Connectomic analysis of the central complex in fruit fly larvae

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I would like to dedicate this thesis to my loving parents …

Declaration

I hereby declare that except where specific reference is made to the work of others, the contents of this dissertation are original and have not been submitted in whole or in part for consideration for any other degree or qualification in this, or any other university. This dissertation is my own work and contains nothing which is the outcome of work done in collaboration with others, except as specified in the text and Acknowledgements. This dissertation contains fewer than 65,000 words including appendices, bibliography, footnotes, tables and equations and has fewer than 150 figures.

Laura Lungu
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Abstract

Abstract

In holometabolous insects such as the fruit fly *Drosophila melanogaster*, the brain central complex (CX) develops 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 analyzed the connectome of the larval brain and, on the basis of neuronal lineages, synaptic connectivity patterns, and anatomy, identified a putative larval CX, comprising 4 key neuropils: the protocerebral bridge (PB), the ellipsoid 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) visual input into the PB and EB; (ii) modulation of CX neuropil inputs by the mushroom body (MB); (iii) reciprocal connectivity between CX neuropils and select MB compartments; and (iv) strong connectivity between CX neuropils. While some neuronal lineages contributing to the larval CX do not contribute to the adult CX, many others are conserved. The characterization of a larval CX brings structure to largely unexamined larval brain circuits, linking with a vast body of literature, and will inform the design of experiments to probe larval brain function.

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Chapter 1

Introduction

1.1 Mapping brains - The Power of Connectomics

- how it helps us unravel fundamental problems about the brain

1.2 The advantage of an insect brain

tiny

1.3 Multisensory integration

Interpreting sensory inputs requires dynamic transformations, the brain must integrate sensory cues into one coherent representation that maximizes sensitivity and reduces ambiguity, before turning it into action via activation of specific muscles. This process is esential for maintaining goal-oriented behavioural programs over long timescales, which require the brain to ignore transient sensory distractions and compensate for fluctuation in the quality of information or its temporal availability.

Representations that persist in absence of sensory input rely on attractor dynamic generated by recurral neural circuits in the deep brain regions rather than at the sensory/motor periphery. Deep-brain circuits' inputs and outputs are usually difficult to identify and characterize, especially those involved in flexible navigation who's circuits have large populations of neurons. The deep-layer connectivity is difficult to determine in large brain animals, such as mammals. Insects are better suited for this purpose, as they have small brains and identified neurons, providing the opportunity to obtain a detailed understanding of their circuits and how they generate behaviour.

1.4 Navigation as a mode to understand multisensory integration

Insects maintain a specific pattern of action selection over many minutes and even hours during behaviors like foraging or migration, and maintain a prolonged state of inaction during quiet wakefulness or sleep (Hendricks et al., 2000; Shaw et al., 2000). Both types of behaviors are initiated and modulated based on environmental conditions (for example, humidity, heat, and the availability of food, nutritive state, hunger,hydration etc) and an insect's internal needs (for example, sleep drive and nutritive state; Griffith, 2013). The context-dependent initiation and control of many such behaviors is thought to depend on a conserved insect brain region called the central complex (CX)

1.5 The Central Complex

1.5.1 Central Comple across insects

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.

1.5.2 Evolutionary origins of Central Complex

Ancestrally, the central complex arises during embryogenesis in hemimetabolous insects - insects that undergo 'incomplete' metamorphosis, whose nymph stages transition slowly over multiple steps into the adult shape, with their brain developing gradually(e.g. beetle or ciccada). In later derived holometabolous insects - those that compress all nymph stages into a sessile stage, the pupa OR insects that undergo 'complete metamorphosis', and have distinct larval, pupal and adult stages designed to restrict developmental resources to those needed for growth - the central complex arises during postembryonic stagese (e.g. flies,moths, bee).

1.5.3 Central Complex in the Drosophila Adult

In Drosophila, the adult central complex starts forming during late larval and metamorphic stages, by the proliferation of pioneer undifferentiated neurons that remain quiescent until the pupal stage. This is in contrast to other holometabolous insects such as the tribolium (Farnworth et al., 2020) whose upper part begins forming during embryogenesis. What is special about the holometabolous insects such as drosophila is the extremely arrested brain development during larval stages the brain development during larval stages (Andrade et al, 2019), when they're essentially free-living, feeding embryos(Truman et al., 1999). The rough connectivity patterns of the central complex neuropils are relatively well known, but what types of navigation require the central complex, whether most central complex neurons are multisensory, or how sensory information from different modalities is organized within the central complex is still not well understood. As stated by Currier et al. in 2020, in-depth studies combining single-cell manipulations of neural activity of gene expression, reconstructed electron microscopy circuits and behavioural assays should be used to better understand the wiring diagram responsible for multisensory interaction in the central complex.

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.

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) [22] and, as of recently, the Assymetrical Body (AB) [83]. The Lateral Accessory Lobe (LAL) reciprocally interconnects with these, making it an important accesory structure and reference point.

1.5.4 PB,EB,FB,NO

1.6 Larval CX as a research opportunity

-developental neuronal diversity (the 3 types)

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 reocgnizable 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 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 [2]; 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 [3], mushroom body [12], and lateral accessory lobe [24]. 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 [76]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.

1.7 What this thesis does

This thesis aims to identify the putative central complex neuropils of the Drosophila larva, characterize their connectivity, neurotransmitter identity, and functional role in behavior, and place these findings in the context of central complex evolution and developmental diversity. To achieve this, I combined connectomic reconstructions, genetic tools, functional imaging, and behavioral assays. The thesis is structured as follows: Chapter 2 describes the methods… Chapter 3 presents results… Chapter 4 discusses…”

Chapter 2

Methods

2.1 Electron Microscopy Reconstructions

2.1.1 Seymour volume

2.1.2 Connectome data

The connectome of a 2-hour old first instar larval brain was used, as reconstructed previously by us [81]. The neuronal reconstructions, synapse labels, and neuron annotations, together with the electron microscopy volume, is available at the [VirtualFlyBrain]

2.1.3 Neurotransmitter Volumes

GABA

Acetylcholine

2.2 Connectomics

2.2.1 Sensory Iformation Flow

2.2.2 Lineage Matching

2.3 Finding Dopaminergic Neurons

2.3.1 Multicolor Stochastic Labelling via FLP-out

We were interested if any of the identified central complex neurons are dopaminergic. To verify that, we used the GAL4/UAS system to tag all the dopaminergic neurons(DANs) in the brain, and focused on those located outside the Mushroom Body (non-MB DANs).

We used the MCFO approach to label dopaminergic neurons inside the brain of *drosophila* larva. To do this we used the TH-GAL4 driver line, which expresses GAL4 in dopaminergic neurons. The GAL4 gene is inserted under the control of the tyrosine hydroxylase (TH) promoter, which is specific to DANs, so it's expressed in TH+ (dopaminergic) neurons.

To achieve stochastic multicolor labeling of individual neurons, we used the Multicolor FlpOut (MCFO) system, which relies on FLP recombinase-mediated excision of FRT-flanked transcriptional stop cassettes placed upstream of multiple fluorescent reporter genes. FLP recombinase expression was driven under UAS control and activated by tissue-specific GAL4 drivers. Upon FLP expression, recombination at FRT sites excises stop cassettes in a random subset of cells, allowing expression of distinct fluorescent proteins from a single transgenic construct. This results in combinatorial multicolor labeling of individual cells, enabling detailed morphological analysis. Our line had three MCFO reporters(smGFPs) HA, FLAG, V5.

2.3.2 Fly Strains

We used TH-GAL4, a line that tags all the dopaminergic neurons in the Central nervous system of the larva, and crossed it with tsh-gal 80 to suppress expression in the VNC.

The following driver line was used: R57C10-FlpL in su(Hw)attP8;;pJFRC210-10XUAS-FRT>STOP>FRT-myr::smGFP-OLLAS inattP2.pJFRC210-10XUAS-FRT>STOP>FRT……

The following effector line was used: w;tsh-Gal80/cyo.Tb.RFP; TH-Gal4 were crossed and the desired selected progeny was the following: R57C10-FlpL / + ; tsh-Gal80 / CyO.Tb.RFP ; TH-Gal4 / UAS-smGFP

To select for larvae that express GLA4 only in the brain, only larvae that had red fluorescent bodies were selected for dissection, as this indicates the presence of tsh-GAL80.

2.3.3 Immunohistochemistry

For Immunohistochemistry, we adapted the protocol from HHMI Janelia Research Campus, in combination with the protocol from [48]. The following primary antibodies were used: **Mouse α-Neuroglian; Rabbit α-HA Tag; Rat α-FLAG Tag** (Sigma). The following secondary antibodies were used: AF488 Donkey **α-Mouse; DL549 Goat α-Rabbit (Sigma)**. The following conjugated antibody was used: AF647 **Mouse α-V5**

Optimization of the protocol required extensive iteration over many months, during which multiple combinations of primary, secondary, and conjugated antibodies were systematically tested. The aim was to obtain strong, specific signals in all imaging channels without cross-reactivity or bleed-through. The final antibody set reported here represents the outcome of this process and was selected because it consistently provided clear labeling across epitopes, enabling reliable multichannel visualization.

The larval central nervous system (CNS) was dissected in cold 1× phosphate-buffered saline (PBS). The tissue was then transferred into 2 mL Protein LoBind tubes containing cold 4% paraformaldehyde (PFA) in 1× PBS, and incubated for 1 h at room temperature (RT) while nutating. The PFA was then removed and tissues were washed in 1.75 mL of 1% PBT (PBS with 0.3% Triton X-100) four times for 15 min each with nutation. Samples were then blocked with 5% Normal Donkey Serum (NDS Jackson Immuno Research; prepared as 95 μL PBT + 5 μL NDS), and incubated for 2 h at RT on a rotator with tubes upright. Primary antibody incubation was carried out in 1% PBT (typically 100 μL per tube) for 4 h at RT, followed by two consecutive overnights at 4°C with continuous rotation. After primary incubation, tissues were washed four times in 1.75 mL of 1% PBT for 15 min each.

Secondary antibody incubation was performed in 1% PBT (100 μL per tube) for 4 h at RT, followed by 1–2 overnights at 4°C with continuous rotation. Post-secondary washes were performed four times in 1.75 mL of 1% PBT for 15 min each. An additional blocking step with 5% Normal Mouse Serum (NMS; Jackson ImmunoResearch, #015-000-120) in PBT was carried out for 1.5 h at RT prior to overnight incubation with at 4°C with the conjugated antibody. Following incubation, samples were washed four times in 1.75 mL of 1% PBT for 15 min each.

For mounting, tissues were placed on poly-L-lysine (PLL)-coated coverslips. Samples were dehydrated through a graded ethanol series (30%, 50%, 75%, 95%, and three changes of 100%), soaking for 10 min at each step. Tissues were cleared by immersion in three sequential 5 min xylene baths. Finally, samples were embedded by applying 4–5 (80 μl) drops of dibutyl phthalate in xylene (DPX) to the mounted tissue, placing the coverslip (DPX side down) onto a prepared slide with spacers, and applying gentle pressure to seat the coverslip. Slides were left to dry in the hood for 1–2 days prior to imaging.

2.3.4 Imaging

For imaging, the Zeiss LSM 780 was used.

2.3.5 Matching LM images with Seymour Data

2.4 Light Sheet Microscopy and Ca Imaging

2.4.1 Fly lines

2.4.2 Sample Preparation

Fly larvae were raised on standard cornmeal-based food. Second instar larvae were selected for live imaging.Individual larvae were dissected in physiological saline. After being pinned dorsal side up in Sylgard-lined Petri dishes, a dorsal incision was made along the larval body with fine scissors. The body wall was pinned flat and internal organs were removed. The isolated Drosophila CNS was then dissected away, preserving the Rh5 photoreceptors which expressed fluorescence. Only CNS samples that expressed irfp were selected.The samples were then embedded in 1% low-melting temperature agarose in physiological saline at 36 °C. The agarose containing the CNS was drawn into a glass capillary with 1.4 mm inner diameter and 2.0 mm outer diameter, where the agarose quickly cooled to room temperature, forming a soft gel. The agarose cylinder was extruded from the capillary so that the CNS was optically accessible outside of the glass.

2.4.3 LSM and Functional Imaging

The SiMView software was used to locate the brain. There are 2 views, one from the back and from the front. The view was set to an angle that is as flat and central as possible. Imaging is with green (561nm) exciting jRGECO(present in the cytosol) which is the Ca reporter expressed pan neuronally, and with red is the IRFP(expressd in the nuclei) brightens the cell nuclei very well which allows you to see the cell position.

2.4.4 Data Analysis

2.5 Behavioural Assays

2.5.1 Fly strains

9 split GAL4 lines were used and crossed with either UAS-TNT and UAS-impTNT effector lines. The cross was set at 25°C on fly food for 3 days. Larvae containing the UAS-TNT or UAS-impTNT transgene were raised at 18°C for 7 days with normal cornmeal food. Foraging third instar larvae were used for all experiments.

2.5.2 Behavioural Experiments

9 split GAL4 lines were used and crossed with either UAS-TNT (effector) or UAS-impTNT (control) genetic driver lines lines. The cross was set at 25^{o}C and the flies were laid on fly food for 3 days. Larvae containing the UAS-TNT or UAS-impTNT transgene were raised at 18^{o}C for 7 days with normal cornmeal food. Third instar larvae were used for all experiments.

Larvae were separated from food by using 15% sucrose and washed with water, then dried and placed in the center of the arena, consisting in 3% Bacto agar gel in a 25 × 25 cm square plastic plate. Experiments were conducted at 25^{o}C. Larvae were monitored with the Multi-Worm Tracker (MWT) software (http://sourceforge.net/projects/mwt); [51].

For light-stimulation experiments, we used approximately 30 larvae for each run. The larvae were presented with green light for 40 seconds, and the amount of larvae turning was monitored before, during and after stimulus presentation. 6 runs were performed for every line.

2.5.3 Behavioural Quantification

Larvae were tracked in real-time using the MWT software. We rejected objects that were tracked for less than 5 seconds or moved less than one body length of the larva. For each larva MWT returns a contour, spine and centre of mass as a function of time. Raw videos are never stored. From the MWT tracking data we computed the key parameters of larval motion, using specific choreography (part of the MWT software package) variables28. From the tracking data, we detected and quantified crawling and rolling events and the speed of peristaltic crawling strides.

2.5.4 Behavioural Data Analysis

Chapter 3

Results

3.1 Connectomics

3.1.1 The Central Complex Adult vs Larva

Protocerebral Bridge

In the adult *Drosophila*, the PB comprises two sets of bilaterally symmetric compartments, sometimes referred to as glomeruli 1–9 [31], positioned at the most posterior-dorsal location possible in the brain.

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; [82]) 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 [82]; and the horizontal neurons (also known as horizontal fibers) derived from a single lineage (PBp1; [2]) 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 ([27]). Then the PB relays this information to the EB compartments.

In addition to visual input, the adult PB also integrates olfactory inputs [31], 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; [77]).

Among neurons of the DALv1 lineagel, 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 ([**winding2023**]) 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 ([31]). 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; [**eschbach2021**]) 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 [**EsbachFushiki2021**]). 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 [**wolff2015neuroarchitecture; wolff2018; hulse2021connectome**]. Beyond the canonical columnar neurons projecting to other Central Complex neuropils, we found some whose axons descend to the SEZ or nerve cord (**??**).

Ellipsoid Body

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) [52].

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 [31]. 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.

Fan-Shaped Body

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 [25]. As one of the biggest CX neuropils, a large variety of lineages contribute to the FB. 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 the adult FB is strong innervation by Mushroom Body Output Neurons (MBONs) [**MISSING**]. In addition, the axons of dopaminergic neurons driven by visual inputs innervate the FB [43].

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.

Noduli

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 [83] [31] 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 [2].

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) [82, 31]. The primary NO inputs outside of the CX are from the LAL, these are known as LNO neurons and are suggested to be inhibitory [83, 31]. 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 the neurons projecting onto this neuropil receive input from LAL, (LAL.d MB2ON-75)

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, clutchy 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.

3.2 Neurotransmitter Identity of Central Complex Neurons

DANs

GABA and Acetylcholine

3.3 Functional Connectivity via Inhibition of CX neurons

NBLAST genetic lines for CX neurons

Behavioural Assays

Chapter 4

Discussion

4.1

[**exampleReference**]

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Appendix A

Appendix title example

example

example

Appendix B

example2