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 60,000 words including appendices, bibliography, footnotes, tables and equations and has fewer than 150 figures.

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Laura Lungu
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Acknowledgements

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

Investigating the origins of human behaviour and mapping it to onto the brain is a mission as old natural philosophy. Hippocrates was the first to identify the brain as the 'analyst of the outside world', the interpreter of consciousness and the center of intelligence and willpower [**breitenfeld2014hippocrates**], and to this day he is considered the forefather of neurology.

At its heart, neuroscience has always been about reverse engineering human behaviour. We attempt to understand the full functionality of the brain from its underlying anatomical features - molecular and cellular - its activity and its circuits. Ultimately, the goal is to infer causality about behaviour using the best tools we have at our disposal [**mckinstry2013connectome**].

Detailed maps of synaptic connectivity (also known as wiring diagrams) are a core to our understanding of the fundamental link between brain and behaviour and, crucially, how malfunctions of it can result in behavioral or neurological disorders. Whilst large-scale circuit reconstructions aren't yet possible with state-of-the-art imaging technology - the human brain has 86 billion cells[**herculano2009human**], with tens of thousands of synapses each, and would take centuries to map - advances in tiny brains mapping are pushing the brain sciences by unraveling causal and correlative relations between structure and function and paving the way to establishing fundamental principles of how neural systems operate.

Connnectomics - the study of complete sets of connections in individual neural systems - is at large responsible for a lot of these advancements. It allowed us to create roadmaps for brain of various animals such as the C. Elegans - the first landmark study of a connectome [**brenner1974genetics**] -, the mouse visual cortex [**microns2025functional**], the *Drosophila* adult [**scheffer2020connectome**] as well as the *Drosophila* larva[**ohyama2015multilevel**, **berck2016wiring**, **larderet2017opticlobe**, **eichler2017complete**, **eschbach2021circuits**, **eschbach2020recurrent**]. All these datasets were obtained via a technique known as electron

Organism	Tissue / region	Neurons	Imaging method	Project / Reference
C. elegans	Entire CNS	302	ssTEM	White et al. (1986); Witvliet et al. (2021, *Nature*)
C. intestinalis (larva)	Entire CNS	177	ssTEM	Ryan et al. (2016, *eLife* 16962)
P. dumerilii (larva)	Entire CNS	9 162	ssTEM	Verasztó et al. (2024, *eLife* 97964)
D. melanogaster (larva)	Entire CNS	∼9 700	ssTEM	Winding et al. (2023, *Science*)
D. melanogaster (adult)	Entire brain	∼135 000	ssTEM	Zheng et al. (2018, *eLife* 16962; *Cell*)
M. musculus	Visual cortex (VISp + HVAs)	∼100 M (per mm^3)	SBF/MB-EM	MICRONS v3 (*Nature*, 2025)

Table 1.1 **Fully-mapped Connectomic Datasets** Comparison of major connectomic datasets across species, from *C. elegans* to mouse visual cortex. All values are directly reported in the cited publications; no extrapolated voxel or volume data are included. Abbreviations: ssTEM, serial-section transmission EM; SBF/MB-EM, serial block-face or multi-beam EM.

microscopy (EM, ssEM, TEM, FIB-SEM) and have become foundational references in neuroscience, ushering in the long-anticipated era of synaptic wiring diagrams (Table 1.1).

EM is, to this day, the highest-resolution technology for brain mapping, allowing us to visualize the smallest neurites (as small as 15 nm; [**meinertzhagen2016connectome**]) and all the synaptic connections—vesicles and clefts (40 nm and 20 nm, respectively)—between them. The great advantage of tiny animal models such as *Drosophila* is that their physical dimensions (600 μm or 0.15 mm^3 for the adult fly brain; 242 μm or 0.001 mm^3 for the larval nervous system) can be reconstructed with synaptic resolution within a manageable timeframe and cost. Their dimensions - no neuropil greatly exceeds 50 μm in depth [**meinertzhagen2016connectome**] — also facilitate the use of multimodal imaging approaches: confocal microscopy for molecular and genetic labeling, light-sheet microscopy for rapid volumetric imaging (sometimes paired with calcium activity), and electron microscopy for ultrastructural detail—on the same specimen or across comparable samples.

The female adult vinegar fly *Drosophila Melanogaster* is one of the largest pieces of nervous tissue collected and imaged by EM (i.e., FAFB; [**zheng2018complete**]). The dataset encompasses 40 teravoxels and 21 million serial section transmission EM images, covering a 995 × 537 × 283 μm volume at 4 × 4 × 40 nm resolution. Complementary to this, a complete EM reconstruction of the *Drosophila* larva central nervous system ([**winding2023connectome**]) spans the entire brain and ventral nerve cord within a roughly 242 μm volume, capturing approximately 9,700 neurons at 3.8 × 3.8 × 50 nm resolution. I have contributed to mapping the larval fruit fly, specifically, in unraveling a new area responsible for navigational decisions. This area is known as the Central Complex in the adult, and, as I will go on to show, I postulate that an analogous, numerically smaller Central Complex exits at the larval stage of development.

Together, these datasets bridge developmental stages of the fly nervous system, enabling both sparse and dense connectomic analyses that inform a growing range of hypotheses

around their relevance to function and behaviour. Collectively, connectomic datasets provide a structural framework through which brain function can be interpreted. They transform anatomy into networks and reveal not only which neurons are connected but also the direction of information flow. This is the exact type of data that is translatable into connectivity matrices — quantitative maps of presynaptic and postsynaptic partnerships— that we can therefore use we can infer circuit logic and predict functional pathways to expose hidden motifs, highlight convergence or divergence across modalities, and delineates subnetworks anatomical areas that underlie specific behaviours. Using *Drosophila* is powerful approach to functionally study these detailed wiring diagram, because it is one of the most experimentally accessible animal model.

1.2   The advantage of the *Drosophila* larva brain

Historically, for connectomes of larger insects of vertebrates, connectomes of isolated areas have been studied and made sense of. However, brain regions don't operate in isolation and connectivity inside the whole brain spans multiple dispersed areas, neurons can converge or diverge towards each other, and understanding any single computation requires understanding its inputs, outputs and relation to other regions of the brain [**huang2020bricseq**]. Functional (Ca activity) studies confirm this to be the case in Drosophila [**lemon2015whole**]as well as in vertebrates [**ahrens2013whole**].

A smaller circuit is therefore advantageous for its accessibility to assess dispersed neural computations. With its nervous system comprising 12 000 neurons and only 2,500 of these inside the brain - all of which have been mapped with synaptic resolution using whole-CNS EM, and its brain networks mostly reconstructed - the fruit fly larva is especially well suited for brain-wide circuit studies. This animal's circuit architecture is stable across larval stages (1st, 2nd and 3rd instar) with neurons only growing in size but not changing synaptic partners [**gerhard2017conserved**]. In addition, the larva vinegar fly has rich but numerically limited adaptive behaviour that is equivalent from 1st to 3rd instar [**almeida2017ol1mpiad**].This means that behaviour observed in later stages(which can be a favourable choice as they are more easily identified by cameras and generally easier to work with) can be mapped onto our circuits from the 1st instar.. Brain structures homologous to adult and other insect species ([**eichler2017complete**, **truman2023metamorphosis**, **carreira2018mdn**])

Not only does the larval fly have a full synaptic-resolution connectome, this animal has a vast library of genetic driver lines (GAL4, LexA) for targeted gene expression in individual neurons, as well as effector line (UAS, LexOp), which allows for controlled expression of

proteins that allow (e.g.) optogenetic manipulation via CsChrimson [**kim2015optogenetics**] or monitoring neural activity via GCaMP7 [**owald2015light**].

Our ability to genetically manipulate protein expression in individual neurons and record their activity in freely behaving larvae, as well as quantify simple output behaviours [**vogelstein2014discovery**], link them directly to their circuit and infer from adult structures, makes it a very tractable option to identify specialised, functionally distinct natural networks from our reconstructed larval circuits.

1.3 Navigation: Fundamental Across Species

When one wants to inspect connectivity maps to understand universal principles of neural computations, one must start with the fundamentals and ask themselves: what are some aspects of the brain that are universally true across species?

Certainly what is true for all species is they need to find resources, shelter and avoid threats by navigating their environment. Navigation is therefore a fundamental and crucial function of biological systems across the board.

Navigation is achieved via multisensory integration, a process wherein sensory cues are integrated into one coherent neural representation, to maximize sensitivity and reduce ambiguity. Interpreting sensory inputs requires dynamic transformations that allow the brain to ignore transient sensory distractions and compensate for fluctuation in the quality of information or its temporal availability. Once sensory inputs are processed, they're followed by action selection via activation of specific muscles.

During navigation, the brain is able to retain a model of the world (i.e. neural representations of the external world persist) in absence of sensory input. This relies on attractor dynamics generated by referral 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 whose circuits have large populations of neurons. Importantly, deep-layer connectivity is nearly impossible to determine in large brain animals, such as mammals. For this reason, smaller animals, such as insects are best suited for such studies, with their numerically smaller, tractable neural circuits, they provide the opportunity to obtain a detailed understanding of deep-layer computations underlying navigation.

Insects are expert navigators. They 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). These 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 (such as sleep drive and nutritive state; Griffith, 2013). The context-dependent initiation and control of many such behaviors has been shown to depend on a conserved insect brain region called the central complex (also known as CX)

1.4   The Central Complex

Parts of this section incorporate text from published work [The central complex of the larval fruit fly brain] (Lungu et al., 2025)

1.4.1   Central Complex across insects

The Central Complex (CX) is a conserved set of neuropils found across arthropods (bumblebee, the red flour beetle, Monarch butterfly) that acts as a sensory integration area and a center for coordinated motor activity [**PfeifferHomberg2014**, **turnerevans2016CX**, **heinze2024variations**].

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). Later derived holometabolous insects - those that compress all nymph stages into a sessile stage(the pupa) - undergo 'complete metamorphosis', and have distinct larval, pupal and adult stages designed to restrict developmental resources to those needed for growth. In this case, the central complex arises during postembryonic stagese (e.g. flies, moths, bee).

This brain region originated more than 400 million years ago and has remained highly conserved across insect species, comprising 4 neuropils: the protocerebral bridge (PB), the ellipsoid body (EB; or central body lower unit/CBL), the fan-shaped body (FB; or central body upper unit), and a pair of noduli (NO). The CX is known for its stereotypical regular neuroarchitecture composed of vertical columns(columnar neurons) and horizontal layers (tangential neurons). Columnar neurons link the PB with the other CX neuropils (EB,FB,NO) forming columnar compartments in each of them. Tangential cells input horizontally across the entire surface of a neuropil, and intersect with culumnar neurons [**honkanen2019insect**].

1.4.2   Central Complex of Adult *Drosophila*

In the fruit fly, the central complex starts forming during late larval and metamorphic stages, via 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 [**farnworth2022atlas**] whose CX cells begin arborizing during embryogenesis.

In *Drosophila melanogaster*, the functions of the CX include multi-sensory navigation, path integration, place learning, allocentric orientation of the head relative to its body, sleep regulation, and providing an internal sense of direction in the absence of stimuli, among others [**hanesch1989neuronal**, **ofstad2011visual**, **seelig2013feature**, **PfeifferHomberg2014**, **Stone2017CXModel**, **franconville2018building**, **heinze2018principles**, **szuperak2018sleep**, **pisokas2020head**, **ShaferKeene2021sleep**, **fisher2022flexible**].

At the adult stage, the fruit fly CX has four well-studied neuropils: the Protocerebral Bridge (PB), the Fan-shaped Body (FB), the Ellipsoid body (EB) and the Noduli (NO) [**hanesch1989neuronal**] plus, as of recently, the Asymmetrical Body (AB) [**wolff2018neuroarchitecture**], in addition to several smaller accessory neuropils often referred to as the lateral complex: the gall (GA), the lateral accessory lobe (LAL) - which is interconnected with all other neuropils - and the bulbs (BU) [**wolff2015neuroarchitecture**, **franconville2018building**, **hulse2021connectome**].

The Central Complex neuropils originate primarily from DM lineages (DM1-DM6) in addition to neuropil specific ones (Table 1.2) [**yang2013diverse**, **andrade2019developmentally**]. The DM1–DM4 lineages generate the small-field columnar neurons that innervate all the four major CX neuropil (PB, FB, EB, NO) in highly ordered isomorphic patterns to establish projections across different CX compartments. Other lineages are more specialised, such as DM5 which exclusively innervates the PB, or DL1 which only innervates the FB.

Protocerebral Bridge (PB)

In the adult *Drosophila*, the PB comprises two sets of bilaterally symmetric compartments, sometimes referred to as glomeruli 1–9 [**hulse2021connectome**], 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, organized into hundreds of types (194 [**wolff2015neuroarchitecture**], see Table 1.3) 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 [**wolff2015neuroarchitecture**]; and the horizontal neurons (also known as horizontal fibers) derived from a single lineage (PBp1; [**andrade2019developmentally**]) 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 ([**heinze2009transformation**]). Then the PB relays this information to the EB compartments (Fig.

In addition to visual input, the adult PB also integrates olfactory inputs [**hulse2021connectome**], suggesting that spatial navigation is not unimodal but integrative across multiple sensory modalities.

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. The EB is made of two main types of neurons: Ring-neurons (RNs; 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 (EPGs, derived from the DM4 lineage) that divide the EB into 16 compartments (the wedges) [**wolff2015neuroarchitecture**]. The EPG (Wedge) neurons are excitatory and synapse with each other, as well as reciprocally with the Ring neurons, which are inhibitory [**franconville2018building**, **hulse2021connectome**].

The adult EB circuit has been modeled as a ring attractor [**Stone2017CXModel**] to, in concordance with its anatomy, reproduce *in silico* the observed "bump" of neural activity in the form of a sole active wedge in the *Drosophila* adult EB [**seelig2015neural**].

EPG (Wedge) neurons form 16 wedges around this ring, and project to both hemispheres of the PB, where they connect to two sets of columnar neurons (PEG and PEN) that project back to the EB, forming recurrent loops. 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 [**Stone2017CXModel**].

In the adult, the EB receives visual inhibitory GABAergic inputs, via two parallel pathways for distinct visual information: 1. Ring neurons that map the visual environment of the fly; 2. tangential neurons that take in information about body rotations and translational velocity. The latter receive input in the LAL and output to the 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 [**hulse2021connectome**]. It receives strong inputs from PB, NO and the LAL, and outputs onto the PB.

Fan-Shaped Body (FB)

The adult FB is a bilaterally symmetric neuropil posterior and dorsal to the EB, with well-defined horizontal and vertical components: 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 [**heinze2017unraveling**]. As the biggest CX neuropils, a large variety of lineages contribute to the FB (see Table **??**). In the adult FB 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; [**hulse2021connectome**]); (2) neurons that relay sleep drive to the FB, whose activity is mandatory for sleep initiation [**ShaferKeene2021sleep**].

The adult FB columnar neurons, or columnar input cells, are known as PFN (PB-FB-NO; [**wolff2015neuroarchitecture**]), with dendrites in the PB and axonic outputs on both the FB and NO. There are 5 main types [**hulse2021connectome**], with their arbors tiling the PB glomeruli, the FB layers, and the NO layers.

The third class of adult FB cells are interneurons with dendrites and axons within the FB. Of these there are 2 main types: FB intrinsic neurons whose arbors lay entirely within the FB, and FB mixed arborisation neurons with additional axonic branches outside the CX and sometimes dendritic branches in the PB [**wolff2015neuroarchitecture**].

A key feature of the the adult FB is strong innervation by MB Output Neurons (MBONs) [**scheffer2020connectome**, **hulse2021connectome**]. In addition, the axons of dopaminergic neurons driven by visual inputs innervate the FB [**lin2013comprehensive**].

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 no columnar organization is known.

The NO neurons present a unique morphology featuring compact, clutchy axons, which set them apart from other CX neurons [**wolff2018neuroarchitecture**, **hulse2021connectome**] 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 [**andrade2019developmentally**].

In the adult *Drosophila* brain, the NO is interconnected with the EB and the FB, with the latter relaying information to the NO 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) [**wolff2015neuroarchitecture**, **hulse2021connectome**]. The primary NO inputs outside of the CX are from the LAL; these are known as LNO neurons and are suggested to be inhibitory [**wolff2018neuroarchitecture**, **hulse2021connectome**]. 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. All columnar neurons (PFNs and PENs) that synapse onto NO (are NO.b) are recurrently connected to the same LNO (LAL-NO) neurons they receive input from.

Neuromodulatory neurons in the CX

In the adult CX numerous neurons express neuromodulators and neuropeptides, and their receptors, particularly in the FB [**kahsai2011neuromodulators**, **kahsai2012distribution**]. These modulatory molecules have been associated with the overall level of motor activity and the regulation of sleep [**donlea2019sleep**], among other roles [**PfeifferHomberg2014**]. Here, we list the subset of neurons with assigned neuromodulators that are monosynaptically connected to CX neurons in the larval brain.

The sVUM2mx and sVUM2md are a pair of octopaminergic ventral unpaired medial (VUM) neurons with somas in the SEZ and bilaterally symmetric arbors, which innervate the larval optic neuropil (LON; [**larderet2017opticlobe**]). These octopaminergic neurons further project their axons into the LAL and also deliver axonic boutons to the EB.

Another octopaminergic VUM (a ladder neuron), named MB2IN-191 [**eschbach2020recurrent**], innervates the FB bilaterally but not exclusively, with its bilateral axon extending further into nearby medial and posterior areas of the brain.

Given the known role of octopamine in controlling sleep/wake in larvae [**szuperak2018sleep**] and the role of the adult CX in modulating motor activity levels and sleep [**PfeifferHomberg2014**, **ShaferKeene2021sleep**], these octopaminergic neurons should be tested experimentally for their potential in mediating a signal for sleep in larvae.

Furthermore, the pair of SP2-1 serotonergic neurons described for the optic lobes [**larderet2017opticlobe**] present ipsilateral dendrites that integrate inputs from multiple PB.b neurons and also FB.b (hs-FB.6), and then project their axons contralaterally, extending across the PB and into the optic lobe, dropping presynaptic connections onto PB.d neurons and then further posterior and lateral until reaching the LON. These neurons are ideally suited for relaying feedback signals onto the optic lobe to modulate the processing of incoming visual inputs as a function of PB and FB activity.

Another serotonergic neuron, CSD [**berck2016wiring**], that integrates inputs from olfactory sensory neurons (ORNs) and the superior lateral protocerebrum (an extended area around

the LH), and projects back to the antennal lobe (AL) to modulate olfactory LN function [**vogt2021internalstate**], integrates strong inputs from the FB intrinsic neuron MB2ON-125.

Taken together, via SP2-1 and CSD neurons the output of the FB may modulate with serotonin both the LON and the AL, presumably to provide context to first-order circuits for sensory processing (vision and olfaction), as has been reported for hunger versus satiation [**vogt2021internalstate**].

Mushroom Body and the CX

In the adult **Drosophila**, the Mushroom Body is known to output onto the Central Complex neuropils through the MB Output Neurons (MBONs). At this stage, adult MBONs primarily target the Fan-shaped Body - tangential neurons from the middle layers (4-6) - and the Noduli - via a direct glutamatergic connection from MBON-30 to LCNOp(LAL–NO) neurons which then target the PFN (PB-FB-NO) neurons [**hulse2021connectome**].

1.5   Larval Central Complex as a research opportunity

What is special about the holometabolous insects such as *Drosophila* is the extremely arrested brain development during larval stages [**andrade2019developmentally**]. This suggests that other larval neurons must take over essential navigational functions performed by the adult central complex, in the form of what we expect to be an earlier stage larval central complex.

In *Drosophila melanogaster*, the functions of the CX include multi-sensory navigation, path integration, place learning, allocentric orientation of the head relative to its body, sleep regulation, and providing an internal sense of direction in the absence of stimuli, among others [**hanesch1989neuronal**, **ofstad2011visual**, **seelig2013feature**, **PfeifferHomberg2014**, **Stone2017CXModel**, **franconville2018building**, **heinze2018principles**, **szuperak2018sleep**, **pisokas2020head**, **ShaferKeene2021sleep**, **fisher2022flexible**].

The fruit fly larva exhibits a range of behaviors that require spatial navigation, including chemotaxis for foraging and escape [**fishilevich2005chemotaxis**, **khurana2013olfactory**, **Ebrahim2015OR49**, **davies2015model**] and aversive phototaxis in response to blue light [**sawin1995phototaxis**, **gong2009phototaxis**, **KeeneSprecher2012photobehavior**]. In addition to responding to an isolated unimodal stimulus, the larva integrates competing stimuli prior to decision making for navigation [**gepner2015computations**]. Furthermore, larvae sleep, which is required for long-term memory [**poe2023sleepmemory**] just like in the adult fly [**donlea2011sleep**, **donlea2019sleep**], where sleep is regulated by the central complex [**ShaferKeene2021sleep**].

Anatomically, the larval central brain shares the set of neuroblasts with the adult [**lacin2016lineage**], and presents neuropil areas that directly correspond to those of the adult brain such as the Antennal Lobe (AL), the Mushroom Body (MB), and the Lateral Accessory Lobe (LAL), in addition to more larval-specific neuropils such as the larval optic neuropil (LON) [**pereanu2010neuropildev**]. The organization and synaptic connectivity of the larval AL, MB, and LON are well understood at present [**berck2016wiring**, **eichler2017complete**, **larderet2017opticlobe**].

Nevertheless, these neuropils constitute only ∼30% of the complete larval brain connectome by number of neurons [**winding2023connectome**]. We postulate that amongst the remaining ∼70% of brain neurons, and given the reported conservation of neuroblasts [**lacin2016lineage**] and neuronal identities from larval to adult [**truman2023metamorphosis**], and the larval behaviors in navigation and sleep that in the adult are associated with the CX, the question of whether the larval brain harbors a simpler yet homologous set of Central Complex neuropils must be examined. Furthermore, in other holometabolous insect orders, neuroblast proliferation arrests at a later point in the stereotyped sequence of neuron types [**truman1999origins**], generating more neurons embryonically and rendering the central complex identifiable at the larval stage, such as in the beetle *Tribolium castaneum* where the CX develops embryonically [**koniszewski2016TriboliumCX**, **farnworth2020TriboliumCXvsFly**]. In the fruit fly larva, hundreds of late embryonic-born neurons known to later on pioneer the development of the adult CX remain undifferentiated throughout larval life [**andrade2019developmentally**].

The larval brain, though, does not present fused brain hemispheres like in the adult. With adult CX neuropils being largely medial structures, any putative larval brain counterparts will differ significantly by morphology alone. The basis of our search for the larval CX has to rely on (1) neuronal lineages generated by the same neuroblasts; (2) the relative spatial location of neurons within the overall neuropil since these are conserved for neuropils known to be homologous such as the AL, MB, LON and LAL; (3) the synaptic circuit architecture organizing the adult central complex neuropils; and (4) the patterns of sensory inputs. For (1) we use embryonic-born neurons that remain identifiable yet undifferentiated throughout larval life, a subset of which will develop into the adult CX [**andrade2019developmentally**]. For (2), (3) and (4) we rely on the fully reconstructed neuronal arbors constituent of the complete connectome of the larval brain [**winding2023connectome**], together with prior publications on larval sensory systems [**mast2014pheromones**, **ohyama2015multilevel**, **berck2016wiring**, **schlegel2016synaptic**, **larderet2017opticlobe**, **miroschnikow2018convergence**, **huckesfeld2021unveilin hernandez2021thermo**]. We use all four in an iterative process to progressively discover the complete list of neurons composing the putative larval CX.

This thesis aims to identify the putative central complex neuropils of the Drosophila larva, characterize their connectivity pathways, 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 analytical and experimental methods used across the study, Chapter 3 presents the main results in the context of specific research questions, and Chapter 4 discusses the implications of these results for our understanding the structure and function of a central complex at an earlier developmental stage of a holometabolous insect.

Neuropil	Lineage
Protocerebral Bridge	DM1 (DPMm1)
	DM2 (DPMpm1)
	DM3 (DPMpm2)
	DM4 (CM4)
	DM5 (CM5)
	DM6 (CM3)
	PBp1 (-)
Ellipsoid Body	DM1 (DPMm1)
	DM2 (DPMpm1)
	DM3 (DPMpm2)
	DM4 (CM4)
	DM6 (CM3)
	EBa1 (DALv2)
	LALv1 (BAMv1)
Fan-Shaped Body	DM1 (DPMm1)
	DM2 (DPMpm1)
	DM3 (DPMpm2)
	DM4 (CM4)
	DM6 (CM3)
	DL1 (CP2)
	EBa1 (DALv2)
	LALv1 (BAMv1)
	AOTUv4
	CREa1 (BAMd1)
	CREa2 (DALCm1)
	SMPad2 (DAMd2/3)
	SIPp1 (DPMpl2)
Noduli	DM1 (DPMm1)
	DM2 (DPMpm1)
	DM3 (DPMpm2)
	DM4 (CM4)
	LALv1 (BAMv1)
	DM6 (CM6)
	SAMPad2 (DAMd2/3)

Table 1.2 *Drosophila* Adult Central Complex Lineags. Individual Central Complex neuropils and their contributing lineages in the nomenclature of ItoLee[**lai2008clonal**] and (Hertenstein)[**lovick2013postembryonic**]

Hulse et al Name	Wolff/Francoville Name
EB	
Ring Neurons	
ER 1–6	BU.s EB.b
Extrinsic Ring Neurons	
ExR1–8 (Extrinsic Ring)	NO/BU/LAL/GA.s EB.b
Wedge Neurons	
EPGt (EB–PB Columnar)	EB.s PB.b GA-t.b
EPG (EB–PB Columnar)	EB.s PB.b GA.b
EL (EB Columnar)	EB.s GAs.s.b
WL–L	Wedge–LAL.s.b
PB	
PEN (EB–PB Columnar)	PB.s EB.b NO.b
PEG (EB–PB Columnar)	PB.s EB.b GA.b
PFN (PB–FB Columnar)	PB.s FB.b NO.b
PFR (PB–FB Columnar)	PB.s FBd.b ROB.b
PFG (PB–FB Columnar)	PB.s FB.s.b GA.b
PFL1–3 (PB–FB Output)	PB.s FB.s LAL.b CRE.b
Delta 7 (PB Intrinsic)	PB.s.b
P–ECG	PB.s EB.b GA.b
P1–9 (Octopaminergic)	PB.s EB.b GA.b
LPsP (Dopaminergic)	PB.b LAL.s PS.s
Sps–P	PB.b SPS.s
IbSpsP	PB.b IB.s.SPS.s
P6–8P9 (intrinsic)	PB.s.b
FB	
vDelta (Intra–FB Columnar)	FB.s.b
hDelta (Intra–FB Columnar)	FB.s.b
FR (FX Columnar)	FB.s ROB.b
FS (FX Columnar)	FB.s SNP.b
FC (FX Columnar)	FB.s CRE.b
FB Tangential (FBt)	FB.s (..)
NO	
LNO–1 (LG–N/GLNO)	LAL.s GA.s NO.b
LNO–2 (LNA)	LAL.s NO.b
LNO–3 (LCNOp/LCNOp)	LAL.s CRE.s NO.b

Table 1.3 ***Drosophila* Adult Central Complex Neurons** This table contains CX neurons nomenclature according to Hulse et.al [**hulse2021connectome**] mapped onto Wolff et al[**wolff2018neuroarchitecture**] and Francoville [**franconville2018building**] nomenclatures which contain information about the location of input regions (.s for dendritic spines) and output regions (.b for axonal boutons)

Chapter 2

Methods

2.1 Reconstructing neurons in Electron Microscopy Volumes

2.1.1 Seymour volume

[**ohyama2015multilevel**]

2.1.2 Connectome data

The connectome of a 2-hour old first instar larval brain was used, as reconstructed previously by us [**winding2023connectome**]. 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 Information Flow

2.2.2   Lineage Matching

2.3   Identifying non-Mushroom Body 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 [**nern2015multicolor**]. 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 $1\times$ phosphate-buffered saline (PBS). The tissue was then transferred into 2 mL Protein LoBind tubes containing cold 4% paraformaldehyde (PFA) in $1\times$ 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   Ca^{2+} Imaging with Light Sheet Microscopy

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^oC and the flies were laid on fly food for 3 days. Larvae containing the UAS-TNT or UAS-impTNT transgene were raised at 18^oC 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^oC. Larvae were monitored with the Multi-Worm Tracker (MWT) software (http://sourceforge.net/projects/mwt); [**Ohyama2013PlosONE**].

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 Identification of Central Complex Neuropils

I used an iterative strategy in finding a putative larval central complex neurons. For this, I pattern matched individual cells based on adult CX neurons lineages, morphology, relative location and connectivity.

We expected to find neurons originating from DM1, DM2, DM3, DM4, DM5 and DM6 hemilineages as these are core lineages contributing to all neuropils(Table 1.2), with the second priority being neuropil specific lineages(e.g. EBa1(DALv2) for the EB see 1.2). 12 of the 15 lineages found to contribute CX neurons in the adult also exist in the larva. Of these, we found that 9 of them contribute neurons to the larval central complex (Table 3.1). We therefore used these 9 lineages as a starting point to find our putative CX neuropils in the larva. In addition to the lineages that contribute to the adult CX, there were several other lineages that sent neurons that satisfied other criteria (connectivity patterns and inputs our outputs) and were therefore classified as central complex neurons. These lineages are found in (Table 3.2).

None of these lineages are exclusive contributors to the Larval CX - i.e al of these lineages contribute neurons to other parts of the brain - however there are a few that contribute more than a fifts (20%) of their neurons to CX neuropils (Figure (3.3)).

We were interested in the connectivity patterns of specific neurons that are expected to belong to the central complex. Specifically, where a neuron receives synaptic input (location of the dendrites) and sends output processes (location of their axons). The central complex is known primarily for visual input processing, therefore in the larva we were interested in neurons downstream of photoreceptors. We thus investigated cells immediately connected to visual projection neurons (vPNs). Several of these neurons have been catalogued as Protocerebral Bridge (see section 3.2.1) or Noduli(see section 3.2.4) neurons. In additon to visual

Adult lineage	Larval lineage
DM1	DPMm1
DM2	DPMpm1
DM3	DPMpm2
DM4	CM4
DM5/DM6	CM1/3
DL1	CP23
EBa1	DALv2
LALv1	BAMv1
CREa1	BAmd1
CREa2	DALCm12
SMPad2	DAMd2/3
SIPp1	DPMpl12

Table 3.1 **Lineges of the Adult CX that also contribute neurons to the Larval CX** Adult CX lineages are listed in the first column, and their corresponding larval lineages in the second.

input, we find that olfactory input is integrated by all the larval CX neuropils indirectly via the Lateral Horn(LH) and the Mushroom Body through convergence neurons(CNs; which integrate learnt valences from the MB and innate valences from the LH [**eschbach2021circuits**]) or MB output neurons and MB input neurons. This is in line with previous findings about the CX being integrative across multiple sensory modalities [**hulse2021connectome**].

Columnar and horizontal neurons have stereotyped connectivity patterns between them, within themselves ot with other accessory structures. To be able to define these, we categorised them similarly to how they have been categorised in the literature. For naming, we adapted Wolff et al., 2015 nomenclature, and named them on the baisis of the location of input synapses (.d for dendrites) and the location of their output synapses (.b for axonal bouttons). Once the the synapses for each neuron was mapped, we categorised them on the basis of their location within the CX neuropils and relative to accesory structures as per Figure 3.1.

3.2 The Central Complex Adult vs Larva

3.2.1 Protocerebral Bridge

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

Larval lineages	PB.b	PB.d	FB.b	FB.d	EB.b	EB.d	NO.b	NO.d	Total
BAla12				2	2				4
BAla34			4					9	13
BAlp_ant		2			2			1	5
bamd1						1		2	3
bamd2_d			1						1
bamd2_v			3					4	7
bamv12_dor	1			1			2	5	9
BAmv12_dor	1			1			2	5	9
Bamv12_ven	1			1	2	2		1	7
BLAd-OL	2								2
BLAl				2					2
BLAV12_ant				2					2
BLP12						2			2
BLVp2		2							2
CM13_lat		2			4	2			8
CM13_med					2	2			4
CM4-vm				2				4	6
CPa			1						1
CPb					8	4			12
CPe				2				4	6
Dalcl12					16	22			38
Dalcm12-m						4		2	6
DALcm12-v		2		2			4	1	9
dall1		2	4						6
Dalv1	8			6					14
Dalv23					14			4	18
dplal1-3			2		3				5
dplc_post_med		4		2			4		10
DPLc5		6	1	2			3		12
DPMl_ant	3	2						3	8
DPMl12		3		2			2		7
DPMl34_post				1			1		2
DPMm1	4		14	2			4		24
DPMm2		3		2		2			7
DPMpl12		5	2	2					9
dpmpl3								1	1
DPMpm1			4	2		4			10
DPMpm2						2		2	4
Total	20	33	36	36	53	47	24	46	295

Table 3.2 **Larval Central Complex Lineages.** A full list of Larval CX lineages is shown in the first column. If a lineage also contributes to the adult CX, the corresponding adult nomenclature [**eckstein2024neurotransmitter**] is shown in the second column. Columns 3–6 indicate the number of neurons from each lineage that contribute axons (".b" for boutons) or dendrites (".d") to individual neuropils: PB, FB, EB and NO.
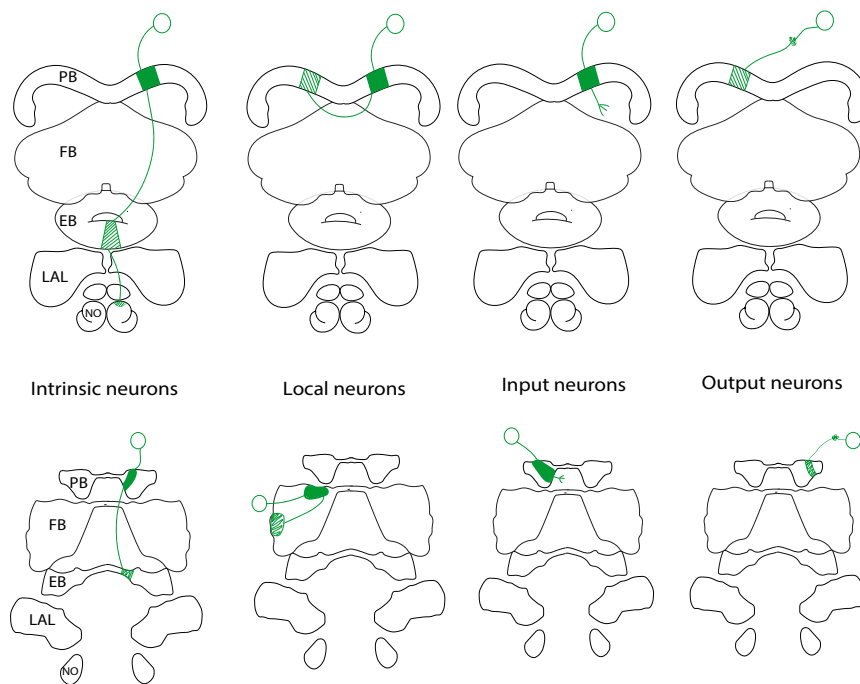
Fig. 3.1 **Classes of CX neurons** This schematic illustrates neuron classes of the adult CX (upper panel) and the larval CX (lower panel). The PB, FB, EB, NO and LAL are shown in each case to illustrate where a neuron arborizes. Neurons are drawn as a sketch, where the soma is represented by a green circle, the arbors are split into dendritic inputs - filled green boxed - and spiny axonal outputs - hashed scribbled green boxes. Cells are classified as either **Intrinsic Neurons**:neurons whose inputs and outputs are located within CX neuropils; **Local Neurons**: cells whose inputs and outputs located within one neuropil; **Input Neurons**: Neurons that have dendrites (receive synaptic input) located within the CX but output outside of it; and **Output Neurons**: Cells that receive inputs outside of the CX but output onto CX neuropils

Lineage	Left Hemisphere	Right Hemisphere	CX neurons	CX % of Lineage
Dalcl12	25	27	38	73.08%
Dalv1	11	11	14	63.64%
dplc_post_med	6	10	10	62.50%
DPMl12	13	2	7	46.67%
Dalv23	18	21	18	46.15%
DPLc5	13	14	12	44.44%
dplal1-3	2	12	5	35.71%
CPb	14	20	12	35.29%
DPMm1	38	47	24	28.24%
dall1	9	18	6	22.22%

Table 3.3 **Lineage contribution to CX** This table illustrates lineages that contribute more that 20% of their neurons ot the CX. the second and third column show their distribution across the brain hemispheres, whilst tha last column shows the percentage of neurons that contribute(axons, dendrites or both) to CX neuropils

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; [**truman2023metamorphosis**]).

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 ([**winding2023connectome**]) 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 ([**hulse2021connectome**]). 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**, **wolff2018neuroarchitecture**, **hulse2021connectome**]. Beyond the canonical columnar neurons projecting to other Central Complex neuropils, we found some whose axons descend to the SEZ or nerve cord (

3.2.2 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) [**omoto2018neuronal**].

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 [**hulse2021connectome**]. 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.

3.2.3 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 [**heinze2017unraveling**]. 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 tan gential 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 [**lin2013comprehensive**].

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.

3.2.4   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 [**wolff2018neuroarchitecture**] [**hulse2021connectome**] 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 [**andrade2019developmentally**].

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) [**wolff2015neuroarchitecture**, **hulse2021connectome**]. The primary NO inputs outside of the CX are from the LAL, these are known as LNO neurons and are suggested to be inhibitory [**wolff2018neuroarchitecture**, **hulse2021connectome**]. 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.

3.3   Visual input into the Larval Central Complex

- 4 graphs showing connectivity between

3.4   Mushroom Body and the Central Complex

Vertical lobe is used by the NO and MB and LAL.

mb2on-c1 has no results(when it comes to aversion), but massively related to EB so it might

mb2in-l1 according to eschback all the compartments of vertical love are for aversive behaviour.only mnbin that is not bilateral.

m2on-p1 noduli itself gets input from(..) (extended data figure 6a - complete connectome of learning memory centre) ipsilateral control via these only mbons and mbins that are ipsilateral as opposed to bilateral.

3.5 Descending Neurons from CX

129 neurons descending to VNC 93 neurons descending to SEZ 216 total descending layer 1 annotated as: 'cx descending l1' (56 neurons) 25.6% 569 second order descending layer 2 annotated as: 'cx descending l2' (71 neurons of 569) 12.4%

3.6 Neurotransmitter Identity of Central Complex Neurons

3.6.1 DANs Confocal Images

3.6.2 GABA and Acetylcholine

3.7 Genetic lines for CX neurons

3.8 Behavioural Assays - Loss of Function Analysis during light stimulation

3.9 Neural activity in response to blue light stimulation

Chapter 4

Discussion

Is there a Navigational Centre (Central Complex) in the Larval Drosophila?

The similarities between the larval Central Complex and the adult Central Complex PB,EB,FB,NO

Relation to accessory structures such as the LAL Sensory Biases: olfaction vs photoreception Central Complex relation to the Mushroom Body (Learning and Memory Centre of the fly) iii. Functions of the Larval central complex 1. Network connectivity predictions 2. Does biological data confirm our intuition? 3. Conclusions about the neural functionality iv. What we now know v. Expectations for the future vi. Can this teach us universal components of multisensory integration

4.1

Appendix A

Appendix title example

example

example

Appendix B

example2