provided by Tanya Wolff at Janelia Research Campus). The controls in our silencing
383 experiments were the progeny of the UAS-Kir2.1 line and an engineered split-GAL4 line
384 in which the two insertion sites carried empty vectors of the two GAL4 domains, but
385 were otherwise genetically identical to the experimental driver lines (30). We generated
386 flies for confocal imaging by crossing UAS-*myr:GFP*; UAS-*red-Stinger* with each of the
387 split-GAL4 lines.

388

389 Fly tethering

390 For sun orientation (Fig. 1) and genetic silencing experiments (Fig. 3), we tethered flies
391 under cold anesthesia and glued them to a tungsten wire (0.13 mm diameter) at the
392 anterior dorsal portion of the scutum with UV-cured glue (Bondic Inc.). We also fixed
393 the head of each fly to its thorax by applying an additional drop of glue. Flies were
394 allowed to recover for at least 10 minutes prior to testing.

395

396 For functional imaging experiments (Fig. 2), we tethered each fly to a specially designed
397 physiology stage (40) that permitted access to the posterior side of the fly's head. We

398 filled the holder with saline, and removed a section of cuticle overlying the region of the
399 central complex. To improve imaging quality, we removed adipose bodies and trachea
400 from the light path. Flies were continuously perfused with saline (41) which was
401 actively regulated to a temperature of 21°C. We allowed flies a minimum of 20 minutes
402 to recover from cold-anesthesia prior to imaging.

403

404 Flight arenas and stimulus presentation

405 For sun-orientation behavior (Fig. 1) and genetic silencing experiments (Fig. 3), we
406 placed tethered flies in an LED flight simulator (42) (Fig. 1A). We displayed patterns on
407 a circular arena of either 12 x 1 (Fig. 1) or 12 x 2 (Fig. 3) LED panels, with each panel
408 consisting of an 8 x 8 array of individual pixels (Betlux #BL-M12A881PG-11, $\lambda=525$ nm).
409 Each pixel subtended an angle of 2.8° at the center of the arena with a 0.93° gap between
410 adjacent pixels. The panels were controlled using hardware and firmware
411 (IORodeo.com) as described previously (42), with slight modifications in current
412 sinking required to display a single bright pixel without generating bleed-through on
413 other pixels in the same panel row. We placed the fly in the center of the arena at a body
414 angle of ~60°, approximating the orientation during free flight (43). For wing tracking,
415 flies were backlit with a collimated infrared source (850 nm, 900mW; Thorlabs Inc.
416 #M850L3). We placed a 45° mirror below the fly and used a firewire camera (Basler
417 A602f-2) or a Point Grey USB 3.0 camera (now FLIR, Blackfly 0.3MP monochrome
418 camera, BFLY-U3-03S2M-CS) for image capture. Each camera was equipped with a
419 Computar macro lens (MLM3X-MP) and IR-pass filter (Hoya B-46RM72-GB) to exclude
420 extraneous light from the LED display.

421

422 To track the wing stroke envelope during flight, we used Kinefly, real-time machine-
423 vision software developed in the lab (44). As in previous studies (12), we used the
424 difference in wing beat amplitude (Δ WBA) as a feedback signal by which the fly could

425 control the angular velocity of the visual stimulus. The gain of this control relationship
426 was set to 14.67, 5.88, or $4.75^\circ \text{ sec}^{-1}$ for each $^\circ\Delta\text{WBA}$ for sun orientation experiments (Fig.
427 1), functional imaging (Fig. 2), and genetic silencing (Fig. 3), respectively. We found that
428 a lower feedback gain was required in our experiments with transgenic lines to generate
429 stable orientation behavior to both sun and stripe stimuli.

430

431 For functional imaging experiments, we presented visual stimuli using a 12 x 4 panel
432 (96 x 32 pixel) arena, which covered 216° of azimuth with a resolution of $\sim 2.25^\circ$. To
433 reduce light pollution from the LED arena into the photomultiplier tubes of the 2-
434 photon microscope, we shifted the spectral peak of the visual stimuli from 470 nm to
435 450 nm by placing two transmission filters in front of the LEDs (Roscolux no. 59 Indigo
436 and no. 39 Skelton Exotic Sangria). We tracked wing stroke angles using Kinefly and
437 presented stimuli in closed-loop as described above, except that we illuminated the
438 wings using four horizontal fiber-optic IR light sources (Thorlabs Inc. #M850F2)
439 distributed in a $\sim 90^\circ$ arc behind the fly.

440

441 For data presented in Figs. 1 and 3, a single pixel served as our ersatz sun. At the plane
442 of the fly, a single pixel subtends a maximum angle of 2.8° . However, because the fly
443 was placed $\sim 30^\circ$ below the plane of the illuminated pixel, the simulated sun subtended
444 a maximum angle of $\sim 2.3^\circ$ at the fly's retina, which is larger than the sun's angular
445 diameter ($\sim 0.5^\circ$), but smaller than the inter-ommatidial acceptance angle of $\sim 5^\circ$ (45). For
446 sun orientation experiments (Fig. 1), we conducted all trials in a 12 x 1 panel (96 x 8
447 pixel) arena. For stripe fixation, we presented a 4 pixel-wide dark stripe (15° wide x 30°
448 high) on a bright background.

449

450 To determine the visual contrast flies experienced during our experiments, we
451 measured the normalized difference between the lightest and darkest parts of the

452 display (Michelson contrast). We placed a small metal tube covered in black electrical
453 tape over a power sensor (Thorlabs S170C, PM100D) to reduce reflections and
454 approximate the acceptance angle of an ommatidium ($\sim 5^\circ$). We held the sensor at the
455 center of the arena and directed it toward a sun or stripe to measure the stimulus light
456 level and then moved the stimulus 45° in azimuth to measure the background light
457 level. The Michelson contrast for all sun stimulus experiments was 0.99 and stripe
458 contrast was 0.75 and 0.74 for the data presented in Fig. 1 and Fig. 3 respectively.

459
460 The behavior of flies from the control line (UAS-Kir; split-GAL4-empty-vector) was
461 generally similar to wild type flies; however, they tended to perform poorly in the
462 stripe-fixation paradigm, as indicated by relatively low vector strength (Fig. 3B) and a
463 smaller proportion of flies that completed the trial without stopping. Given that flies'
464 azimuthal control of a stripe stimulus improves as a function of increasing stripe height
465 (24), we doubled the height of the visual display to a stripe of $\sim 58^\circ$ (12 x 2 panels, 96 x 16
466 pixels) for our genetic silencing experiments (Fig. 3). We noted that reflections
467 generated by a single bright pixel on the faceted inner surface of the arena generated a
468 faint dark stripe on the column of panels on which the sun stimulus was displayed. To
469 guard against the possibility that the fly would orient to this reflection feature, we
470 fabricated cylindrical inserts of black velvet that obscured the surface of the display
471 except for a narrow slit ($9 \text{ mm} \times 360^\circ$) that contained the LED row in which the sun
472 stimulus was displayed. The insert could be quickly removed without disturbing the fly
473 for trials using a stripe stimulus. To facilitate the collection of large sample sizes for the
474 genetic silencing experiments, we constructed two identical arenas, which we operated
475 in parallel.

476
477 In the functional imaging experiments (Fig. 2) we compensated for a larger arena and
478 dimmer LEDs by using a $3.6^\circ \times 3.6^\circ$ spot (2 x 2 pixels) as our sun stimulus, with a

479 Michelson contrast of 0.92. The stripe stimulus consisted of a 12.6° wide x 64° high
480 vertical bright stripe presented on a dark background, with a Michelson contrast of 0.93.
481 Using a bright stripe on a dark background was necessary in order not to saturate the
482 PMTs. Flies exhibit less robust fixation under these conditions (42) but nevertheless
483 performed the behavior, allowing us to compare sun- and stripe- fixation during
484 functional imaging.

485

486 Sun orientation and time gap experiments

487 To test the persistence of flight headings, we presented flies with the sun stimulus in
488 closed loop, provided a rest period between flights for either 5 minutes, 1 hour, 2 hours,
489 or 6 hours, and then tested flies in a second bout with a sun stimulus. Before and after
490 the sun stimulus trials, we presented flies with a stripe for 1 minute. For 5-minute inter-
491 trial intervals, we left the fly in the arena and stopped flight by presenting a small piece
492 of paper. To prevent dehydration during longer inter-trial intervals (1, 2 and 6 hours),
493 we removed the fly from the arena and placed it on a small foam ball floating in a
494 microcentrifuge tube filled with water. Following this rest period, we returned the fly to
495 the arena and the second flight was initiated by providing a small puff of air. We
496 discarded trials in which any fly stopped flying more than once during any of the stripe
497 or sun presentations.

498

499 2-photon functional imaging

500 We imaged at an excitation wavelength of 930 nm using a galvanometric scan mirror-
501 based two-photon microscope (Thorlabs, Inc., Newton, NJ, USA) equipped with a
502 Nikon CFI Plan Fluorite objective water-immersion lens (10x mag., 0.3 N.A., 3.5 mm
503 W.D.). With the addition of a piezo-ceramic linear objective drive (P-726, Physik
504 Instrumente GmbH and Co. KG, Karlsruhe, Germany) we imaged two x-y planes
505 separated by 25 µm along the z axis. Within the resulting volume we recorded

506 tdTomato and GCaMP6f fluorescence in those glomeruli of the protocerebral bridge
507 (PB) that contain terminals of E-PG neurons. We scanned in a boustrophedon pattern
508 from ventral to dorsal to align the piezo drive descent during each plane scan with the
509 anatomical inclination of the PB, maximizing the volumetric capture of the target
510 glomeruli. We acquired the 142 x 71 μm images with 128 x 64 pixel resolution at 13.1
511 Hz. The 2-plane scan with one fly-back frame resulted in a 4.36 Hz volumetric scan rate.
512 To correct for motion in the x-y plane, we registered both channels for each frame by
513 finding the peak of the cross correlation between each tdTomato image and the trial-
514 averaged image (46). Subsequently, we collapsed the two planes with a maximum z-
515 projection. Based on known anatomy, we manually assigned a region of interest (ROI)
516 to each PB glomerulus with E-PG neuron innervation. For each volumetric frame, we
517 computed fluorescence (F_t) of the GCaMP6f signal by subtracting the mean of the
518 background pixels from the mean of the ROI pixels for each glomerulus. The
519 background was defined as the 10% dimmest pixels across the entire z-projected image
520 for each fly. We normalized the fluorescence in the ROI of each glomerulus to its
521 baseline fluorescence (F_0) as follows: $\Delta F/F = (F_t - F_0)/F_0$ and defined F_0 as the mean of the
522 10% lowest GCaMP6f fluorescence in the ROI of each glomerulus. Under closed-loop
523 conditions, we presented each fly with a sun stimulus twice for five minutes, separated
524 by a minimum of 5 minutes. A 2-minute presentation of the stripe stimulus followed the
525 second sun stimulus trial.

526

527 Functional silencing of E-PG neurons in sun navigation behavior

528 We tested all control and experimental flies with a paradigm consisting of 5 minutes of
529 sun stimulus presentation, a 5-minute break, 5 minutes of sun presentation, and 5
530 minutes of stripe presentation. We discarded trials in which flies stopped more than
531 twice per stimulus presentation.

532

533 Immunohistochemistry of split-GAL4 lines

534 We dissected and stained brains of flies expressing UAS-myr:GFP, UAS-redStinger and
535 each of the three split-GAL4 lines (SS00096, SS00131, SS00408) using modifications to
536 standard laboratory immunohistochemistry protocols (44). We dissected brains in 4%
537 formaldehyde fixative. After a 20-30 minute fixation, we washed tissue 2 x 20 minutes
538 in phosphate buffered saline (PBS) followed by a permeabilization step of 2 x 20 minute
539 washes in phosphate buffered saline with 0.5% Triton-X (PBST). We incubated tissue
540 with primary antibodies anti-GFP AlexaFluor™ 488 conjugate (1:1000 concentration,
541 Invitrogen # A21311) and anti-nc82 to label neuropil (1:10 concentration, Developmental
542 Studies Hybridoma Bank, AB 2314866) in 5% normal goat serum in PBST overnight on a
543 nutator at 4°C. The following day, we washed with PBST 3 x 20 min and incubated
544 with a secondary antibody to anti-nc82 (AlexaFluor™ 633, 1:250 concentration,
545 Invitrogen # A21050) overnight at 4 °C. Brains were washed 3 x 20 min with PBST and 2
546 x 20 min with PBS the following day. We dehydrated brains through an ethanol series
547 (30%, 50%, 70%, 90%, 100%, 100%, each for 10 min), cleared tissue with xylene (2 x 10
548 min) and mounted in DPX (47). Using a Leica SP8, we imaged brains under a 63x
549 objective (Leica #506350, 1.4 N.A.). Maximum intensity projections were generated in
550 Fiji (48, 49).

551

552 Quantification and statistical analysis

553 We processed and analyzed all data in Python 2.7 and Matplotlib (50). Before making
554 pairwise comparisons of mean heading direction in separate flights (as in Fig 1E, F), we
555 excluded trials with a vector strength under 0.2 (36.2% of all trials). Mean headings for
556 flights with very low vector strength are not meaningful, as this indicates that the fly
557 did not select a heading during the trial. However, including all data did not
558 qualitatively change the relationship between first and second flights.

559

560 To assess whether heading fidelity was maintained over time gaps, we bootstrapped
561 random pairings of first and second sun presentation trials 10,000 times. We compared
562 the distribution of the mean absolute value of heading difference between the flights for
563 these simulated data sets to the mean absolute value of heading difference of the
564 observed data. We calculated the p-value as the proportion of simulated data sets that
565 had a mean heading difference smaller than that of the observed data. We conducted a
566 similar analysis for the results of our behavioral genetics experiments. In that case we
567 bootstrapped subsamples (N=50) of our control dataset with replacement 10,000 times
568 and calculated the circular variance of each dataset. As above, we then reported the
569 proportion of bootstrapped data sets with a smaller variance than each experimental
570 group. We selected a resample size of 50 as this approximated the sample size of our
571 datasets (N=49, 54, 64). A systematic analysis of p-values showed that they decreased
572 asymptotically to a constant level at resample sizes greater than 20.

573

574 Data and software availability

575 Data will be uploaded to Dryad.

576

577 **Movie S1**

578 **E-PG activity correlates with azimuthal position of sun and stripe visual objects**
579 **under open-loop flight conditions.** All panels are time-synchronized and sampled at
580 the two-photon volumetric imaging rate. Upper panel: Two-photon Ca^{2+} imaging in the
581 protocerebral bridge of a flying fruit fly. The fly is presented with sun and stripe stimuli
582 that move in azimuth at a constant speed (open loop). Upper 10% GCaMP6f
583 fluorescence ($\Delta F/F$) in green, lower 90% $\Delta F/F$ in grey; Gaussian filtered. Middle panel:
584 wing tracking (red) in the machine vision camera view of the tethered fly. This ventral
585 view is flipped to represent the anatomical right wing on the right. Stimulus position
586 (blue) and E-PG neuron activity bump position (green) represented in azimuthal space
587 (not to scale). Lower panels: $\Delta F/F$ during 20 seconds of sun and 20 seconds of stripe
588 stimuli presentations. White vertical stripe indicates current frame. Azimuthal position
589 of E-PG activity bump (blue dots and probability distribution) and stimulus position
590 (computed as in (1), red) co-vary. Visual stimuli are only presented on the centered
591 216° -wide LED arena.

592 **Movie S2**

593 **E-PG activity correlates with azimuthal position of sun and stripe visual objects**
594 **under closed-loop flight conditions.** Identical representation to Movie S1, but with
595 stimuli presented under closed loop conditions (see Materials and Methods section for
596 details).