Neuroarchitecture of the *Drosophila* central complex: A catalog of nodulus and asymmetrical body neurons and a revision of the protocerebral bridge catalog

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Abstract
The central complex, a set of neuropils in the center of the insect brain, plays a crucial role in spatial aspects of sensory integration and motor control. Stereotyped neurons interconnect these neuropils with one another and with accessory structures. We screened over 5,000 *Drosophila melanogaster* GAL4 lines for expression in two neuropils, the noduli (NO) of the central complex and the asymmetrical body (AB), and used multicolor stochastic labeling to analyze the morphology, polarity, and organization of individual cells in a subset of the GAL4 lines that showed expression in these neuropils. We identified nine NO and three AB cell types and describe them here. The morphology of the NO neurons suggests that they receive input primarily in the lateral accessory lobe and send output to each of the six paired noduli. We demonstrate that the AB is a bilateral structure which exhibits asymmetry in size between the left and right bodies. We show that the AB neurons directly connect the AB to the central complex and accessory neuropils, that they target both the left and right ABs, and that one cell type preferentially innervates the right AB. We propose that the AB be considered a central complex neuropil in *Drosophila*. Finally, we present highly restricted GAL4 lines for most identified protocerebral bridge, NO, and AB cell types. These lines, generated using the split-GAL4 method, will facilitate anatomical studies, behavioral assays, and physiological experiments.

KEYWORDS
AB_1549585, AB_1625981, AB_2314866, AB_915420, asymmetrical body, central complex, *Drosophila* brain, GAL4, MCFO, nodulus, protocerebral bridge

1 | INTRODUCTION

Located at the center of the insect brain, the central complex is a set of highly interconnected neuropils that processes complex, multisensory information from the environment, integrates it with information about the insect’s internal state and past experiences, and guides motor outputs that drive appropriate behavioral responses. A comprehensive review of the functional roles of the central complex in diverse insects can be found in Pfeiffer and Homberg (2014).

One of the most studied roles of the insect central complex is the integration of sensory information, predominantly from visual input. The output of this sensory processing encompasses diverse motor and behavioral responses. In this capacity, the central complex regulates locomotor behaviors such as handedness, turn direction, initiation and termination of walking (Buchanan, Kain, & de Bivort, 2015; Guo & Ritzmann, 2013; Martin, Guo, Mu, Harley, & Ritzmann, 2015; Martin, Raabe, & Heisenberg, 1999; Poeck, Triphan, Neuser, & Strauss, 2008; Ritzmann, Ridgel, & Pollack, 2008; Seelig & Jayaraman, 2013); flight (Illus, Wolf, & Heisenberg, 1994); courtship (Sakai & Kitamoto, 2006); sleep (Donlea, Pimentel, & Miesenbock, 2014; Liu, Liu, Tabuchi, & Wu, 2016); hunger (Park et al., 2016); and gravitaxis (Baker, Beckingham, & Armstrong, 2007). The central complex is thought to play a key role in migration, navigation, and orientation using input such as celestial cues (el Jundi et al., 2015; Kakaria & de...
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memories (Liu et al., 2006; Neuser, Triphan, Monz, Poeck, & Strauss,
2008; Ofstad, Zuker, & Reiser, 2011), in the processing of olfactory
(Heisenberg, Borst, Wagner, & Byers, 1985) and gustatory inputs
bouhouche, Vaysse, & Corbiere, 1993) and in maintaining information
about the fly’s satiety state (Dus, Min, Keene, Lee, & Suh, 2011).

Understanding the core principles of operation of the central com-
plex has been greatly enabled by the dissection of behavior at a single
neuron level and the neuron-by-neuron assembly of circuits. A com-
prehensive anatomical atlas and genetic lines that enable manipulation
individual cell types are invaluable tools for this strategy. In this study,
we describe the neuronal composition of the NO and the AB, neither of
which has been extensively studied in Drosophila. An understanding of
the function of the noduli (Figure 1a–d) in behavior lags far behind that
of the other central complex structures: the protocerebral bridge (PB),
fan-shaped body (FB), and ellipsoid body (EB; Figure 1a,b; see Table 1
for abbreviations). The only documented roles for the NO in Drosophila
are in the time course of walking activity (Strauss & Heisenberg, 1993)
and in influencing handedness during locomotion (Buchanan et al.,
2015). The locust neurons that connect the PB, EB, and NO and the PB,
FB, and NO are sensitive to polarized light (Heinze & Homberg, 2009).
Most recently, recordings from optic-flow-sensitive neurons that con-
nect the LAL to the NO and other neurons that link the NO to the FB in
the bee have demonstrated the NO are involved in path integration
(Stone et al., 2017). Finally, the fact that this structure appears to be pre-
sent only in the subclass of winged insects has led to the speculation
that the noduli may regulate flight (Pfeiffer & Homberg, 2014).

Structural conservation of the central complex across insect spe-
cies is strong, but not absolute. The discussion that follows focuses on
the anatomy of the Drosophila neuropils (Figure 1), unless otherwise
noted. The PB, FB, and NO are midline structures and exhibit a strati-
ﬁed organization (Heinze & Homberg, 2008; Homberg, 2008; Straussfeld, 1999; Williams, 1975). The PB is a handlebar-shaped struc-
ture in the posterior dorsal brain (Figure 1a,b). The EB is shaped like a
torus and is tilted on its dorsoventral axis such that its ventral border
defines the anterior margin of the central complex (Figure 1a,b). The
FB lies between the PB and EB and represents the largest of the four
central complex structures (Figure 1a–c). The bilateral noduli, histori-
cally called ventral tubercles (Hertweck, 1931), are the most ventral
neuropil of the central complex and are nestled beneath the FB
(Figure 1a–c). There are three pairs of noduli neatly stacked on top of
one another from dorsal to ventral; each pair is bisected by the midline
(Figure 1a–c; one set is shown in panel d). The dorsal nodulus (NO1) dis-
plays some hint of a transverse division (see Wolff, Iyer, & Rubin, 2015)
whereas the medial (NO2) and ventral (NO3) noduli exhibit longitudinal
segmentation. NO2 is divided into dorsal and ventral subdomains
(NO2D and NO2V; D and V, respectively, in Figure 1d) and NO3 has
three subdomains (NO3A, NO3M, and NO3P; A, M, and P, respectively,
in Figure 1d; see Wolff et al., 2015 for details).

The neuropils considered to be components of the central com-
plex have evolved over time. Power’s 1943 description of the central

![FIGURE 1](https://bmcbioinformatics.biomedcentral.com/articles/10.1186/s12859-017-1694-9 or (Wan, Otsuna, Chien, & Hansen, 2009,2012)
and were aligned in the JRC 2013 brain. The color coding is as follows.
PB: dark blue; FB = green; EB = orange; NO = teal; AB = rose (dorsal
to NO); LAL = lilac; GA = burgundy (“shoulders” of LAL); crepine
(CRE) = light blue; round body (ROB) = yellow; brain = gray. Scale
bar = 20 μ. (b) Frontal (left) and sagittal (right) views of central complex
neuropils. Anterior is to the right in the sagittal view. PB = purple;
FB = green, EB = orange; NO = cyan; AB = rose. Scale bar = 20 μ.
(c) The same images as shown in (b), with the PB and EB removed to
better visualize the location of the AB (rose). Scale bar = 20 μ.
(d) Sagittal view of one set of noduli. The dorsal nodulus, NO1 (1), is
not horizontally segmented. The medial nodulus, NO2, has two
subcompartments, dorsal (D) and ventral (V). The ventral nodulus, NO3,
has three subcompartments, anterior (A), medial (M), and posterior (P)

Bivort, 2017; Seeig & Jayaraman, 2015; Kuntz, Poeck, & Strauss,
2017) and displays responses to looming stimuli suggestive of an
involvement in generating escape responses in the locust and fly
(Rosner & Homberg, 2013; Weir, Schnell, & Dickinson, 2014). The
central complex has been suggested to contain a ring attractor net-
work (Kim, Rouault, Druckmann, & Jayaraman, 2017; Seeig &
Jayaraman, 2015) that maintains a representation of the fly’s heading
direction that may be useful for navigation and orientation in visual
conditions as well as in darkness (Green et al., 2017; Seeig & Jayara-
man, 2015; Turner-Evans et al., 2017). The central complex is also
involved in the formation and recall of short- and long-term visual
memories (Liu et al., 2006; Neuser, Triphan, Monz, Poeck, & Strauss,
2008; Ofstad, Zuker, & Reiser, 2011), in the processing of olfactory
(Heisenberg, Borst, Wagner, & Byers, 1985) and gustatory inputs
bouhouche, Vaysse, & Corbiere, 1993) and in maintaining information
about the fly’s satiety state (Dus, Min, Keene, Lee, & Suh, 2011).
complex includes the FB, EB, and "ventral tubercles" (Power, 1943), or NO. By the mid-1970s, the modern view of the central complex had emerged: Williams included the PB within the locust central complex, alongside the FB, EB and NO (Williams, 1975).

The asymmetrical body (AB) is a relatively inconspicuous structure located at the midline, adjacent to the ventral FB. It was first described in the fly as a round, almost exclusively right-hemisphere structure (Pascual, Huang, Neveu, & Preat, 2004). The AB was observed in both hemispheres in just 7.6% of 2,250 brains immunolabeled with an antibody against the Fasciclin II (Fas II) protein, which is expressed in this structure. Flies with bilateral ABs were reported to have disrupted long-term memory, leading to the suggestion that asymmetry of this structure is important for long-term, but not short-term, memory (Pascual et al., 2004). Although thousands of GAL4 lines that drive expression in small subsets of neurons in the larval and adult fly brains have been examined (Jenett et al., 2012), the AB is the only reported instance of an asymmetric structure in the adult fly brain.

Elements with likely homology to the Drosophila AB have also been described in the grey flesh fly Neobellieria bullata and the blowfly Calliphora erythrocephala (Phillips-Portillo & Strausfeld, 2012). In both species, these bodies occur bilaterally, and one of the two is consistently smaller and less densely innervated than the other. In addition, the smaller of the two appears fragmented.

Jenett et al. (2012) identified five GAL4 lines that show asymmetric innervation of the AB. Their analysis revealed lines ranging from a strong right hemisphere bias in innervation to those with asymmetric but bilateral expression, with more conspicuous expression in the right AB. This study builds on Jenett et al. (2012) by providing a systematic characterization of the neurons that target the AB, and leads us to propose that the AB be added as the fifth neuropil of the Drosophila central complex.

In this work, we present a characterization of cell types of the NO and AB, including morphology, presumed polarity, and population size. We also generated and characterized a set of split-GAL4 lines for NO and AB cell types, reagents that will greatly facilitate functional studies. These lines are presented here in Table 2.

In addition, since publication of our description of the neurons that arborize in the PB (Wolff et al., 2015), we have gained several new insights into the PB neurons. These include (a) one new PB neuron family has been identified; (b) a neuron identified by Lin et al. (2013) has since been found in the GAL4 collection and is characterized here using the multicolor flip-out technique (MCFO; Nern, Pfeiffer, & Rubin, 2015); and (c) we generated and characterized a set of split-GAL4 lines for PB cell types; these are presented in Table 2.

## 2 MATERIALS AND METHODS

### 2.1 Split-GAL4 methodology and lines

In the split-GAL4 method, the DNA-binding domain (DBD) and activation domain (AD) of the GAL4 transcription factor are separately linked to complementary leucine zippers and placed under the control of different gene enhancers to create “hemidriver lines” (Luan, Peabody, Vinson, & White, 2006; Pfeiffer et al., 2010). Each enhancer drives gene expression in an empirically determined population of neurons. In cells in which both enhancers are active, both the AD and DBD are expressed and can dimerize via their leucine zippers to reconstitute transcriptionally active GAL4 protein. The reconstituted GAL4 protein binds to the upstream activating sequence (UAS) and activates expression of the UAS-transgenes. In this case, the UAS-transgenes used are “reporters” (e.g., GFP) that visually identify those cells that express GAL4.

Adult brain expression patterns of approximately 8,200 GAL4 lines, 7,000 from the Janelia collection (Jenett et al., 2012; Pfeiffer et al., 2008) and close to 1,200 from the Vienna collection (Tirian & Dickson, 2017), were screened for enhancer fragments that drive expression in the central complex. Hemidriver lines in which these

| TABLE 1 Abbreviations |
|-----------------------|
| PB                    | protocerebral bridge |
| FB                    | fan-shaped body      |
| EB                    | ellipsoid body       |
| EBc                   | canal (EB domain)    |
| EBt                   | tile (EB domain)     |
| NO                    | nodulus              |
| NO1                   | nodulus 1, dorsal nodulus |
| NO2                   | nodulus 2, medial nodulus |
| NO2D                  | dorsal compartment of NO2 |
| NO2V                  | ventral compartment of NO2 |
| NO3                   | nodulus 3, ventral nodulus |
| NO3A                  | anterior compartment of NO3 |
| NO3M                  | medial compartment of NO3 |
| NO3P                  | posterior compartment of NO3 |
| AB                    | asymmetrical body    |
| LAL                   | lateral accessory lobe |
| GA                    | gall                  |
| GA-t                  | gall tip             |
| CRE                   | crepine              |
| ROB                   | round body           |
| SMP                   | superior medial protocerebrum |
| SLP                   | superior lateral protocerebrum |
| SCL                   | superior clamp       |
| VES                   | vest                 |
| WED                   | wedge                |
| G                     | glomerulus of PB     |
| G#                    | subset of glomeruli designated by number/s |
| ℓ                     | layer (of FB)        |
| s                     | spines/spiny         |
| b                     | boutons              |
| D                     | dorsal               |
| V                     | ventral              |
| P                     | posterior            |
| i                     | ipsilateral          |
| c                     | contralateral        |
| SS                    | stable split         |
| MCFO                  | multicolor flip-out  |
enhancer fragments drive expression of either the GAL4 activation domain (AD; p65ADZp) or DNA-binding domain (DBD; ZpGAL4DBD) were subsequently selected from a collection of transgenic lines (Dionne, Hibbard, Cavallaro, Kao, & Rubin, 2018; Tirian & Dickson, 2017). AD and DBD hemidriver lines judged to drive expression in common cell types were crossed to one another. The progeny of these crosses was screened to identify combinations in which the GAL4 AD and DBD domains are both expressed in the desired cell type and can combine to produce functional GAL4 protein. Stable genetic stocks containing both AD and DBD hemidriver constructs (stable split, or SS, lines; Table 2) were generated for the most specific lines for each cell type and these split-GAL4 lines were analyzed using the multicolor flip-out method (MCFO; see below) and polarity markers. Details on the methodology of this approach can be found in Dionne et al. (2018). The split-GAL4 lines generated for each cell type are shown in Table 2 and the hemidriver constructs used to make each line are listed in Table 3.

### Table 2: Stable split GAL4 lines and cell number per cell type

| Cell type | Cell#/hem 1/C14 | SS line | SS line | SS line | SS line |
|-----------|-----------------|---------|---------|---------|---------|
| PB neurons |                  | SS52244 | SS00191 | SS00425 |         |
| PB neurons |                  | SS02255 | SS00081 | SS00043 | SS0190  |
| PB neurons |                  | SS52245 | SS02303 | SS00154 | SS00159 |
| PB neurons |                  | SS00078 | SS52669 | SS52338 |         |
| PB neurons |                  | SS52577 | SS52628 | SS53161 | SS53191 |
| PB neurons |                  | SS54295 | SS52285 | SS04912 | SS02233 |
| PB neurons |                  | SS27853 | SS02191 | SS53233 |         |
| PB neurons |                  | SS02195 | SS53236 | SS56252 |         |
| PB neurons |                  | SS00090 | SS50574 | SS00408 | SS00098 |
| PB neurons |                  | SS02254 | SS50594 | SS50576 |         |
| PB neurons |                  | SS52590 | SS53175 | SS52547 |         |
| PB neurons |                  | SS54549 | SS02270 | SS02293 |         |
| PB neurons |                  | SS02239 | SS02215 | SS53185 |         |
| PB neurons |                  | SS52578 | SS52604 | SS52684 |         |
| PB neurons |                  | SS00117 | SS52272 | SS52347 |         |
| PB neurons |                  | SS52578 | SS52604 | SS52684 |         |
| PB neurons |                  | SS52578 | SS52604 | SS52684 |         |
| PB neurons |                  | SS52578 | SS52604 | SS52684 |         |
| NO neurons |                  | SS46517 | SS46524 | SS46512 | SS46515 |
| NO neurons |                  | SS47398 | SS47399 | SS47397 | SS47405 | SS47354 |
| NO neurons |                  | SS47398 | SS47399 | SS47397 | SS47405 | SS47354 |
| NO neurons |                  | SS47342 | SS47436 | SS47449 | SS47406 |         |
| NO neurons |                  | SS47384 | SS47392 | SS47467 | SS47356 | SS47410 |
| NO neurons |                  | SS546525 | SS46528 | SS46507 | SS46523 |         |
| AB neurons |                  | SS50464 | SS50498 | SS50502 | SS50510 |         |
| AB neurons |                  | SS50420 | SS50417 | SS50477 | SS50517 |         |

Cell types and number of cells per hemisphere are provided for each line. One primary (best) and several good lines/cell type are provided. Some 2 lines are equally as good as the 1 lines; these are indicated in bold.

- PBG2-9.s-FB1.b-NO2.P.b contains PBG2-9.s-FB1.b, PBG2-9.s-FB1.b-NO2.P.b, PBG2-9.s-FB1.b-NO2.P.b, and PBG2-9.s-FB1.b-NO2.P.b.
- Also has weak expression in PBG2-9.s-FB1.b-NO2.P.b.
- Also has weak expression in PBG2-9.s-FB1.b-NO2.P.b.
- This line targets P-EN2 cells (Dan Turner-Evans, personal communication; see Green et al., 2017, for description of cell type). The identities of the P-EN cells in the first two lines have not been characterized. Also has weak expression in PBG2-9.s-FB1.b-NO2.P.b.
- This line is stochastic—expression may occur in only one hemisphere.

#### 2.2 Using the MCFO method to screen for and characterize cell types

Of the 8,900 GAL4 and split-GAL4 lines screened for expression in the central complex, as described above, approximately 1,200 were selected for analysis by MCFO. The MCFO technique stochastically labels a small number of cells in various colors (Nern et al., 2015), making it possible to analyze the morphology of individual cells at high resolution using confocal microscopy. The MCFO strategy was used to identify and characterize the NO and AB cell types described here. Briefly, the fly stocks used for MCFO contain three UAS reporter constructs (UAS-FRT-STOP-FRT-epitope tag), each with a different
| Parental AD and DBD Hemidriver lines |
|---------------------------------------|
| SS00078 GMR_16D01_XA_21-x-GMR_15E01_XD_01 |
| SS2669 GMR_68A10_BB_21-x-GMR_75H10_XD_01 |
| SS5338 GMR_75H10_XA_21-x-GMR_83D12_AV_01 |
| SS00090 GMR_16D01_XA_21-x-GMR_15E01_XD_01 |
| SS52628 GMR_68A10_BB_21-x-GMR_75H10_XD_01 |
| SS5338 GMR_75H10_XA_21-x-GMR_83D12_AV_01 |
| SS00098 GMR_19G02_XA_21-x-GMR_93G12_XD_01 |
| SS52628 GMR_68A10_BB_21-x-GMR_75H10_XD_01 |
| SS5338 GMR_75H10_XA_21-x-GMR_83D12_AV_01 |

(Continues)
TABLE 3  (Continued)

| Line ID         | Source                           | Description                                      |
|-----------------|----------------------------------|--------------------------------------------------|
| SSS2267 JBD_122F02_BB_21-x-GMR_72C10_AV_01 |                                      |                                                  |
| SSS2265 JBD_122F02_BB_21-x-GMR_37H01_AV_01 |                                      |                                                  |
| SSS6247 JBD_122F02_BB_21-x-BJD_122C09_AV_01 |                                      |                                                  |

The lines shown in Table 3 correspond to those shown in Table 2. Lines are arranged numerically by primary SS line number and in ascending order. They are further arranged by neuropil region, starting with the PB, followed by the NO and last by the AB. Each text box in Table 3 includes all lines for a given cell type that are presented in Table 2. SS lines indicated in bold are the same lines shown in bold in Table 2 and identify the best lines for each cell type.

2.3 | Split-GAL4 expression patterns

The expression patterns of split-GAL4 lines are visualized in Figure 2 using 20XUAS-CsChrimson-mVenus in attP18 (Klapoetke et al., 2014) as the neuronal marker and anti-BRP to label the neuropil. Expression patterns of additional lines are shown at www.janelia.org/split-GAL4 and confocal stacks corresponding to all split-GAL4 lines described here can be downloaded from that site. Instructions for requesting split-GAL4 lines can also be found at this site. To supplement the anatomy-based assignments of neuronal polarity, split-GAL4 lines were used to drive the expression of two reporter constructs, one targeted to membranes (pJFRC225-5XUAS-IVS-myr::smGFP-FLAG in VK00005); (Nern et al., 2015; Viswanathan et al., 2015) and a second that localizes to synaptic vesicles in presynapses (pJFRC51-3XUAS-IVS-Syt::smGFP-HA in su(Hw)attP1). Synaptotagmin (Syt) is a presynaptic protein (Littleton, Bellen, & Perin, 1993). Immunolabeling was performed as described in Aso et al. (2014). Additional details on reporter constructs are also described in this publication. A minimum of two female brains and three CNSs (a CNS is a brain plus ventral nerve cord, or VNC) per stable split line were immunolabeled and mounted in DPX. Detailed protocols are available at: https://www.janelia.org/project-team/flylight/protocols (see “IHC—Anti-GFP,” “IHC—Polarity Sequential,” and “DPX mounting”). Imaging conditions for each brain and corresponding VNC are identical, so the intensity seen in the brain and VNC for each line accurately reflects the relative levels of expression in these two regions. Microscope settings were adjusted as necessary in different lines.

2.4 | Antibody characterization

Primary antibodies used in this study are presented in Table 4. Several assays document the specificity of the anti-Bruchpilot (a presynaptic active zone protein; BRP) antibody, nc82 (RRID: AB_2314866). Wagh et al. (2006) demonstrate (a) nc82 localization at presynaptic active zones, (b) specificity of anti-nc82 in Western blot analysis, and (c) correspondence in nc82 localization and GFP label in tissues in which untagged and GFP-tagged bruchpilot were ectopically expressed in the wing imaginal disc, trachea and epidermal cells.
FIGURE 2 Expression patterns of PB, NO, and AB split-GAL4 lines in brain and VNC. Expression of 20xUAS-CsChrimson-mVenus (insertion in attP18; labeled with anti-GFP antibody, green) driven by split-GAL4 lines indicated in panels. Neurorips was visualized using anti-Brp, shown in magenta. The sparsest lines for each cell type are shown. Confocal stacks for these and additional lines noted in Table 2 can be viewed and lines can be ordered at www.janelia.org/split-GAL4. This website will be updated with additional clean lines as they become available. Scale bar = 100 μ.
Furthermore, they show a correspondence between GFP and endogenous BRP patterns when a panneural driver is used to drive GFP-tagged bruchpilot. Brp expression is lost in brp mutants and rescued by expression of BRP in these mutants (Kittel et al., 2006). Hamanaka and Meinertzhagen (Hamanaka & Meinertzhagen, 2010) report the distribution of nc82 at neuromuscular junctions and Drosophila photoreceptor synapses.

The antibodies used to detect the three epitope-tagged smGFPs (Viswanathan et al., 2015) used for the MCFO studies (rat anti-FLAG, rabbit antihemagglutinin, and mouse anti-V5) are widely used commercial antibodies. Their specificity is indicated by the fact that the observed expression patterns differ between GAL4 lines, arguing against staining being directed against endogenous epitopes, but are reproducible for a given GAL4 line. Two secondary antibodies were used in the polarity protocol: ATTO647N goat antirat IgG (1:150; H&L; Rockland #612-156-120) and CY3 goat antirabbit (1:1,000; Jackson Immuno Research #111-165-144).

2.5 Immunohistochemistry, clearing, and mounting

A detailed protocol for MCFO immunohistochemistry and mounting is presented in Nern et al. (2015) and, for polarity, in Aso, Hattori,
et al. (2014). There are subtle differences between the two protocols. The specifics are omitted here; the referenced articles should be consulted for successful tissue preparation. Following dissection in Schneider’s medium, tissue was fixed in paraformaldehyde (PFA) diluted in Schneider’s medium. Samples were then rinsed in PAT3 (Triton X-100/bovine serum albumin/phosphate-buffered saline), blocked in normal goat serum (NGS) and then, for MCFO brains, incubated in: mouse anti-nc82 (Developmental Studies Hybridoma Bank, University of Iowa; RRID: AB_2314866) (Hofbauer et al., 2009; Wagh et al., 2006); rabbit anti-HA (1:300; Cell Signaling Technology; RRID: AB_1549585); and rat anti-Flag (1:200; Novus Biologicals; RRID: AB_1625981). Antibodies were diluted in NGS/PAT3. For MCFO, following rinses in NGS/PAT3, samples were incubated in secondary antibodies, including in Alexafluor-488 donkey antimouse (1:400; Jackson Labs), Alexafluor-594 donkey anti-rabbit (1:500; Jackson Labs), and Alexafluor-647 donkey anti-rat (1:300; Jackson Labs) in 3% NGS/PAT3. Following additional rinses in PAT3 and a block in normal mouse serum/PAT3, tissue was incubated in DyLight-549 mouse anti-V5 (1:500; AbD Serotec; AB_915420). For the tissue prepared for polarity, primary antibodies rat anti-Flag (1:200; Novus Biologicals, Littleton, CO), rabbit anti-HA (1:300; Cell Signaling Technology, Danvers, MA), and anti-nc82 (1:33) were used, and secondary antibodies AlexaFluor-647 donkey anti-rat (1:300; Jackson Labs) and Cy3 donkey antirabbit (1:500; Jackson Labs). Brains were subsequently washed in PAT3, then in PBS, and postfixed in PFA. Finally, tissue was rinsed in PBS, followed by a PAT3 rinse, and mounted within 3–5 days.

Tissue was dehydrated through an ethanol series, placed on poly L-lysine-coated No. 1 coverslips and then further dehydrated. Following xylene treatment, tissue was mounted in DPX (Sigma-Aldrich, St. Louis, MO). DPX was allowed to dry for 2 days before imaging.

2.6 | Image acquisition and analysis
A Zeiss LSM 710 confocal microscope was used to collect images, using a Plan-Apochromat 63×/NA1.4 oil immersion objective. Frame sizes were 1,024 × 1,024 pixels, voxel size 0.19 × 0.19 × 0.38 μm, zoom factor 0.8, and one frame average. While reference channel intensity was adjusted throughout the depth of the sample to maximize image quality, the gain and power were maintained at constant levels within each sample. The “Janelia Workstation” image-viewing software (Murphy et al., unpublished data) was used to analyze confocal stacks.

2.7 | Neuropil masks and brain alignment
Registration of data collected from individual brains into a standardized frame of reference can distort the morphology of neurons, so unaligned data was used to characterize the morphology of neurons...
described here. On the other hand, since aligning the data to a standard brain allows a comparison between brains, registered data was used for the color depth mask searches. Details for preparation of the standard brain used in this work (JFRC2013) can be found in Aso, Hattori, et al. (2014). Neuronal masks were generated using FluoRender (software that generates three-dimensional images: https://bmcbioinformatics.biomedcentral.com/articles/10.1186/s12859-017-1694-9 or Wan et al., 2009, 2012), brains labeled with anti-Brp, and a standard brain (JFRC2013). For details, refer to Aso, Hattori, et al. (2014).

2.8 | Figure preparation

In cases in which the neuron intensity in MCFO images was below the threshold required for visibility in a publication, brightness, and contrast were increased in the Janelia Workstation. In several cases, anti-nc82 label and neuron fragments from neurons that were not the focus of the figure, were “erased” using Adobe Photoshop (San Jose, CA). Brightness and contrast for each pair of brains and VNCs shown in Figure 2 were individually optimized; settings are identical for brains and corresponding VNCs within each sample.

3 | RESULTS

3.1 | Identification and characterization of split-GAL4 lines

Most GAL4 lines drive expression in multiple cell types, sometimes making it difficult to isolate individual cells for anatomical analysis using the MCFO technique. Their activity in multiple cell types also makes these lines problematic for behavioral assays. To circumvent these limitations, the split-GAL4 intersectional method (Luan et al., 2006) in combination with optimized vectors (Pfeiffer et al., 2010) was used to reduce the number of cell types targeted in a given line. A detailed description of this method and of the generation of the hemidriver collection of lines is provided in Materials and Methods.

Transgenic hemidriver lines (Dionne et al., 2018; Tirian & Dickson, 2017) carrying unique enhancer fragments that drive expression in cell types of interest were selected from two GAL4 collections (Jenett et al., 2012; Pfeiffer et al., 2008; Tirian & Dickson, 2017). Parental AD and DBD hemidriver lines judged to have overlapping expression patterns were crossed. A subset of these crosses is successful in producing lines in which the UAS reporter is expressed in a small number of cell types. The number of cell types that is observed in each intersection is cross-specific and highly reproducible among the progeny of a given cross. The most useful combinations were made into stable split-GAL4 lines that carry both the AD and DBD hemidriver constructs (see Methods). The “cleanest” split-GAL4 lines for each cell type (i.e., those that show expression in the least number of cell types in the brain, optic lobes, and VNC) are presented in Table 2 and Figure 2. Images and confocal stacks for all lines listed in Table 2 can be accessed at www.janelia.org/split-GAL4.

The split-GAL4 lines provided in Table 2 all drive expression in fewer cell types than the original GAL4 lines, but not all are specific for a single cell type. In some cases, this is due to the limited number of AD and DBD hemi-drivers (the parent lines) in the available collections. In the case of the NO3M and NO3P cells, we were able to design many split-GAL4 lines that are expressed in both of these cell types, but we could not identify enhancers that are uniquely expressed in each of these cell types; either they do not exist or the lines in the GAL4 collection do not target them.

Three analyses were used to characterize the morphology, polarity, and population size of the cells targeted by these lines. The first, the MCFO method, is a selective approach that stochastically labels small populations of cells, providing insight into cellular morphology and neuropils targeted by a given cell type; it does not, however, reveal the population-wide projection pattern for that cell type.

Polarity data, generated in the second type of analysis in which cell type-specific split-GAL4 lines are used to drive expression of reporter constructs that target membranes and presynapses, supplements the MCFO data in that it provides molecular confirmation of the morphology-derived polarity of neurons obtained using the MCFO methodology (not shown). These polarity data rely on the correct targeting of an exogenous fusion protein and can overpredict presynaptic sites in cases where expression levels are too high (Aso, Hattori, et al., 2014). Although the expression levels of this protein are titrated

### TABLE 5 Neuron abbreviations

| Neuron Abbreviation | Abbreviation |
|---------------------|--------------|
| PB neurons          |              |
| PBg2.9s-FBepsilon1b-NO3Pb | P-FNp |
| PBg2.9s-FBepsilon1b-NO3Mb | P-FNM |
| PBg2.9s-FBepsilon2b-NO3Ab | P-FNa |
| PBg2.9s-FBepsilon3b-NO3Db | P-FN0 |
| PBg2.9s-FBepsilon3b-NO3Yb | P-FNv |
| PBg2.9s-EBtlb-NO1b | P-EN1, P-EN2 |
| PBg1.9s-EBtlb-D/V GA b | P-EG |
| PBg1.8b-EBwV-D/V GA b | P-Eg |
| PBg1.9s-EBcb-D/V GA b | P-EcG |
| PBg3b-EB-PS-GA-tb | P-EG |
| PBs-FBepsilon6b-epsilon3s-V GA ss b | P-F-Gs |
| PBg1s-epsilon3s-epsilon4s-5b-NO2Vb-ROb | P-F-R |
| PBg1-7s-FBepsilon2s-LALb-CRE.b | PF-LC |
| PBb-LALs-PS.s | LPs-P |
| PBg6-8sGsb | P-6a-P9 |
| PB18s-Gx7Gyb | Delta7 |
| Bg12.9-1b-SPSi.s | Sps-P |
| PBg2.9b-1b-PS.s-SP5s | Sps-PS-P |
| NO neurons          |              |
| LALs-GA5s-NO3li.b | LG-N |
| LALs-NO3li.b | L-N |
| LALs-NO3Ai.b | L-NA |
| LALs-CREc5s-NO3P/MI.b | LC-NM |
| LALs-CREc5s-NO3Pc.b | LC-Np |
| AB neurons          |              |
| SLPs-AB.b | SLP-A |
| AB.s-FBepsilon8b.b | A-F |
| SLPs-AB.b-FBepsilon8b.b | SLP-AF |
FIGURE 3   Nodulus neurons. (a) LAL.s-GAi.s-NO1i.b, the only NO1 neuron identified to date. The arbors in the GA and LAL are predominantly input, populate the entire gall (length is highlighted by green line) and just the lateral margin of the LAL. Inset, sagittal view of noduli. NO1, the dorsal nodulus, receives the output from this neuron. GA = gall. Scale bar = 30 μ. [MCFO image from line R76E11]. (b) LAL.s-NO2i.b neuron. The LAL arbor is more robust than the NO1 cells shown in (a). Inset, both dorsal and ventral compartments of NO2, the medial nodulus, are filled with boutons. Scale bar = 20 μ [MCFO image from line R41H08]. (c) LAL.s-GAi.s-CREi.s-NO2i.b-FBℓ3i.b-CREi.b neuron. The presumed output arbor in the crepine is substantial whereas just a few fine, thin terminals project into a different region of this neuropil and represent apparent offshoots from the LAL arbor, which tracks primarily along the lateral margin. The GA arbor fills both domains of the GA, CRE = crepine. Scale bar = 20 μ [MCFO image from line SS04682]. (d) LAL.s-GAi.s-CREi.s-NO2i.b-FBℓ3i.b neuron. The FB arbor most likely should extend the full span of FBℓ3. The CRE arbor in this cell is similar to the input CRE arbor of the cell in (c) in that it is minimal and appears to extend from the more elaborate LAL arbor. CRE = crepine; WED = wedge. Scale bar = 20 μ [MCFO image from line SS04682]. (e) LAL.s-NO2i.b-SCLi.b-CREc.b neuron. Minimal fine terminals extend into the GA and only one bouton populates the contralateral CRE. SCL = superior clamp. Scale bar = 20 μ. [MCFO image from line SS04682]. (f) LAL.s-NO3Ai.b neuron. This LAL arbor’s flocculent texture distinguishes it from the other NO neurons. Inset, as indicated by the cell’s name, only the anterior compartment of NO3 (NO3A) is targeted by this cell. Scale bar = 20 μ [MCFO image from line R12G04]. (g) LAL.s-CREi.s-NO3P/Mi.b neuron. The distribution of the spiny arbor is widespread throughout the LAL and reaches into the CRE, which lies anterior to the bulk of the LAL arbor and is therefore not identified in the figure. As with all of the NO cell types described here, the nodulus arbor is predominantly composed of boutons, and, in this case, fills both the medial and posterior NO3 compartments, as shown in the inset. The asterisk identifies NO3A in the inset. Scale bar = 20 μ [MCFO image from line SS04689]. (h) LAL.s-CREi.s-NO3P/Mi.b neuron. This LAL.s-CREi.s-NO3P/Mi.b neuron is unique among the identified NO neurons in that it crosses the midline between the LAL and NO. It is from the same brain as the neuron shown in (h); both neurons are shown in left inset. The distinction between these two neurons, particularly the ipsilateral versus contralateral circuitry, is evident in the left inset. Right inset illustrates this neuron’s absence from NO3M [note the gap between the filled NO3P neuropil and NO3A, marked by an asterisk, a gap that is filled with the yellow arbor in the inset in (h)]. Scale bar = 20 μ [MCFO image from line SS04689].
in this system, caution should still be exercised in interpreting these patterns. Similarly, while the MCFO anatomical data also has its limitations, it can reliably identify the predominant arbor type. Since neither of these methods provides the accuracy that can be achieved using electron microscopy (EM), and since arbors often have both inputs and outputs, EM-level resolution will be required to establish the true percentage of spines and boutons for each terminal.

In the third method of analysis, brains were prepared using the Chrimson-Venus marker to reveal the expression patterns (Figure 2 and www.janelia.org/split-GAL4) of the GAL4 lines listed in Table 2. These samples reveal both the approximate population size of a given neuron type as well as each cell type’s projection pattern. We observed minimal brain-to-brain variability in the number of cells in some lines. The variability is due to loss of cell bodies during dissection, stochasticity in gene expression, difficulty in resolving individual soma when they were tightly clustered, and perhaps some variability in neural composition between individuals. The cell counts provided are therefore not precise population sizes. Taken together, these three labeling techniques provide a comprehensive description of each cell type analyzed.

3.2 | Nomenclature

The nomenclature system used here includes three features that unambiguously identify a neuron so that it can be recognized by its name: the structures targeted by the neuron; the primary neurite’s path relative to the midline; and the polarity, deduced from the anatomy of the arbor. A detailed description of this nomenclature system can be found in Wolff et al. (2015); several points are reiterated here to assist in interpreting the neuron names presented in the results.

Central complex neurons connect up to three structures within the central complex and, in some cases, more if they also project outside the central complex. Each class of neurons follows a stereotyped projection pattern as it links these structures. Some neuron classes maintain ipsilateral paths through their targeted neuropils whereas others cross the midline. The crossover point—between the first and second or second and third neuropils—is a conserved feature of the neuron class. This midline crossover point and the locations of the arbors relative to one another are important anatomical features of the neuron and are therefore described within the neuron’s name. The abbreviations “i” for ipsilateral, “c” for contralateral, and “ic” for instances when a projection arborizes in both the ipsi- and contralateral counterparts of a structure, are appended to all but the first neuropil in the neuron’s name to indicate their location relative to the first neuropil. For example, in the hypothetical neuron “A-Bi-Ci-Dc,” the arbors in neuropils B and C are on the same side of the midline as A’s arbor, the primary neurite crosses the midline between neuropils C and D, and the arbor in structure D is in the contralateral hemisphere.

It is generally possible to distinguish between the two basic arbor morphologies of a neuron at confocal microscope-level resolution, although EM studies will be required to quantitatively describe all arbors. These two morphologically distinct arbors, which resemble thin fibers and blebs, provide insight into the neuron’s polarity and direction of information flow: the fine, thin arbors are typically associated with functional input whereas the bleb-like boutons are typically recognized as the functional output of a neuron. These anatomical features are included in the nomenclature system used here. The abbreviations “s,” for terminals that appear to be predominantly thin spines, “b” for terminals that appear to consist predominantly of boutons, and “sb” for obviously mixed terminals, are appended to each structure in the neuron’s name. In the hypothetical neuron A-s-Bi.s-b-Ci.b-Dc.b, neuropil A has primarily spines in its arbor, B is a mixture of both spines and boutons, and the arbors in neuropils C and D consist primarily of boutons.

One change to the nomenclature system used in Wolff et al. (2015) is being adopted here. To conform to the standard used in other species, neuropils with arbors that are predominantly spiny in morphology (presumed input regions) are listed first, obviously mixed terminals are next, followed last by presumed output regions.

Finally, the boundaries between some adjacent neuropils were defined based on subtle morphological features (Ito et al., 2014) that
may not necessarily coincide with functional differences. The arbors of some neurons extend beyond these nebulous boundaries. For example, some neurons discussed below have extensive arbors in the

3.3 | Recommended abbreviations for neuron names
The nomenclature used in this publication and in Wolff et al. (2015) is comprehensive but too cumbersome for reiterative use in papers and talks, so a set of standardized abbreviations is presented in Table 5. In an effort to keep the abbreviations as brief as possible, only critical information is retained. For example, FB layers and NO designations (1, 2, and 3) are excluded from the PB-FB-NO contractions since compartment information, such as anterior, dorsal, etc., uniquely identifies these cell types and is included. This abridged nomenclature is consistent with, and builds upon, a loose convention established by several labs working on central complex neurons, and it evolved from discussions among several individuals working on these neurons (see Acknowledgments). The following rules were used to derive the abbreviations in Table 5.

1. Central complex structures are abbreviated with a single capital letter that is the first letter of the neuropil’s name (PB = P, FB = F, EB = E, NO = N, AB = A). Structures that are targeted by central complex neurons and that have unique first letters are similarly abbreviated (LAL = L, GA = G, CRE = C, ROB = R).

2. The remaining neuropils follow the three-letter designations presented in Ito et al. (2014). To avoid confusion with the single letter notations of sequential neuropils in the abbreviation (see rule 5), only the first letter of the three-letter code is capitalized (e.g., Slp, which, if SLP, could be confused for an unknown neuropil “S,” followed by the LAL, followed by the PB).

3. The field has established P-EG and E-PG as the abbreviations for the neurons PBG9.b-EB.P.s-GA-t.b, is abbreviated E-PGT. (Note the original nomenclature system did not list input neuropils first, but the gall tip E-PG cell, PBG9.b-EB.P.s-GA-t.b, is abbreviated E-PGt. Note the original nomenclature system did not list input neuropils first, but the gall tip cell is, in fact, an E-PG cell.) In these two cases, the subscripted capital letter is appended as a tip cell is, in fact, an E-PG cell.) In these two cases, the subscripted capital letter is appended as a

4. Neuropil subdomain information is included only when necessary to distinguish between otherwise identical abbreviations. In these cases, the first letter of the subdomain’s name is appended as a subscripted capital letter. For example, the canal P-EG, PBG1-9.s-EB.b-D/V GA.b, is abbreviated P-EGb, and the gall tip E-PG cell, PBG9.b-EB.P.s-GA-t.b, is abbreviated E-PGt. (Note the original nomenclature system did not list input neuropils first, but the gall tip cell is, in fact, an E-PG cell.) In these two cases, the subscripted “C” and “T” disambiguate these two cell types from the P-EG and E-PG noted in #3, above.

5. Apparent input (spiny morphology) neuropils are listed first followed by neuropils with mixed input and output followed last by apparent output neuropils (boutons).

6. Input, mixed, and output neuropils are separated with hyphens.

7. Variants that are not morphologically distinct are assigned arbitrary, nonsubscribed numerals that follow the designated abbreviation (e.g., P-EN1 and P-EN2).
Cells with formal names that convert to pronounceable acronyms will adopt those acronyms (e.g., Delta7).

3.4 NO neurons

The classification of a neuron as a PB, FB, EB, or NO cell is somewhat arbitrary since central complex neurons arborize in one to three structures of the central complex and could therefore be classified by any one of the three targeted neuropils. We began our studies of the central complex with the PB (Wolff et al., 2015), so all neurons that were found to arborize in the PB were assigned to the PB class, even though many of them project to additional structures within and outside of the central complex. Neurons presented here as “NO neurons” exclude both those described previously as PB neurons that also arborize in the NO, for example, the PB-FB-NO and PB-EB-NO neurons (Lin et al., 2013; Wolff et al., 2015), as well as tangential FB neurons that arborize extensively throughout one layer of the FB and also send more restricted projections to the NO (for example, see Hanesch, Fischbach, & Heisenberg, 1989). In the discussions that follow, “NO neuron” refers exclusively to those neurons described in the results.

This work identified as many as nine distinct “nodulus neurons” (Figure 3), with the most frequently seen types illustrated in Figure 4. The common themes of the NO neurons described here are: (a) they project to only one nodulus, the “left” or “right,” of a pair; (b) the arbors in the noduli appear to be predominantly presynaptic: only boutons are evident in light-level, confocal images; (c) presumed input, based on anatomy, comes almost entirely from the LAL; (d) four of the five most commonly seen classes of NO neurons are exclusively ipsilateral, targeting structures only in either the left or right hemisphere. The morphology of the LAL arbors and the
subregions they occupy within the LAL are largely distinct for each of the NO neuron types.

### 3.4.1 NO1-targeted neuron

Just one cell type that targets the dorsal nodulus (NO1) has been identified, the ipsilateral LAL.s-GAi.s-NO1i.b neuron (n > 81; Figures 3a and 4a). As indicated by this neuron’s nomenclature, light-level analysis indicates that this neuron has primarily spiny arbors in the LAL and GA and predominantly boutons in NO1, suggesting information is relayed from the LAL and GA to the NO. In some cases, one to two boutons extend from NO1 into NO2 or NO3 (Figure 3a, arrow). The bulk of the LAL arbor is confined to the lateral margin of the LAL (Figure 4a) and its length is somewhat variable. The gall arbor fills both its dorsal and ventral compartments.

### 3.4.2 NO2-targeted neurons

The NO2-targeted neurons identified to date project to both the dorsal and ventral subcompartments of the medial nodulus, NO2. This distribution is implied in the names of each of these neurons by the absence of the ”D” and ”V” (for dorsal and ventral) designations. Five NO2 cell types are described, although four are rare. Although the sample size for these four neurons is small, their morphologies differ significantly from the most common cell type, LAL.s-NO2i.b (below), and they display features that distinguish them from one another. These morphological criteria suggest that these four neurons are strong candidates to be bona fide distinct neuronal types; they are therefore described here.

**LAL.s-NO2i.b**

The ipsilateral LAL.s-NO2i.b neuron has predominantly spiny branches in the LAL whereas the NO2 arbor is a densely packed bundle of boutons (n > 52; Figures 3b and 4b). The LAL arbor is more proliferative than the NO1 neuron’s LAL arbor. Its dorsal boundary often creeps slightly into the adjacent CRE (Figure 4b). The arbor’s intrusion into the CRE is minimal and therefore not included in the neuron’s name.

### Four putative cell types

Four additional ”putative” NO2 cell types are described below. Each of these possible cell types has been seen just one or two times unless otherwise noted, all in the same split-GAL4 line, SS04682, so we cannot be certain that they represent bona fide cell types rather than cells with aberrant projections. If this is the case, it suggests the commonly seen LAL.s-NO2i.b cell is highly prone to errors in line SS04682 and these putative cells may represent developmental mistakes. However, this possibility seems less likely since the morphological features of these cells are robust and elaborate—not typical of the projections we have occasionally seen in other lines that resemble developmental miswiring. Alternatively, the scarce number of sightings may simply reflect that these neuron types are rare or infrequently targeted with...
The LAL.s-GAi.s-CREi.s-NO₂i.b-FB³i.b-CREi.b cell described above is the extensive array of boutons that is absent in the CRE of this cell (LAL.s-GAi.s-CREi.s-NO₂i.b-FB³i.b).

LAL.s-NO₂b-SCLi.b-CREc.b

This cell type is obviously distinct from the other four putative NO₂ neurons. The most distinguishing features are the long projection to the contralateral crepine (CRE.c) that terminates in a single bouton and the bifurcating trajectory to the ipsilateral superior clamp (SCL), where it terminates in a small number of boutons (Figure 3e). The LAL arbor of this cell is much less robust than that of LAL.s-NO₂b (Figure 3b). The LAL arbor is primarily spiny and a few branches reach into the gall.

The LAL.s-GAi.s-CREi.s-NO₂i.b-FB³i.b-LALi.b cell.

This putative cell type (Figure 3f) shares several features in common with the other three putative cell types: bouton-rich arbors in both subcompartments of NO₂, a spiny arbor in a large portion of the LAL and the entire GA, and boutons in a fraction of FB³. This cell type also has a separate, small arbor of primarily boutons in the LAL, close to the FB arbor, and its NO₂ arbor appears to send small shoots into NO₂A and the vest (a large bilateral neuropil ventral to the FB), although more samples are needed to confirm this observation. This cell closely resembles LAL.s-GAi.s-CREi.s-NO₂i.b-FB³i.b (Figure 3d), and while it is plausible that they are variants of the same cell, this seems less likely given the absence of the dorsal-projecting shoot that targets the CRE as well as the greater number of fine branches in the LAL arbor in the LAL.s-GAi.s-NO₂i.b-FB³i.b-LALi.b cell.

3.4.3 | NO₂-targeted neurons

Three subcompartments comprise the large, ventral nodulus, NO₂: NO₂A, NO₂M, and NO₂P (Figure 1d) (Wolff et al., 2015).
neurons that arborize in NO3 have been identified. Based on morphology, likely input regions for these three neurons include the LAL and, in two cases, a small amount of input from the CRE. The nodulus arbors for these cells are rich in boutons, suggesting this neuropil is primarily receiving input from the NO3 neurons.

**NO3A**

The LALs-NO3Al.b neuron is the only currently identified cell type that targets NO3A (n > 86; Figures 3g and 4c). As with LALs-GAI.s-NO3i.b (Figure 3a) and LALs-NO3b (Figure 3b), this is also an ipsilateral neuron. Similar to the LALs-GAI.s-NO3i.b cell type, the LAL arbor is confined to the lateral margin of this neuropil, running the length of the LAL (Figure 4c). An occasional bouton projects from the NO3 arbor into the NO2 subcompartment (not shown).

**NO3P/M**

The ipsilateral LALs-CREi.s-NO3P/Mi.b (n > 107; Figures 3h and 4d) resembles LALs-NO3i.b (Figure 3b) in its gross morphology, primarily in its expansive dendrite-rich LAL network. The NO3P/M neuron also has dendrites in the CRE and occasional spines reach into the vest. This neuron has a dense population of boutons in both the posterior and medial compartments of NO3 (inset).

**NO3P**

The LALs-CREc.s-NO3Pc.b neuron (n > 73; Figures 3i and 4e) exhibits one notable feature that distinguishes it from the other four classes of common NO-LAL neurons described here: it is not an ipsilateral neuron. Instead, with predominantly spiny arbors in the LAL and boutons in the contralateral posterior domain of NO3 (NO3P), the morphology suggests the predominant input comes from the LAL (with a smaller contribution from the CRE) on one side of the brain and is delivered to the contralateral nodulus. Unlike the LALs-CREi.s-NO3P/Mi.b neuron described above, only the NO3P subcompartment is targeted by this cell (Figure 3i, right inset). The LAL arbor in this cell type is more sparse than that in the LALs-NO3i.b and LALs-CREi.s-NO3P/Mi.b cell types, but it covers roughly the same region (Figure 3i, left inset).

### 3.5 AB anatomy and neurons

#### 3.5.1 The asymmetrical body is a distinct, bilateral neuropil that is asymmetric in size and bilaterally innervated

The analysis presented here indicates that the AB, while asymmetric in morphology, is present in both hemispheres in all brains (n > 167; Figure 5). Furthermore, both the left and right ABs are innervated (Figure 5a, right member of each pair). The AB is evident in confocal micrographs of both male and female wild-type brains immunolabeled with anti-Brp, including assorted GAL4 lines (n = 135 female plus 20 male) as well as the Berlin (n = 5 female), DL (Dickinson Lab; n = 3 female), and Oregon R (n = 4 female) wild-type strains.

The AB is positioned adjacent to layer 1 of the FB and juxtaposes the medial–ventral boundary of the FB (Figure 5a,b). These structures have round profiles mediolaterally and dorsoventrally, but are oblong in the anteroposterior axis (Figure 5b). The restriction of neuronal arbors to either the FB or AB suggests the AB is a distinct neuropil rather than an extension of the FB. This respect for the AB/FB boundary is typical. For example, Fas II staining is restricted to the AB (Pascual et al., 2004), as are arbors of the AB neurons (below).
The PBG1-9.s-EBt.b-D/V GA.b cells shown in (b1). The PB in (b2) is aligned with the PB in (a1). This panel illustrates the lateral location of the neurons in the PB. (a3) The yellow and pink EB arbors from the G9 cells shown in (a1) and (a2) are confined to the posterior shell, seen in this sagittal view. Anterior is to the right. Scale bar = 10 μ; also applies to panel a2 [from line SS02191].

(c1) Projections of the G1 (yellow) and G6 (pink) cells in c1 to their respective gall domains. Cells that target the odd glomeruli, G1 (yellow), and G6 (pink), project to the dorsal gall domain (D), whereas the cell that targets the even-numbered glomerulus, G6 (pink), projects to the ventral gall domain (V). Images are rotated to reveal that these arbors fill their respective compartments. D = dorsal gall, V = ventral gall. Scale bar = 10 μ; also applies to (c4). (c4) Same as described for c3, but in this case, the dorsal gall is arborized by the PB cell that targets G3 (orange).

Likewise, FB (unpublished) and PB-FB (Wolff et al., 2015) neuron arbors are confined to the FB. Despite the separation of arbors innervating the AB and FB, these neuropils are situated in close proximity to one another: The presynaptic active zones of the two neuropils, revealed by anti-Brp immunolabeling, are only distinctly separate at confocal microscope resolution in the posterior region of the AB, not in the anterior region.

Several instances indicate a possible compartmentalization of this neuropil. In the two examples shown, two neurons stochastically labeled using MCFO target different regions of the AB (Figure 5c).

Although this work shows the AB is a bilateral structure, the two neuropils are asymmetric in size, with the right AB having a larger volume than the left. The right AB measures 10 μ in diameter at its widest point (posterior) versus 7 μ for the left, and 590 μ³ versus 205 μ³ in volume for the JRC2013 standard brain. The variance across brains and hemispheres is illustrated in the paired dot plot (Figure 5d). Of the 21 brains measured, the right AB volume ranged from 412 μ³ to 651 μ³ and the left from 49 μ³ to 276 μ³. On average, the right AB is four times larger in volume than the left AB. The median volumes are 126 μ³ (left AB) and 522 μ³ (right AB [n = 21], as illustrated in the box plot (Figure 5d). Given the asymmetry in size, the term "asymmetrical body" still appropriately describes this neuropil.

### 3.5.2 | AB neurons and neuron families

Three AB neurons or neuron families have been identified and are described below. These classes of neurons are primarily, but not exclusively, a conduit for information flow between the superior lateral protocerebrum (SLP), FB, and AB. No intrinsic AB neurons have been identified. The AB cell types described in this report innervate both the left and right AB. One or both ABs can be targeted by a single neuron, depending on the cell type. One cell type only innervates the left AB if the right AB is also innervated by the same neuron (see below). The AB neurons appear, based on anatomy, to primarily provide input to the AB, with two of the three AB neurons or families of neurons exhibiting predominantly boutons in the AB. Since the small, left AB is innervated, it is reasonable to conclude it is a functional neuropil.
The SLP-AB neuron

The SLP.s-AB in the AB. The arbors are predominantly spiny in the SLP and consist overwhelmingly of boutons in the AB. This neuron type exhibits three trajectories: an ipsilateral path (SLP.s-ABi.b; Figures 6a and 7a1), a path that crosses the midline (SLP.s-ABc.b; Figures 6b and 7a2), and one that arborizes in both the left and right ABs and the SLP in either the left or right hemisphere (SLP.s-ABic.b; Figures 6c and 7a3). Interestingly, neurons exhibiting the first two pathways arborize exclusively in the right AB, so all SLP.s-ABi.b information is relayed within the right hemisphere whereas SLP.s-ABc.b-transduced information crosses the midline from the left SLP to the right AB. The left AB receives information from the SLP only when the right AB also receives the same information, via the SLP.s-ABic.b pathway.

The SLP.s-ABic.b trajectory is less common than either the SLP.s-ABi.b or SLP.s-ABc.b trajectories; the latter two occur in approximately equal numbers. Furthermore, there is no strong bias toward the left or right SLP for the SLP.s-ABic.b trajectory. One hundred fourteen MCFO-labeled SLP-AB neurons in line SS04682 were characterized as follows: right SLP, right AB (SLP.s-ABi.b), n = 46; left SLP, right AB (SLP.s-ABc.b), n = 40; left SLP, both ABs (SLP.s-ABic.b), n = 18; and right SLP, both ABs (SLP.s-ABi.b), n = 10.

These data indicate that the SLP-AB neuron exhibits a strong asymmetric bias toward the right AB, but a similar strong right/left preference does not extend to the SLP. Furthermore, a single AB (always the right) is targeted more frequently than both simultaneously (75% vs. 25%, respectively, for SS04682). An additional 23 neurons labeled using MCFO, distributed among six stable split lines (SS04691, SS04692, SS04683, SS02822, SS004429, and SS04458), fell into the following classes: 7 SLP.s-ABc.b (left SLP, right AB), 10 SLP.s-ABi.b (right SLP, right AB), and 6 SLP.s-ABic.b. Imaging data for these lines in which the entire population is labeled support the right AB bias for this neuron (not shown).

The AB-FB8 neuron family

The AB.s.b-FB8.b neuron comprises three sometimes subtly morphologically distinct forms (Figure 8). These differences can be so subtle, for example, one bouton in FB81, that they may simply reflect the range of variation or stochastic nature of this cell type. In an effort to encompass the diversity in forms of this neuron, we designate it a
FIGURE 13  The canal cell: PB$_{G1,5}$-EBc.b-D/V GA.b. (a1) Line SS02198 shows expression in both the canal and tile cells and enables a direct comparison between the two cell types. Shoots from the EB arbors of the two green canal cells enwrap the EB canal (yellow asterisk; see also a2) whereas such filamentules are notably absent from this red tile cell (and most tile cells). Note the distinct difference in density of the gall arbors: the red tile cell arbor has more boutons than the sparse canal arbors (green asterisks). G3, G4, and G9 identify the glomeruli of the PB that are targeted by these cells. Scale bar = 20 μ [from line SS02198]. (a2) Footprints of the tile (red) and canal (green) cells differ in radius (top, frontal view) and depth (bottom, sagittal view). Both cell types occupy the posterior shell of the EB; the canal cell projects even deeper, into the medial and sometimes even the anterior shells, although only a few boutons reach the anterior shell. Anterior is to the right in bottom panel. (a3) Canal cell gall arbors (green) are sparser than tile cell gall arbors (red) and generally even less dense than in this sample, for example, as seen in Figure 13b,d. (b) A canal cell that targets G4 in the PB. As noted for the tile cell, PB arbors from canal cells can also spill into neighboring glomeruli, shown in the right inset, in which G9’s arbor extends fine, sparse shoots into G8 (asterisk). The canal cell was named for the filamentules that project from the EB arbor toward the central canal (top right). Sagittal view of the EB (lower right) illustrates that the bulk of the canal arbor is concentrated at the posterior of the EB, with a few branches reaching more anteriorly (anterior is to the right). Note that this cell, which targets an even-numbered PB glomerulus, projects to just the tip of the ventral gall and sends a small projection more ventrally (arrows). The canal cell gall arbor is not as robust as the tile cell’s gall arbor in that it does not fill the gall. The dorsal gall is in a different plane of view than is shown in the inset and does not receive input from this cell [from line SS02195]. (c) The distribution of the canal cell’s EB arbor is variable; in some cells, boutons almost completely enwrap the canal (G5 and G6, in this figure), in others, a uniform swath extends the radius of the EB (G7), and in still others, the greatest density of boutons is offset toward the canal (G1). Several additional examples and the depth to which they penetrate the EB are shown in the lower panel; only the orange cell (G6) reaches the anterior shell, as seen in the side view (anterior is to the right). The projections of these cells to the dorsal/ventral gall are shown in (d) [SS02195, SS02198, SS04776]. (d) The profile of the canal cell’s gall arbor is distinct from that of the tile cell, it follows the odd/even glomerulus to D/V gall projection rule. D = dorsal gall. V = ventral gall. (d1) The mint green G6 cell from (c) projects to the ventral gall (V) in panel d1 but rather than filling this neuropil, as does the tile cell (e.g., red cell in Figure 13a3), it appears to track along this neuropil’s surface [SS02198]. (d2) The red G1 cell shown in (c) also exhibits this tracking behavior in the gall. This three-panel series from posterior (top panel) to anterior illustrates that this red arbor snakes between the dorsal
“family.” All members of the family share several features in common: they all arborize in the AB and FB, the FB arbors are columnar, and the AB terminals are mixed, consisting of both boutons and spines. The presence of spines in the AB of this family is unique among the three AB cell types characterized to date and identifies them as the only output pathway (based on morphology) so far identified from this neuropil. Family members differ in the number of arbors in FB and in whether or not they arborize in FB.

Three forms have been identified. (a) AB.s.b-FB8i.b, which has mixed terminals in the AB and apparent output in the ipsilateral layer 8 of the FB (n = 77, 33 of which arborize in the left AB, 43 in the right AB, and one in both ABs; Figures 8a and 7b1). (b) AB.s.b-FB8i.b-FB8c.b (shown, Figure 8b), although sometimes lacks the contralateral FB8 arbor (AB.s.b-FB8i.b-FB8i.b; not shown). This form is similar to that shown in Figure 8a but in addition, it has sparse output in the ipsilateral FB1 and sends two projections to FB8 (n = 11; 7 arborize in the left AB, 3 in the right AB, and 1 with output in the contralateral FB layers 1 and 8). (c) AB.s.b-FB8i.b-FB8c.b, which has mixed terminals in the AB and two apparent output arbors in FB8, one in each “shoulder,” or lateral margin (n = 14; 9 target the left AB and 5 the right AB; Figures 8c and 7b2). The differences between these forms are subtle and may simply reflect the stochasticity of this cell type. (Cell counts are from lines SS00241, SS02218, SS02718, SS02738, SS02742, SS04417, and VT020016.)

SLP-AB-FB8 neuron family

The SLP.s-AB.b-FB8.b neuron family has the least stereotyped morphology of all PB, NO, and AB neuron classes studied to date. The term family is again applied here to encompass the range in morphology of this cell. The core morphology is conserved, with the SLP, AB and layer 8 of the FB constituting the backbone of this cell type. Several examples of this family are shown in Figure 9 to illustrate both the “familial” characteristics as well as the range of morphologies seen in this group of neurons.

The suggestion that this cell type’s morphology is not highly stereotyped, but instead exhibits a conspicuous degree of variability (in other words, that these are all variations of the same cell type), is supported by the observation that the various forms occur in each of the GAL4 lines and split-GAL4 lines examined, as well as in both split half parents of a given split-GAL4 line. For example, a range of morphologies has been seen in VT006486 and two split-GAL4 lines for which VT006486 is a parent line, SS04457 and SS04423. It is possible that results from behavior and physiology assays, as well as type-specific split-GAL4 lines, will indicate these forms are distinct cell types.

Arbors of these family members are predominantly spiny in the SLP and the remaining arbors are composed primarily of boutons and are therefore presumed to be output. The SLP.s-ABic.b-FB8.b neuron, the “core” member of the family, is shown in Figures 7c and 9a. The remaining projections in cells in this family seem to exhibit some level of stochasticity, as there is a great deal of variability in the layers of the FB that are targeted. Some cells in this family project neurites that terminate in boutons in various combinations of layers 1, 2, 4, and 5 of the FB, and one bouton was seen in the EB. Typically, just one to several layers receive a bouton or two. The neuron shown in Figure 9b strongly resembles that shown in Figure 9a, but in addition to the relatively robust arbors in the SLP, FB8, and AB, it also extends a few boutons into FB layers 1, 4, and 5 (e.g., red asterisk).

The arbor in FB8 in this family always spans the midline but varies in the degree to which it permeates this layer; as few as five medi ally located boutons have been seen. The cells shown in Figure 9c.d provide examples of more restricted FB8 arbors. The cells shown in Figure 9b,c are largely similar, except for the extent of the FB8 arbor. Although the neurons in Figure 9b–d have diverse projections, given the presumed stochasticity of these projections we simply refer to them by their family name, SLP-AB-FB8.

3.6 | PB neurons

Since publication of Wolff et al. (2015), several advances have been made in our understanding of neurons that target the PB. First, we have identified several new PB neurons that have features in common with a published cell type, the octopaminergic OA-AL21 cell (Busch, Selcho, Ito, & Tanimoto, 2009). In addition, new insight has been gained into a previously described neuron, PBG1-9.s-EBt.b-D/V GA.b (Wolff et al., 2015). Finally, a neuron discovered by Lin et al. (PB1_gnomes-EBc->EBc-1DFDSSB (Lin et al., 2013) has now been identified in GAL4 lines, providing additional characterization of this cell type, which we call PBG1-9.s-EBc.b-D/V GA.b to be consistent with our previously described nomenclature system. The latter two cells, PBG1-9.s-EBt.b-D/V GA.b and PBG1-9.s-EBc.b-D/V GA.b, share many features in common, as described below. In addition, split-GAL4 driver lines for the PB neurons have been generated and are presented in Table 2; these lines are generally highly specific for a particular cell type.

3.6.1 | A family of PB neurons with morphologically similar PB arbors

We have identified a number of PB neurons that share features in common with one another and with a published PB octopaminergic neuron, the OA-AL21 cell (Busch et al., 2009); see Figure 10 for several examples. Many of these cells have complex morphology, sprouting elaborate networks of projections. In addition, the GAL4 lines in which these cell types were identified show expression in many cells.
The combination of the intricate morphology of the cells and crowded GAL4 lines has made it difficult to label individual cells in unique colors, so the descriptions and images that follow are best approximations of the neuronal morphology.

Although all the projections could not be unambiguously traced, there is clearly a wide scope of neuropils targeted by this collection of neurons. Their projections proliferate throughout the brain and some appear to send projections to the optic lobes (Figure 10a–c) and the ventral nerve cord (VNC; not shown). The common feature among these neurons is a sinuous arbor of variable density that courses through the PB (Figure 10d1–d4), suggesting that some PB functions may be modulated by octopamine. In some neurons, the PB arbor traverses just half of the PB (Figure 10b, inset). In other instances, it appears to run the full extent of the PB (Figure 10a, inset; 10c, inset; 10d1–d4), but since there may be two labeled neurons in these specimens the limits of single PB arbors cannot be definitively determined. Since the anatomy of these cells is not well-defined in our images, we have not named them.

### 3.6.2 A revised account of the "tile cell," PBG1-9.s-EBt.b-D/V GA.b

The functional and anatomical unit of the PB is the glomerulus (G), which derives its name from the Latin "glomus," meaning "ball of yarn." In keeping with the standard established in Hanesch et al. (1989) for *Drosophila*, glomerulus is used here rather than the generic term "slice," which refers to a transverse division of a structure (Ito et al., 2014). The glomeruli are numbered G1–G9, from medial (G1) to lateral (G9). The specific glomeruli targeted by a given cell type are indicated following the neuropil designation PB. The EB is segmented along both its transverse and longitudinal axes. Two distinct transverse volumes are revealed by the arbors that fill them and were termed tiles ("EB.t") and wedges, based on their geometry (Wolff et al., 2015). A third volume, the "canal," is introduced here.

We identified a cell that bears a strong morphological resemblance to the tile cell, a PB-EB-GA cell (Wolff et al., 2015), in seven split-GAL4 and several generation 1 GAL4 lines. Two significant differences suggested it was a new cell type, but the analysis outlined below indicates they represent the same cell type.

We originally reported that the tile cell targets only eight of the nine glomeruli, skipping the most lateral glomerulus, G9, and that its EB arbor, characterized in GAL4 line R33A12, is confined to the posterior ring of the EB. In contrast, the apparently new cell targets all nine glomeruli (Figures 11a1.a2.a2.b1.b2 and 12a3 right). And third, the GA arbors consist primarily of boutons, which fill either the dorsal or ventral subcompartment of the GA, depending on the glomerulus of origin (Figures 11c3.c4 and 12a4; cells that arborize in the odd glomeruli navigate to the dorsal gall and those that target even glomeruli track to the ventral gall, as described in Wolff et al. (Wolff et al., 2015).

Two approaches were taken to determine if the variants represent the same cell type. First, additional R33A12 MCFO brains were examined for cells with tendrils and cells that target G9. Of 66 MCFO-labeled PBG1-9.s-EBt.b-D/V GA.b cells in line R33A12, just one exhibits a single spindle projection terminating in a bouton and only three cells project from G9, a lower than statistically expected frequency. The presence of this cell type in G9, combined with the strong morphological resemblance of the two variants, suggests the variants are the same cell type.

Second, flies from hemidriver lines representing each variant were crossed and the progeny examined for cells that both arborize in G9 and elaborate tendrils. AD and DBD split half parents from the tile cell line, R33A12, were crossed to complementary split half parents that drive expression in a "tile-like" cell in line VT040589. The tile cells present in the progeny of this cross (line SS27853; Figure 11c) frequently target G9 (not shown), confirming the two variants are not only one and the same type, but also that this class of neurons arborizes in all nine glomeruli. Furthermore, the three SS lines that include VT040589 as a split half parent (SS02191, SS27853, and SS16814), all drive expression in the EB tile cell in all nine glomeruli (e.g., SS27853, Figure 11). This cell's name is therefore being changed from PBG1-9.s-EBt.b-D/V GA.b to PBG1-9.s-EBt.b-D/V GA.b to reflect its presence in G9 (note the change in the name is from G1-G8 to G1-G9).

The 1:1 projection pattern between G1 and G8 in the PB and corresponding domains in the EB and GA for the tile cell is described in detail in Wolff et al. (2015, Figure 14b). Briefly, cells that arborize in G1R/G1L project to the ventral tile of the EB, at approximately 6:00 on a clock face; cells in G2R/G8L project to 7:00–8:00; G3R/G7L project to 8:00–10:00; G4R/G6L to 10:00–11:00; G5R/G5L to 11:00–1:00; G6R/G4L to 1:00–2:00; G7R/G3L to 2:00–4:00, and cells in G8R/G2L project to 4:00–5:00. Cells that target G9 were not previously characterized. Analysis of the G9 subset of tile cells in GAL4 and SS lines revealed they project to the same EB tile as G1R and G1L, so the 6:00 tile receives input from the medial-most and lateral-most glomeruli of the PB (Figure 11a1.a2.b1.b2). These data also show that tile cells that target G9 strictly adhere to the previously described PB glomerulus:GA wiring pattern, so cells that arborize in G9 target the dorsal gall (not shown).

### 3.6.3 The canal cell, PBG1-9.s-EBc.b-D/V GA.b, strongly resembles the tile cell

Since publication of Wolff et al. (2015), MCFO performed on additional GAL4 lines revealed a PB-EB-GA cell not reported in that study. Also in Wolff et al. (2015), an effort was made to correlate the PB cell types described in that report with PB cell types described in Hanesch et al. (1989) and Lin et al. (2013); see Table 3A,B in Wolff et al. (2015). At the time, the closest match to the tile cell (PBG1-9.s-EBt.b-D/V GA.b) was PB1 glomerulus > EBc.idFB58 from the Lin et al. (2013) paper.
We now believe the "canal cell," not the tile cell, is equivalent to the PB
glomerulus* > EBc-IDFBosb cell. (Currently, there is therefore no Lin
et al., 2013 correlate for the tile cell.) Since MCFO enables a detailed
analysis of circuitry and morphology, here we build upon the descrip-
tion provided in Lin et al. (2013) and compare the canal cell to the
morphologically similar tile cell.

The canal cell's name is derived from a prominent anatomical fea-
ture of this neuron: its EB arbor partially enwraps the canal, or central
hole, of the ellipse (Figure 13). Following the nomenclature system
adopted in Wolff et al. (2015), this cell's formal name is the PBG1-8.s-
EBc:b-D/V GA.b cell, where the "c" that follows "EB" stands for canal.
The canal and tile cells share many features in common, including
overall morphology, putative input and output regions, and circuitry.
First, light-level analysis reveals that the canal cell has primarily bou-
tons in the EB and gall ("b" for boutons; Figure 13a–d) and predomi-
nantly spiny arbors in the PB ("s" designates a spiny morphology; Figure
13b, right inset), as described for the tile cell. Furthermore, the
PB arbors for both cells often occupy more than one glomerulus, spilling
over into the neighboring glomerulus (Figure 13b, right inset, asterisk). Occasionally, the arbor completely fills two neighboring glo-
eruli and a small portion of a third (not shown). Second, the canal
cell also targets all nine glomeruli of the PB (Figure 12b1,b2). Third,
canal cells also respect the odd/even glomerulus:D/V GA wiring pat-
tern (Figure 13c,d for several examples). Fourth, the circuitry between
individual glomeruli and the corresponding regions in the EB is identi-
cal between the two cell types [not shown; see Figure 14b from
(Wolff et al., 2015) for visual depiction of this circuitry].

The key differences between the tile and canal cells are the distri-
bution and morphology of the EB and GA arbors. The canal cell EB
arbor projects shoots of variable length around a portion of the cir-
cumference of the canal, partially enwrapping the canal (Figure 13a1,
a2,b,c). Relative to the tile cell, the bulk of the canal cell EB arbor is
shifted from the periphery to the center. The density of boutons in
the canal EB arbor and the area over which the arbor is dispersed
from periphery to center varies significantly from cell to cell (Figure
13a1,a2, green arbors; 13b, top right: 13c). Consequently, in
some instances it is difficult to distinguish the tile and canal cells from
one another.

The depth of the EB arbor also differs between the two cell types.
Whereas the tile cell EB arbor is confined to the posterior ring
(Figure 12a3, right), the canal cell EB arbor occupies not only the pos-
terior ring, but also invades the medial and sometimes even the ante-
rior ring of the EB (sagittal views in Figures 12b3 and 13a2,b,c).

The gall arbors in the tile and canal cells are usually distinct,
although sometimes they, too, are hard to differentiate. The tile cell's
gall arbor is robust and fills the entire volume of the dorsal or ventral
gall (Figures 11c3,c4 and 12a4). In contrast, the canal cell's gall arbor
is much sparser (Figures 12b4 and 13a1, green asterisks; 13a3, green
arbor; 13b, left inset and arrows; 13d) and appears to either creep
along the surface of the gall or snake between the dorsal and ventral
compartments of the gall (Figure 13d1,d2). Sometimes it seems to
hover at the dorsal or ventral tip (Figure 13d3, pink cell).

While the variability in morphology can make it difficult to distin-
guish between the tile and canal cells at the single cell level, distinc-
tions between the two cell types are unmistakable when the entire
population of each cell type is labeled in split-GAL4 lines specific for
each cell type (compare tile cell in Figure 12a to canal cell in
Figure 12b). These images confirm the MCFO-based observations
noted above, as follows. First, all nine glomeruli are targeted by both
cells (Figure 12a1,a2,b1,b2). Second, the tile cell EB arbor does not extend to
the canal (Figure 12a3, left), it fills only the posterior shell of the
EB (Figure 12a3 right; anterior to the right), and the arbors in the
dorsal and ventral gall are dense and uniform (Figure 12a4). In con-
trast, the canal cell's EB arbor exhibits a significant concentration
around the canal (Figure 12b1,b3 left) and is dispersed throughout the
posterior, medial, and anterior rings of the EB (Figure 12b3, right),
and the gall boutons are not densely concentrated throughout this neuro-

3.6.4 A renamed PB cell

The PBG1-8.s-FBc3,4,5.s.b-rub.b cell is being renamed PBG1-8.s-
FBC3,4,5.s.b-ROB.b. We misidentified the rubus (RUB) in the original
classification; instead, this arbor targets a structure named the round
body by Lin et al. (2013). While this structure is abbreviated “RB” by
Lin et al., K. Shinomiya proposes ROB to avoid confusing it with RUB
(personal communication).

4 DISCUSSION

There is increasing evidence that many complex insect behaviors, such
as navigation and sleep, rely crucially on the central complex, yet our
understanding of this key brain region is in its infancy—even the funda-
mental building blocks of its neuropils are, for the most part,
uncharacterized. With the current tools available in Drosophila,
comprehensively cataloging these neurons and generating the tools to
manipulate them is a logical and feasible first step, the results of which
will facilitate a more sophisticated understanding of the circuit com-
putations and the behaviors controlled by neurons of the central com-
plex. With a catalog of neurons, a set of genetic driver lines that
target each of these cell types and a battery of behavioral assays
through which to run these targeted fly lines, the tools exist to quan-
tify the behavioral effects of manipulating the activity of defined,
small subsets of neurons. Such experiments provide a way to infer the
involvement of different neuron types and neuronal networks in spe-
cific behaviors. The work described here provides such a descriptive
catalog of neurons and genetically targeted GAL4 driver lines for two
structures, the noduli and the asymmetrical body, as well as GAL4
driver lines for PB neurons. The availability of these tools will facilitate
studies of the roles of the NO, AB, and PB and the neuronal cell types
that populate these brain structures.

4.1 The asymmetrical body: The fifth central
complex structure

The neuropils considered to be constituent components of the central
complex have changed over the decades. Power (1943) defined the
neuropils of the central complex to include the "central body, the ellip-
soid body and the pair of ventral tubercles." The central body (Flögel,
1878), included what became known as the ellipsoid body (Lowne, 1892) and the fan-shaped body (Dietl, 1876); reviewed in (Strausfeld & Seyfarth, 2008). The noduli were known as the ventral tubercles. More recently, the central complex has been defined as “a group of modular neuropils across the midline of the insect brain” (Pfeiffer & Homberg, 2014), “…interconnected neuropils and nuclei that populate the midline of the forebrain-midbrain boundary region” (Strausfeld & Hirth, 2013), and “a system of interconnected neuropils lying at, or about, the midline of the protocerebrum” (Ito et al., 2014). Although the modular architecture of the central complex structures is conspicuous (e.g., the glomeruli of the PB and the trajectory patterns of neurons that project to, from, and within the central complex structures), it is the assigned boundaries that encompass the central complex that seem to be the feature that defines these structures as members of the central complex.

Here, we illustrate that the Drosophila AB, which appears to be a structure that is distinct from the FB, meets the criteria outlined above for central complex neuropils: It is a midline neuropil; it falls within the boundaries defined by Power (1943) and Strausfeld and Hirth (2013); and it is interconnected (to the FB and SLP) by a network of previously undocumented (with one exception) neurons. Since the AB meets all the criteria previously used to define neuropils as components of the central complex, we propose that the AB be added as a fifth neuropil of the central complex of Drosophila.

The AB is not unique to Drosophila. Phillips-Portillo and Strausfeld (2012) describe the presence of likely homologous bilateral, asymmetrically sized ABs in N. bullata and C. erythrocephala. Their work also identifies a tangential FB neuron that bears a resemblance to the SLP-AB neuron described here. It remains to be determined if the AB is more widely represented in other insect orders.

4.2 | A mostly asymmetric neuron

The right AB is significantly larger than the left. At a minimum, this difference is likely due to a combination of smaller arbors in the left AB and the lower frequency with which the left AB is targeted: only the ipsilateral-contralateral-projecting form of the SLP-AB neuron, which arborizes in both the left and right ABs, targets the left AB, whereas the ipsilateral and contralateral-projecting forms of the SLP-AB neuron target exclusively the right AB. Thus, the right AB appears to receive a disproportionately larger share of information from the SLP, although the right and left hemispheres appear to be equally represented as sources of input. Notably, this left–right bias is restricted to the AB, as a parallel preference is not shown for the SLP. The availability of genetic lines that target AB-specific cell types will enable experiments aimed at revealing the relevance of this left–right bias.

4.3 | Unusual properties of the NO neurons

Three unusual features distinguish the five most commonly seen NO neurons described here from other central complex neurons. First, in contrast to the majority of PB neurons described to date, the projections of four of these five NO neurons are ipsilateral. Second, while anatomical features identify distinct input and output neuronal populations in other central complex neuropils, the noduli appear to be sites for receiving primarily input from other neuropils (boutons appear to be the predominant anatomical feature in the noduli in confocal micrographs of NO, PB-FB-NO, and PB-EB-NO neurons). Golgi preparations and data from the likely locust equivalent of the PB-FB-NO neuron (the CPU4 neuron), however, indicate these NO arbor are mixed (Hanesch et al., 1989; Heinez & Homberg, 2008; Stone et al., 2017); perhaps the intensity of the dense populations of boutons masks the presence of spines in confocal preparations. Third, although the noduli do receive input from central complex neuropils (e.g., via the PB-FB-NO and PB-EB-NO neurons, from FB tangential neurons, etc.), the majority of direct input for this new set of neurons is provided by just one neuropil, the LAL. Such a restricted thoroughfare of communication is in stark contrast to the PB neurons, for example, which have a much broader and more diverse network of direct communication.

The LAL-s-CREc.s-NO3Pc,b cell type is distinct from the other four common NO neurons in that it delivers contralateral, rather than ipsilateral, input from the LAL and CRE to NO3P. The posterior compartment of NO3 is therefore unique in that it is the only nodulus subcompartment to communicate directly with the contralateral hemisphere. Given that NO3P (and NO3M) also receives ipsilateral terminals from the LAL via LAL-s-CREc.s-NO3P/Mi,b, this subcompartment may act as a limited integration center between the fly’s left and right sensory fields.

4.4 | Potential roles of the NO neurons

Physiological data from two neurons in the sweat bee offer insight into a likely role for the NO neurons described here. The TN1 and TN2 neurons (“noduli tangential neurons”) share a high degree of anatomical homology with the LAL-NO neurons; TN1 and TN2 are ipsilateral neurons with input branches in the lateral central brain and blebbed branches in the noduli (Stone et al., 2017). Recordings from these two cells reveal they fire in response to simulated backward and forward flight, respectively, and that the rate of firing is dependent on the stimulus velocity, suggesting these neurons encode speed using optic-flow and can thereby track the distance traveled by the bee. Similar physiological features and path integration functions would not be unexpected for the apparent homologous Drosophila neurons.

The LAL is the primary source of input for the NO neurons described here and its activity may provide additional insight into the roles of the LAL-NO neurons. It is a large, bilateral neuropil that is highly interconnected with neuropils of the central complex. Functionally, the LAL is considered to be a sensorimotor integration center, based on several lines of evidence in various insect species. For example, in crickets and moths, activity in LAL neurons is associated with walking (Iwano et al., 2010; Mishima, 1999; Zorovic & Hedwig, 2013). In the locust, assorted LAL neurons exhibit changes in activity in response to various aspects of flight, implicating this brain region in flight control (Homberg, 1994). In Drosophila, LAL neurons involved in walking backwards have been documented (Bidaye, Machacek, Wu, & Dickson, 2014).

It has been suggested that the noduli are involved in walking and motor control in Drosophila (Buchanan et al., 2015; Strauss & Heisenberg, 1993). The neurons implicated in left–right turning bias in
locomotion are the PB-FB-NO neurons (Buchanan et al., 2015), which have presumed input (fine terminals) in the PB, and presumed output (boutons) in the FB and NO. The authors speculate that the bias to turn in one direction or the other is influenced by an interplay between the nodulus subdomains that are targeted by the different PB-FB-NO cell types. Direct communication between the PB-FB-NO neurons and the LAL-NO neurons is not unexpected, as Stone et al. (2017) have shown synaptic contacts between the bumblebee equivalents of these two cells, the CPU4 and TN cells, respectively. Considering the sensorimotor contribution made by the LAL in various types of movement, the LAL-NO neurons described here are strong candidates to contribute to the circuits involved in turning.

4.5 | NO neurons: An incomplete picture

The catalog of NO neurons described here is incomplete. Our analyses of other GAL4 lines have identified several large-field FB neurons that also arborize in the noduli, as well as other brain regions that we are currently characterizing; some of these neurons are illustrated in the Golgi stains of Hanesch et al. (1989). Hanesch et al. (1989) also describe other cell types with arbors in the noduli that have so far eluded our identification. Finally, it seems likely that there would be output neurons from the NO, although we have not yet identified such neurons in our studies. Electron microscopic-level analysis should provide a path to identifying these neurons.

4.6 | Defining “cell type” and neuropil boundaries

The debate continues to swirl over what constitutes a distinct cell type. Morphology and function have long been accepted as reliable criteria to distinguish cell types. While morphology is a straightforward and easy means of classifying cell types, it can be misleading in that cells that appear identical may have functional differences. For example, Green et al. (2017) describe clearly distinct physiological roles for two PB neurons that appear to have indistinguishable morphology at the light level. Morphological features evident with light microscope-level resolution will therefore likely be insufficient to distinguish all cell types, so knowledge of some combination of synaptic connectivity, functional properties and the genetic programs used to specify these attributes will be necessary to fully define cell types.

Similar limitations confound the assignment of neuropil boundaries and subcompartments. Synaptic density varies considerably across brain regions and this variation has provided landmarks used to define the neuropils of the fly brain (Ito et al., 2014). While the boundaries of some structures are unambiguous (e.g., the PB and EB), neuropil margins are not universally so clear-cut, with many neuropils appearing to meld seamlessly with adjacent neuropils. The opportunity to map the domains of arbors within neuropils identifies distinct regions that are not revealed by differences in synaptic density (e.g., wedge and tile domains in the EB). For example, the mushroom body lobes can be divided into a series of nonoverlapping compartments with distinct functions by the extent of the arbors of dopaminergic input neurons and mushroom body output neurons (Aso, Hattori, et al., 2014; Aso, Sitaraman, et al., 2014). The LAL provides an example of one neuropil that may have functionally distinct subregions. It is a large neuropil with no obvious boundaries revealed by anti-Brp staining, yet the arbors of many neurons that target this neuropil exhibit strong regional preferences. Mapping the domains of these arbors may identify regions that are functionally distinct.

4.7 | Obtaining a comprehensive description of cell types, circuits, and the tools to manipulate them

Three major efforts aimed at cataloging all the neurons in the Drosophila brain are in progress. One, typified by this and others’ work (Aso, Sitaraman, et al., 2014; Tuthill, Nern, Holtz, Rubin, & Reiser, 2013; Wu et al., 2016), characterizes one structure at a time using light microscopy in combination with the generation and analysis of highly specific GAL4 driver lines. The second method is a modern implementation of the Golgi approach of randomly labeling small numbers of neurons in order to describe their morphology (Chiang et al., 2011). And the third, which is now becoming practical at the required scale, involves reconstruction of neuronal morphology and circuits through analysis of image volumes collected using electron microscopy (see, for example, Takemura et al., 2017, Takemura et al., 2013). We believe that such light and electron microscopic-level analyses will be highly synergistic. Light microscopy, with genetically marked cells, provides the ability to observe the morphology of hundreds of individual cells of the same cell type in many different individuals, providing insights on stereotypy. However, its dependence on GAL4 drivers means that completeness of coverage cannot be assured. Conversely, electron microscopic analysis, while usually limited to a single sample, not only ensures completeness but also enables visualization and quantification of synaptic connectivity. Moreover, since EM samples do not carry transgenes expressing ectopic membrane proteins that can interfere with development, wiring errors may be less likely. While only electron microscopy is likely to provide the complete wiring diagram of a circuit, light level analysis of genetic driver lines will be needed to provide the critical bridge between circuit maps and the tools required to precisely manipulate the activity of their individual components.

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