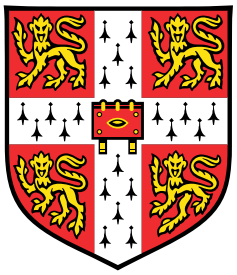

A centre for multisensory integration in Drosophila Larva

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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
August 2025

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Abstract

Multisensory integration is cool, why not study it via the central complex, and it's the best thing to ever study, how great. TEST

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Chapter 1

Introduction

1.1 Mapping brains

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1.3 Multisensory integration

1.4 The Central Complex

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

At the larval stage, this animal exhibits similar behaviours to those observed in the adult: it demonstrates chemotaxis during foraging and performs aversive phototaxis in response to blue light. In addition to individual stimulus response, *Drosophila* larva is able to integrate competing stimuli into a coherent representation(Gepner et al.,2015) prior to decision making. The larval brain shares similar set of neuroblasts with the adult, and presents neuropils with direct correspondance to the adult such as the Antennal Lobe(AL), the Mushroom Body(MB), and the Lateral Accessory Lobe(LAL).

The underlying connectivity of AL, MB, LAL is well understood at present(Winding et al., 2023), as well as the Lateral Horn(LH; a larvae specific neuropil). Nevertheless, these structures constitute only up to 25% of the larval brain connectome. We postulate

that amongst the remaining 75% of neurons, a multitude should be devoted to navigational decisions, and may constitute the putative larval Central Complex neuropils. These are unlikely to be 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 adult *Drosophila* central complex has five neuropils: the Protocerebral Bridge (PB), the Fan-shaped Body (FB; or central body upper), the Ellipsoid body(EB; or central body lower), the Noduli (NO) [**?**] and, as of recently, the Assymetrical Body (AB) [**?**]. The Lateral Accessory Lobe (LAL) reciprocally interconnects with these, making it an important accesory structure and reference point.

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

Chapter 2

Methods

2.1 Electron Microscopy Reconstructions

2.1.1 Seymour volume

2.1.2 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 dopamin-

ergic 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 Flp-Out (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 Janelia, incombination with the protocol from Nern et al. 2015.

The following primary antibodies were used: Mouse α-Neuroglian; Rabbit α-HA Tag; Rat α-FLAG Tag

The following secondary antibodies were used: AF488 Donkey α-Mouse; DL549 Goat α-Rabbit; AF594 Donkey α-Rat.

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

(https://www.nature.com/articles/ncomms8924Sec11)

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 1.4mm inner diameter and 2.0mm 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

Behavioural Apparatus The apparatus comprises a video camera (DALSA Falcon 4M30 camera) for monitoring larvae, a ring light illuminator (Cree C503B-RCS-CW0Z0AA1 at 624 nm in the red), a computer and two hardware modules for controlling vibration and temperature (Oven Industries PA, Model 0805). Both hardware modules were controlled through multi worm tracker (MWT) software (http://sourceforge.net/projects/mwt).

Before the experiments, the larvae were separated from food by using 15% sucrose and washed with water. The larvae were then dried and placed in the center of the arena. The substrate for the behavioural experiments was 3% Bacto agar gel in a 25 × 25 cm square plastic plate for experiments involving thermal activation and vibration stimuli, or a 10 × 10 cm plate for those involving thermal activation alone. We tested approximately 15–50 larvae at once in the behavioural assays. The temperature of the entire rig was kept at 25 °C (for optogenetic activation experiments) or 30 °C or 32 °C for thermogenetic activation experiments. The agar plates were also kept at the room temperature prior to experiment. The MWT software64 (http://sourceforge.net/projects/mwt) was used to record all behavioural responses and to control the presentation of vibration stimuli.

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

Chapter 4

Discussion

4.1 first part

Appendix A

How to install LaTeX

Windows OS

TeXLive package - full version

1. Download the TeXLive ISO (2.2GB) from
   https://www.tug.org/texlive/
2. Download WinCDEmu (if you don't have a virtual drive) from
   http://wincdemu.sysprogs.org/download/
3. To install Windows CD Emulator follow the instructions at
   http://wincdemu.sysprogs.org/tutorials/install/
4. Right click the iso and mount it using the WinCDEmu as shown in
   http://wincdemu.sysprogs.org/tutorials/mount/
5. Open your virtual drive and run setup.pl

or

Basic MikTeX - TeX distribution

1. Download Basic-MiKTeX(32bit or 64bit) from
   http://miktex.org/download
2. Run the installer
3. To add a new package go to Start » All Programs » MikTex » Maintenance (Admin)
   and choose Package Manager

4. Select or search for packages to install

TexStudio - TeX editor

1. Download TexStudio from
   http://texstudio.sourceforge.net/#downloads

2. Run the installer

Mac OS X

MacTeX - TeX distribution

1. Download the file from
   https://www.tug.org/mactex/

2. Extract and double click to run the installer. It does the entire configuration, sit back and relax.

TexStudio - TeX editor

1. Download TexStudio from
   http://texstudio.sourceforge.net/#downloads

2. Extract and Start

Unix/Linux

TeXLive - TeX distribution

Getting the distribution:

1. TexLive can be downloaded from
   http://www.tug.org/texlive/acquire-netinstall.html.

2. TexLive is provided by most operating system you can use (rpm,apt-get or yum) to get TexLive distributions

Installation

1. Mount the ISO file in the mnt directory

   ```
   mount -t iso9660 -o ro,loop,noauto /your/texlive####.iso /mnt
   ```

2. Install wget on your OS (use rpm, apt-get or yum install)

3. Run the installer script install-tl.

   ```
   cd /your/download/directory
   ./install-tl
   ```

4. Enter command 'i' for installation

5. Post-Installation configuration:
   http://www.tug.org/texlive/doc/texlive-en/texlive-en.html#x1-320003.4.1

6. Set the path for the directory of TexLive binaries in your .bashrc file

For 32bit OS

For Bourne-compatible shells such as bash, and using Intel x86 GNU/Linux and a default directory setup as an example, the file to edit might be

```
edit $~/.bashrc file and add following lines
PATH=/usr/local/texlive/2011/bin/i386-linux:$PATH;
export PATH
MANPATH=/usr/local/texlive/2011/texmf/doc/man:$MANPATH;
export MANPATH
INFOPATH=/usr/local/texlive/2011/texmf/doc/info:$INFOPATH;
export INFOPATH
```

For 64bit OS

```
edit $~/.bashrc file and add following lines
PATH=/usr/local/texlive/2011/bin/x86_64-linux:$PATH;
export PATH
MANPATH=/usr/local/texlive/2011/texmf/doc/man:$MANPATH;
export MANPATH
```

```
INFOPATH=/usr/local/texlive/2011/texmf/doc/info:$INFOPATH;
export INFOPATH
```

Fedora/RedHat/CentOS:

```
sudo yum install texlive
sudo yum install psutils
```

SUSE:

```
sudo zypper install texlive
```

Debian/Ubuntu:

```
sudo apt-get install texlive texlive-latex-extra
sudo apt-get install psutils
```

Appendix B

Installing the CUED class file

LaTeX.cls files can be accessed system-wide when they are placed in the <texmf>/tex/latex directory, where <texmf> is the root directory of the user's TeXinstallation. On systems that have a local texmf tree (<texmflocal>), which may be named "texmf-local" or "localtexmf", it may be advisable to install packages in <texmflocal>, rather than <texmf> as the contents of the former, unlike that of the latter, are preserved after the LaTeXsystem is reinstalled and/or upgraded.

It is recommended that the user create a subdirectory <texmf>/tex/latex/CUED for all CUED related LaTeXclass and package files. On some LaTeXsystems, the directory look-up tables will need to be refreshed after making additions or deletions to the system files. For TeXLive systems this is accomplished via executing "texhash" as root. MIKTeXusers can run "initexmf -u" to accomplish the same thing.

Users not willing or able to install the files system-wide can install them in their personal directories, but will then have to provide the path (full or relative) in addition to the filename when referring to them in LaTeX.