

The central complex of the larval fruit fly brain

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Abstract

The Central Complex (CX) is a conserved set of arthropod brain neuropils that integrates multisensory information and mediates spatial navigation and sleep. In holometabolous insects such as the fruit fly *Drosophila*, the CX forms during metamorphosis and serves the adult stage. Whether a form of the CX exists in the brain of the evolutionarily novel larval stages is not known. Here, we analysed the connectome of the *Drosophila* larval brain and, on the basis of neuronal lineages, synaptic connectivity patterns, anatomy, and neuronal-behaviour maps, we identified a simplified larval CX, comprising 4 key neuropils: the protocerebral bridge(PB), the elipsoid body(EB), the fan-shaped body(FB) and the noduli (NO). Consistent with our interpretation, we found in the larval brain synaptic connectivity patterns characteristic of the adult, including (i) strong, direct connections from MBONs to the FB and NO; (ii) strong visual input into the PB and EB; and (iii) strong connectivity between FB and NO. Interestingly, we find that some neuronal lineages contributing to the larval CX do not contribute to the adult CX, whilst many others remain conserved. The characterisation of a larval CX brings structure to larval brain circuits, linking with a vast body of literature, and will inform the design of experiments to probe brain function.

1 Introduction

The Central Complex(CX) is a morphologically conserved set of neuropils found across insects that acts as navigational centre and sensory integration area for coordinated motor activity. This region is best described and understood in *Drosophila* adult where its core functions include multisensory navigational decisions, path integration, allocentric orientation of the head relative to its body - convergence of head and body direction - and providing an internal sense of direction in the absence of stimuli.

At the larval stage, this animal exhibits similar behaviours to those observed in the adult: it demonstrates chemotaxis during foraging and performs aversive phototaxis in response to blue light. In addition to individual stimulus

response, *Drosophila* larva is able to integrate competing stimuli into a coherent representation(Gepner et al.,2015) prior to decision making. The larval brain shares similar set of neuroblasts with the adult, and presents neuropils with direct correspondance to the adult such as the Antennal Lobe(AL), the Mushroom Body(MB), and the Lateral Accessory Lobe(LAL).

The underlying connectivity of AL, MB, LAL is well understood at present(Winding et al., 2023), as well as the Lateral Horn(LH; a larvae specific neuropil). Nevertheless, these structures constitute only up to 25% of the larval brain connectome. We postulate that amongst the remaining 75% of neurons, a multitude should be devoted to navigational decisions, and may constitute the putative larval Central Complex neuropils. These are unlikely to be reognizable morphologically at this stage of development, since the brain lobes aren't yet fused at the midline, and the larval brain presents a commisure. The basis of our search has to be, in turn, based on lineage membership, relative spatial location and circuit architecure specific to adult central complex neuropils. We use all three in an iterative process to progressively find putative CX neurons.

The adult *Drosophila* central complex has five neuropils: the Protocerebral Bridge (PB), the Fan-shaped Body (FB; or central body upper), the Ellipsoid body(EB; or central body lower), the Noduli (NO) (?) and, as of recently, the Assymetrical Body (AB) (?). The Lateral Accessory Lobe (LAL) reciprocally interconnects with these, making it an important accesory structure and reference point.

The central complex has been associated with a set of functions - spatial navigation decisions, directed locomotion and sleep - some of which are shared by the larva. The neuroblasts that give rise to the neuronal lineages populating the adult CX also exist in the larval brain. A subset of embryonic-born neurons remain undifferentiated throughout larval stages and delineate the structures of the adult CX, acting as pioneer neurons during metamorphosis (?); however, earlier-born, differentiated neurons of the same lineages contribute to structures in the larval brain. The question remains as to what structures. Furthermore, the larval brain presents readily recognizable neuropils of accepted homology with the adult brain, including the antennal lobe (?), mushroom body (?), and lateral accessory lobe (?). In the adult brain, the central complex neuropils are primarily medial structures, suggesting that any putative larval counterpart will be necessarily split across the midline given the lack of fusion of the larval brain hemispheres. With all the above in mind, and considering the evolution of the larval stage in holometabolous insects (?)and the presence of a central complex-like structure in the larva of the holometabolous beetle *Tribolium castaneum*, we set out to identify the putative central complex neuropils of the fruit fly larva on the basis of: neurons contributing to the larval CX neuropils that share lineage of origin with the adult CX neurons;the synaptic connectivity present across the putative larval CX neuropils is at least a subset of that of the adult CX neuropils; the spatial position and overall morphology of the arbors of larval CX neuropils is similar to that of the adult CX neurons.

2 Methods

2.1 Nomenclature

We used an adapted version of the nomenclature system proposed by Wolff et al. 2015, a convention that provides a description of individual cell types. The first neuropil in a neuron's name is the neuropil closest to the cell body. The second component of a cell's name is the predominant morphology of its arbors, abbreviated as either "d" for dendrites, or "b" for boutons(axon terminals). Letters that are used in the neuron names are highlighted in color. Ovals in the schematics represent the cell bodies, solid and hatched fills denote spine and bouton arbor morphologies, respectively.

PB: protocerebral bridge; EB: ellipsoid body; FB:fan-shaped body; NO: noduli; d:dendrites; b: boutons.

2.2 Connectivity

Using stereotypical Central Complex connectivity patterns identified in the adult, such as visual input processing of polarized light into the Protocerebral bridge and the Ellipsoid Body Ring neurons (??), connectivity between the Mushroom Body and the Fan-Shaped Body, and more (see results).

2.3 Lineage

The Drosophila central brain consists of stereotyped neural lineages, developmental-structural units of macrocircuitry formed by the sibling neurons of single progenitors called neuroblasts. Using the currently identified lineages contributing to the Central Complex of the Adult Drosophila (?)we filtered the neurons satisfying imposed connectivity rules (e.g of those neurons connecting to the LAL, how many come from a known CX lineage) and continued to constrain these iteratively via a back and forth manual analysis of these neurons.

3 Results

3.1 Correspondence between larval and adult neuronal lineages

On the basis of neurons from lineages known to contribute to the adult central complex and the spatial arrangement of their arbors in the brain, we will now examine each larval central complex neuropil individually.

3.2 The larval Protocerebral Bridge (PB)

In the adult *Drosophila*, the PB comprises two sets of bilaterally symmetric compartments, sometimes referred to as glomeruli 1–9 ?, positioned at the most posterior-dorsal location possible in the brain.

Larval lineages	Adult lineages	PB .b	PB .d	FB .b	FB .d	EB .b	EB .d	NO .b	NO .d
Bamv12-ven	LALv1					2	2		
Dalv23	EBa1					12			
Dalcl12	-					2			
BAla12	-					2			
CPb	-					8	4		
BAlpant	-					2			
CM13lat	DM6/CM3					4			
CM13med	DM6/CM3					2	2		
DPMm1	DM1		4	14				4	
DPMpm1	DM2						4		
DPLc5	-							2	
CPa	-				2				
dall1	-				4				
BAla34	-				4				
bamd2-d	-				2				
bamd2-v	-				2				
dplal1-3	-					2			
DPMpl12	SIPp1				2				
DPMpm2	DM3						2	2	
dplcpostmed	-							4	
DPMl34post	-							2	
bamv12dor	LALv1							2	
DPMl12	-		2					2	
Dalv1	-		8						
BLAd-OL	-		2						
BLAV12ant	-				2				
BLVp2	-		2						
DPMm2	-		2				2		
DPMl-ant	-		2					2	
DALcm12-v	CREa2		2		2			10	
BLAl	-				2				
CPe	-				2			4	
BAmv12-dor	-				2			6	
CM4-vm	DM4				2			4	
Dalcm12-m							4	4	
BAmd1	CREa1		2				2		
BLP12	-								
dpmpl3	-							2	

Table 1: Central complex lineages corresponding to each neuropil. The second column lists the corresponding lineage in the adult brain, as per Eckstein et al. 2020, listing only the subset of adult lineages known to contribute to the adult CX (note that we don't list further adult lineages that contribute to the CX whose larval corresponding lineage doesn't). The numbers are of neurons belonging to each lineage (rows) that contribute either axons ("b" for "bouton") or dendrites ("d") to the CX neuropils (columns).

Neuropil	PB	FB	EB	NO	LAL
PB		*	*		*
FB	*			*	*
EB	*				
NO		*	*		
LAL					
MBONs		*			

Table 2: Synaptic connectivity between CX neuropils, with a row neuropil synapsing onto a column neuropil.

Neuropil	Sensory Inputs Adult	Sensory Inputs Larva
PB	visual	visual
	olfactory	olfactory
FB	olfactory	olfactory
	thermosensory	
EB	visual	visual
	olfactory	olfactory
	mechanosensory	
NO	proprioceptive	proprioceptive
	olfactory	olfactory

Table 3: Sensory inputs into the Central Complex neuropils in adult vs larva

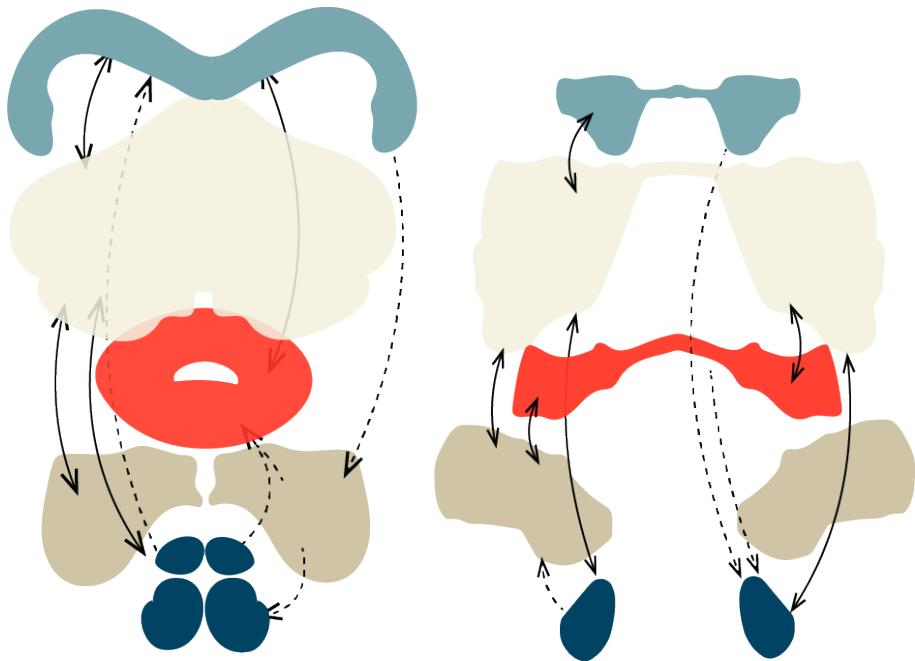


Figure 1: The Central Complex Neuropils and their inter-connectivity.
 Dotted-line arrows represent unidirectional connections, filled-line arrows indicate bidirectional connections **A**. The adult *Drosophila* CX. Bidirectional connectivity is seen between PB-FB, PB-EB, FB-LAL and FB-NO, with unidirectional connections from NO-PB, PB-LAL, LAL-NO and NO-EB; **B**. The larval *Drosophila* CX. Bidirectional connections are PB-FB, FB-EB, FB-LAL, FB-NO and EB-LAL; unidirectional connections are seen from NO-LAL, PB-NO and EB-NO

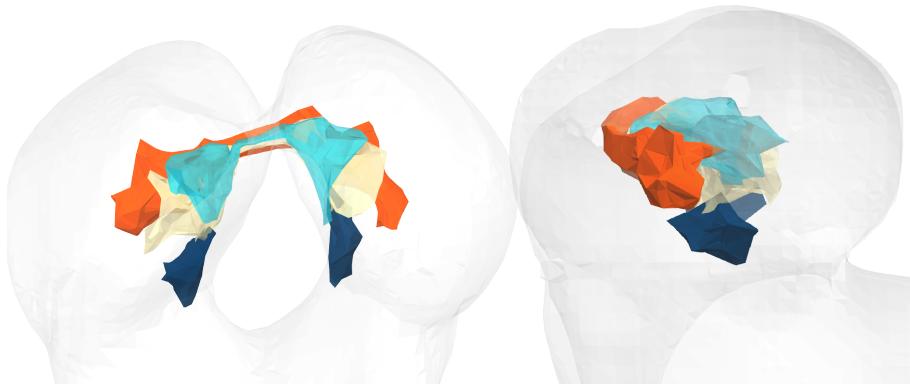


Figure 2: The Central Complex Neuropils. Posterior and Lateral views of the Larval Central Complex neuropils.

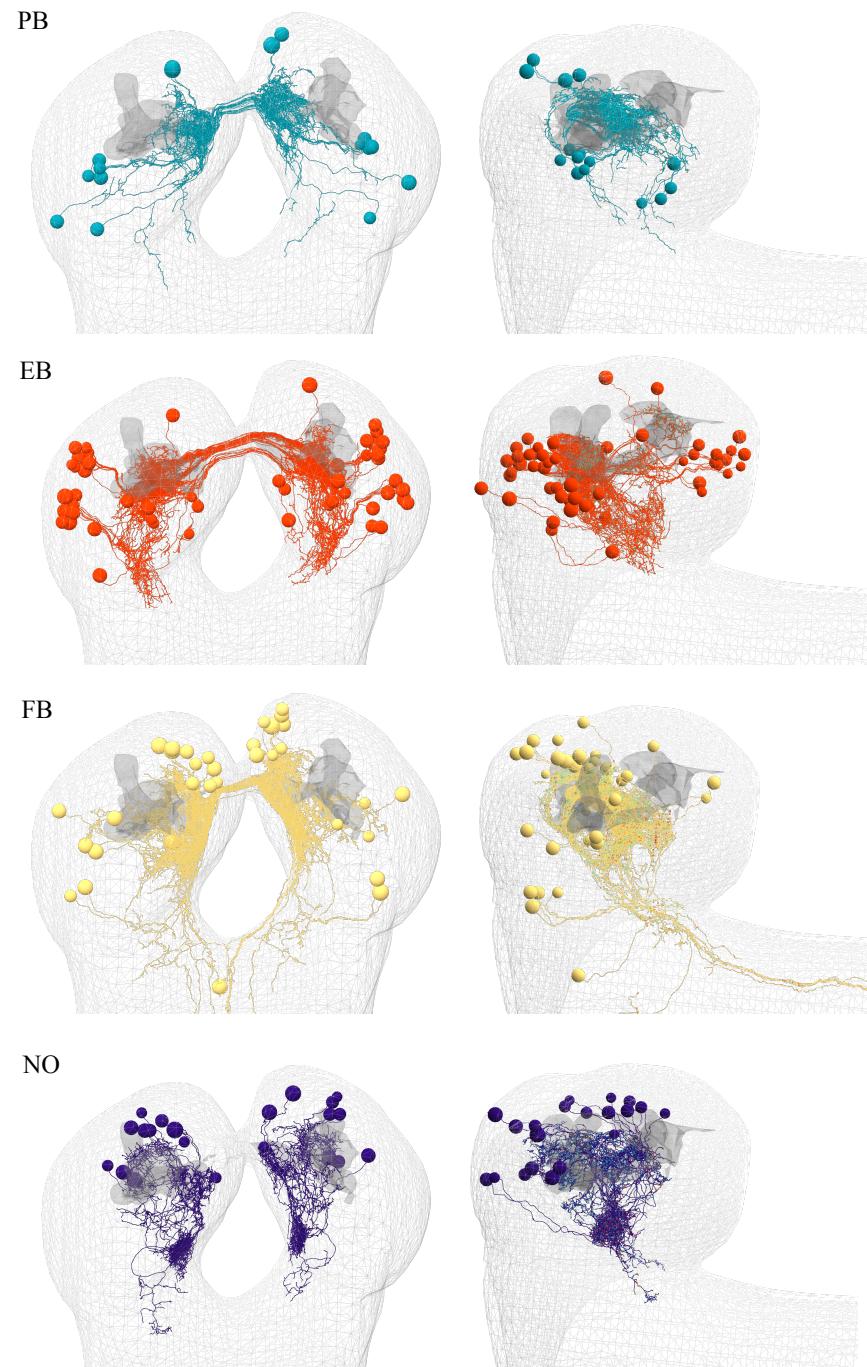


Figure 3: Larval CX neuron boutons define the volumes of the 4 main CX neuropils. Posterior and lateral views of neurons that contribute boutons (".*b*") to each of the 4 neuropils. In concordance with the adult, the PB is the most dorsal and posterior neuropil of the whole brain; the FB occupies an intermediate medial position; the EB is the most medial anterior neuropil (same dorso-ventral level as the mushroom body medial lobe but even more anterior); and the NO are medial and ventral.

These compartments are arranged in a continuous manner medio-laterally, contacting at the midline. In the adult, about 600 neurons innervate the PB, organised into hundreds of types (194; (?)) that are split into two main general groups: the columnar neurons (from lineages DM1, DM2, DM3, DM4 and DM6) whose dendrites innervate one or more of the 9 + 9 compartments of the PB (?); and the horizontal neurons (also known as horizontal fibers) derived from a single lineage (PBp1; (?)) whose axons innervate many or all PB compartments.

In the adult, the PB receives visual input via relay neurons (POL neurons) conveying information on polarized light, in a highly structured pattern across its compartments that binarizes the continuum of angles of polarized light ((?)). Then the PB relays this information to the EB compartments.

In addition to visual input, the adult PB also integrates olfactory inputs (?), suggesting that spatial navigation is not unimodal but integrative across multiple sensory modalities.

In searching for the larval PB, we expected two sets of neurons: columnar and horizontal. In larva, four central complex lineages contribute columnar neurons, a subset of which position their dendrites at a posterior-dorsal location. We could not find a central complex lineage that would contribute horizontal fibers at a posterior-dorsal location necessary to intersect and synapse onto the dendrites of the PB columnar neurons, but we found a larval lineage (DALv1) whose axons are bilateral and project to the appropriate area, and is developmentally related to another central complex lineage (DALv23).

This suggests that neurons from non-central complex lineages may be recruited temporarily during the larval period, in a pattern reported so far for the mushroom body (see Discussion; (?)).

Among neurons of the DALv1 lineage, 4 left-right pairs (named HF-PB for "Horizontal Fiber PB") project their axons bilaterally and across the dendrites of the columnar neurons. 3 of the 4 pairs present an unusual axon configuration: first, they project contralaterally to drop their first output synapses, with the axon then crossing the midline a second time to return back to the same ipsilaterally corresponding location to again drop presynaptic sites (??).

This peculiar axon configuration is unique among all neurons of the entire brain of the larva ((?)) and suggestive of potentially a delay line for comparing left-right sensory inputs. The 4th pair first drops presynaptic sites ipsilaterally and then its axon crosses the midline until reaching the corresponding contralateral location to synapse again (??).

The presynaptic outputs of DALv1 neurons are symmetric, in that they contact the same homologous pairs of left-right neurons which are predominantly neurons of the columnar system (??). The axons of these 4 pairs of HF-PB neurons are tiled dorso-ventrally, falling into two bilaterally symmetric groups which we interpret as defining 2 + 2 bilaterally arranged PB compartments, each innervated by 2 pairs of axons.

The dendrites of these 4 pairs of DALv1 neurons (HF-PB) are ipsilateral and dorsal, receiving polysynaptic inputs from vision and olfaction, like in the adult PB ((?)). In the larva, we found that these multi-sensory inputs to the

horizontal fibers of the PB are mediated by Convergence Neurons (CN-53 and CN-54, among others; ?) that, as their name indicates, integrate inputs from both Mushroom Body Output Neurons (MBONs) and from the Lateral Horn (LH) such as olfactory and visual PNs (?)). This circuit architecture indicates that sensory inputs arriving to the larval PB will have been modulated or gated by previously established associative memories, with implications for spatial navigation.

In the larva, the columnar system consists of neurons from 4 central complex lineages (DPMpm1, DPMpm2, DPMm1 and CM4) that also generate the columnar neurons of the adult (DM1, DM2, DM3 and DM4, correspondingly). Larval columnar neurons present small, narrow dendrites circumscribed within the 2 + 2 compartments defined by the axons of the horizontal fibers (DALv1 neurons), with whom they synapse. Among the columnar neurons, a subset project their axons directly to the Noduli (NO; ??), and another subset project directly to the larval Ellipsoid Body (EB; ??). We did not find in the larva columnar neurons whose axons would project to more than one Central Complex neuropil, despite such types being common in the adult (?). Beyond the canonical columnar neurons projecting to other Central Complex neuropils, we found some whose axons descend to the SEZ or nerve cord (??).

3.3 Ellipsoid Body(EB)

The adult Ellipsoid Body(EB) is a ring-shaped structure situated between the Fan-Shaped Body(FB) and the Mushroom Body horizontal lobes, facing anterodorsally. Its circuit is made up two types of neurons: ring-neurons (derived mainly from the EBAa1/DALv2 and LALv1/BAmv1/2 lineages) that spread their axons across the length of the EB, and reciprocally connected wedge neurons(derived from the DALcl12 lineage) that divide the EB into 16 compartments (aka. wedges) (?).

Its underlying circuit follows the ring attractor architecture (Zhang, 1996) which, as predicted by its anatomy, is shown to yield neural activity in the form of a topological ring in *Drosophila* adult (Seeling & Jayaraman 2015) with all nodes being connected via inhibitory connections, complemented by local recurrent excitations that maintain activity at each node once they escape inhibition.

The wedge neurons(EPG) form eight wedges around this ring, and project to both hemispheres of the PB, where they connect to two sets of columnar neurons that project back to the EB, forming recurrent loops. These are PEG and PEN neurons. The anatomical offset between EPG and PEN neurons is key to how the fly head direction system translates angular motion into an updated position of the activity bump in the ring attractor.

The EB receives visual inhibitory GABAergic inputs, via two parallel pathways for distinct visual information: 1. Ring neurons that deconstruct the visual environment of the fly; 2. tangential neurons that take in information about body rotations and transnational velocity. The latter receive input in the LAL, output to NO. Mechanosensory input also enters the CX via the second

order projection neurons to the EB. These neurons code head direction; some proprioceptive input has also been observed (?). It receives strong inputs from PB, NO and the LAL, and outputs onto the PB.

In the 1st instar larva, we found a group of 8 pairs of reciprocally connected neurons from lineage DALcl12 known to produce wedge-neurons in the adult, and categorised these together with one other pair of lineage Dalv23 (which produces ring neurons in adult) with the same connectivity pattern as wedge-neurons. Both their dendrites and axons are very small, and tiled medio-laterally, defining 8 compartments with one single neuron pair contributing to each. These are the intrinsic set of neurons, fully enclosed within the putative larval EB.

Similarly, we found one pair of neurons of the BAmv1/2 lineage - known to contribute to ring neurons in adult flies - that receive visual input via PB neurons, and reciprocally interconnects with the previously mentioned wedge-neurons, and whose axons are fully contained within the space defined by the wedge neurons. We categorised these as larval "ring" neurons.

3.4 Fan-shaped Body (FB)

The adult FB is a bilaterally symmetric neuropil anterior to the PB, with well-defined horizontal and vertical components: it has 6 horizontal layers stacked dorso-ventrally that are defined by distinct sets of horizontal neurons(FB tangential neurons); and 9 vertical columns stacked medio-laterally are defined by column-specific columnar neurons. Both horizontal and vertical neurons innervate the FB in a layer- and column-restricted manner (?). As one of the biggest CX neuropils, a large variety of lineages contribute to the FB (see Table ??). The FB does not receive input along only one clearly defined input pathway, but it is connected to many regions of the surrounding protocerebrum via tangential neurons.

There are 2 types of FB tangential cells: (1)neurons that relay the presence of an attractive odor to the FB, originating in the MB or the LH (learnt or innate valences); (2) neurons that relay sleep drive to the FB, whose activity is mandatory for sleep initiation.

The FB columnar neurons, or columnar input cells are known as PFN (PB-FB-NO) and they receive information both in the PB and in the Noduli output cells with dendritic fibers mainly in the FB;

There are 5 types of PFNs, they form a p they all receive the same head direction input from the PB, which is integrated with different input signals received in the NO. The PFN outputs are located in distinct layers of the ventral/posterior FB, essentially mapping the noduli layers onto corresponding regions of the FB. PFN cells have a columnar projection pattern that is offset from the default projection scheme between the PB and the central body. This offset generates a head direction bump in the FB that is contralaterally shifted relative to the PB by one column, i.e., 45° of azimuthal space, thus separating right and left cells originating in corresponding PB columns by 90° in the FB.

The third class of FB cells are interneurons which input and output within the regions of the FB. There are 2 types: FB intrinsic neurons; FB mixed arborisation neurons with additional output branches outside the CX and sometimes input fibers in the PB.

A key feature of the adult FB is strong innervation by Mushroom Body Output Neurons (MBONs) (?). In addition, the axons of dopaminergic neurons driven by visual inputs innervate the FB (?).

In the larva, we found a number of putative FB horizontal/tangential cells originating in lineages known to contribute neurons to the adult FB. Characteristically, most present a bilateral axon closely wrapping around the midline, and an ipsilateral dendrite positioned within the superior dorsal protocerebrum (dorsal anterior neuropil) where they integrate numerous inputs from MBON axons. Among the various neurons with dendrites within this very medial neuropil, we find neurons from lineages known to contribute to the adult FB and whose axons project to the putative larval NO, EB, PB and LAL.

3.5 Noduli (NO)

The noduli are small, bilaterally symmetric spherical neuropils located medially and ventrally to the FB. In the adult *Drosophila* brain, each hemilateral neuropil is divided in 3 subunits: nodulus 1, 2 and 3 (NO1, NO2, NO3), with NO1 having the highest synaptic density of the three. There are notable variations across insect species, with the number of noduli ranging from two to four per brain hemisphere. While the stacked noduli subunits have been referred to as horizontal layers, no vertical subdivisions have been reported for these structures. Therefore there isn't any columnar organisation known.

The NO neurons present a unique morphology featuring compact, clutchy axons, which set them apart from other CX neurons (?) (?) and greatly ease their identification even in the absence of the typical conspicuous anatomical neuropil region present in adult insects. In the adult fruit fly, these neurons primarily originate in the DM1, DM2 and DM3 lineages (?).

At the larval stage of this animal, we found a set of neurons with highly compact, clutchy axons situated in the posterior ventral area of the brain, coming from lineages DM1 and DM3, as well as a few other larval lineages, and postulate this as the putative Noduli of the *Drosophila* larva.

In the adult *Drosophila* brain, the NO is interconnected with the EB and the FB, to which they relay information from tangential input neurons via several PB columnar cells such as PEN-neurons(PB-EB-NO; from the Head Direction System) and PFN-neurons(PB-FB-NO) (??). The primary NO inputs outside of the CX are from the LAL, these are known as LNO neurons and are suggested to be inhibitory (??). LNOs send inputs to and receive feedback from columnar neurons. FB tangential neurons make weak reciprocal connections to LNOs and columnar neurons in the NO. NO is synaptically interconnected with the other CX neuropils. All columnar neurons (PFNs and PENS) that synapse onto NO (are NO.b) are recurrently connected to the same LNO neurons they receive input from.

In the putative larval NO, we find that recurrent connections exist, nevertheless, they are axo-axonic. Most NO neurons output to second order mushroom body output neurons (MB2ONs), convergence neurons (10 pairs) as well as other MB related neurons. The NO receives mostly MBONs input in from multiple compartments

In the adult Drosophila, the NO receives optic flow-based self-motion information and wind direction information via the columnar neurons.

In Drosophila larva, we found a set of neurons with highly compact, clutched axons situated in the posterior ventral area of the brain - similarly to the adult NO - coming from lineages DM1 and DM3, as well as a few other larval lineages. We observe that these neurons are highly interconnected with the PB and FB, with strong inputs from PB and strong outputs to FB, and many of these neurons receive inputs in the LAL. Their highly distinctive morphology, location as well as similarities in connectivity to the adult noduli, make these neurons an excellent candidate for the putative larval noduli.

3.6 The new LAL

3.7 MB to CX

3.8 CX into CNs and DNs

4 Discussion

In the adult fruit fly, the PB is strongly associated with the formation of celestial maps from polarized light (??). Directional clues encoded in the ellipsoid body (EB) are maintained in the PB and further represented in further CX neuropils.

5 The PB is critical to the formation of internal compass based on an internal representation of a map of the azimuth (?).

The PB is thus a major area of the CX for visual processing, and correspondingly integrates strong visual excitatory inputs. In addition to visual inputs, the adult PB also integrates olfactory input (?), suggesting a more general role in the internal representation of sensory space.

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The neuropils of the putative larval CX present a connectivity pattern (FB-LAL, FB-NO, PB-FB, PB-NO, high levels of MBONs inputs onto FB) similar to the adult, with some neuropils showing strikingly similar relative spatial position and neuron morphology, such as the PB and the NO, respectively (Figure ??). The lineages that generate the neurons of the larval CX neuropils mostly overlap with the lineages for the adult CX. Like the adult FB, the larval FB integrates many inputs from the centre for learning and memory, the mushroom

body, via MBONs. The pattern of sensory input integration in larval CX neuropils is similar to that of the adult, as per Table ?? and the neuropils interconnect in a similar fashion (FB-LAL, FB-NO, PB-FB, PB-NO), as shown in Table ?? with some neuropils showing strikingly similar relative spatial position and neuron morphology.

The circuits of the putative larval CX are mostly downstream of the Mushroom Body – a sensory convergence unit - thus, given that inputs are integrated upstream of the decision to act on them, the larval CX likely acts as a navigational decision center responsible for the relative movement of the this animal.