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The mapping of neurons and lineage classification of the larvae and adult *Drosophila* brain
in several Gal4 transmitter lines

A thesis submitted in partial satisfaction
of the requirements for the degree Master of Science in
Physiological Science

By

Sally Ahad

2015

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ABSTRACT OF THE THESIS

The mapping of neurons and lineage classification of the larvae and adult *Drosophila* brain
in several Gal4 transmitter lines

By

Sally Ahad

Master of Science in Physiological Science

University of California, Los Angeles, 2015

Professor Mark Frye, Co-Chair

Professor Volker Hartenstein, Co-Chair

In *Drosophila*, neurons within the central nervous system are grouped into units called lineages. Each lineage contains cells derived from a single neuroblast. A neuroblast is a stem cell that divides and forms lineages of neurons. In flies, the lineage can be subdivided into different parts; the neurons that are born first are closest to the neuropile (Spindler and Hartenstein, 2010). There is a birth ordering of neurons. In the embryo, the neuroblasts divide 5 to 6 times and are called primary neurons. In the larvae, we are able to still see primary neurons, but each neuroblast divides further and undergoes about 50 divisions and these cells are termed secondary neurons. There is a small population of primary neurons and a huge population of adult secondary neurons in *Drosophila*. The secondary neurons are known to use the primary neurons as their guiding path.

My project addresses the relationship of nerve fibres (axons and dendrites) formed by primary and secondary neurons in the brain neuropile. I am using a marker expressed in a subset of primary lineages, called Fasciclin II (FasII), and another marker expressed in all secondary neurons, called Neurotactin. In one set of experiments I am using a Gal4 driver line expressed in the FasII-positive neurons to ablate these cells, and study the effect on the pathfinding of BP106-positive secondary neurons. To that end I have crossed the FasII-Gal4 line to a UAS-hid;rpr construct that will induce cell death. My preliminary results show that the animals die already as embryos, possibly due to neural defects. I am currently investigating the neuronal basis for this early cell death (which precludes me from analyzing secondary neurons). A second project is to assign a large collection of published single neuron clones (Chiang et al., 2011) to the groups of primary vs. secondary neurons. The clones were induced using driver lines expressed in neuron populations expressing different neurotransmitters (such as TH-Gal4, Cha-Gal4). I will use the lines and express a GFP reporter, in addition to BP106, at different developmental stages. This will allow me to determine whether a given line/transmitter is expressed in primary neurons, secondary neurons, or both; and will also allow me to map the neurons to specific lineages.

The thesis of Sally Ahad is approved.

Peter Narins

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Volker Hartenstein, Committee Co-Chair

University of California, Los Angeles

2015

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Introduction

In *Drosophila*, neurons within the central nervous system are grouped into units called lineages. Each lineage consists of 100-200 neurons produced by a single neuronal stem cell called a neuroblast. It is known that the central brain and the ventral nerve cord in flies are generated by roughly 30,000 neurons that are formed through a lineage mechanism. A neuroblast is a stem cell which then divides and forms lineages of neurons. Each neuroblast gives rise to a group of neurons that share several fundamental structural characteristics. Lineages can be subdivided into different parts: in the embryo, the neuroblasts divide 5 to 6 times and generate so called primary neurons that differentiate into the nervous system of the larva. During the larval stage, each neuroblast divides further and undergoes about 50 divisions which produce the secondary neurons of the adult brain.

Cell bodies of neurons of one particular lineage form a cluster in the outer layer (called cortex) of the brain. Neurons project their axons in one cohesive bundle. In the embryo, primary neurons are born first and form the primary axon tracts (PATs); axons of secondary neurons follow the PATs and add to them the secondary axon tracts (SATs) during larval stages (Figure 1). The axonal projections of a particular lineage form arborizations that typically spread within discrete, invariant compartments of the brain (Hartenstein et al., 2008). The axonal and dendritic branches of a certain tract of a lineage, with its arborizations generate compartments that can be followed from the larva into the adult (Hartenstein et al., 2008).

To better understand the mechanisms underlying brain development, genetic manipulation tools can be utilized within lineages to visualize, knock down, or over-express proteins. *Drosophila* presents favorable experimental conditions for such projects. Lineages have a well-described developmental timeline, originate from a single stem cell, and contain groups of bundled neurons that project in a unified direction and have specific genetic identification, which can be easily visualized with antibodies or lineage-specific GAL4 lines, and can be manipulated. *Drosophila* has a short life cycle and offers easy access to the brain (Spindler and Hartenstein, 2010). Future implications of this study will hopefully lead us to be able to understand the development and circuit formation within vertebrate systems, including the human brain.

My thesis project entails the lineage-focused characterization of 8 Gal4 reporter lines inserted in the regulatory domain of genes that encode enzymes in neurotransmitter synthesis/uptake pathways.

Group 1: TRH=Tryptophanhydroxylase, TH(ple)=tyrosine hydroxylase, Tdc2=tyrosine carboxylase, npf=neuropeptide F.

Group 2: VGlut=Vesicular glutamate transporter, Cha=Cholineacetyltransferase, Gad1=Glutamic acid decarboxylase 1.

Previous studies, summarized in detail in the pertinent sections of the Results part (see below) have demonstrated the role of these neurotransmitters in neural function and fly behavior. The reporter lines, to various degrees, faithfully report the expression of the corresponding genes. These lines had been used in a recent, large study to induce more than 10,000 single cell clones in the adult fly brain (Chiang et al., 2011). The resulting data

are documented in an interactive, searchable database, called “Fly circuit”. Searching criteria are the lines used for clone visualization (i.e., the 8 lines corresponding to distinct neurotransmitters), as well as anatomical subdivisions of the brain. Information currently lacking is the lineage association of brain neurons. As mentioned above, lineages represent suitable subunits of the fly brain, similar to brain stem nuclei, or Broca’s areas in the vertebrate brain. When addressing a particular neuron (of which there are more than 10,000 in the central brain alone), it is advantageous if one is able to refer to the subdivision the neuron belongs to. Lineages are the “natural” units to which individual neurons can be assigned.

In my project I used the 8 Gal4 lines to drive UAS-mcd8GFP, a membrane-targeted GFP that labels both cell body and processes (neurites) of neurons. I analyzed expression at two stages, the late larval (L3) and adult stage. To assign the GFP-positive neurons to lineages, I counterstained preparations with antibodies against the adhesion protein Neuroglian (BP104) and DN-cadherin. Neuroglian highlights the axon tracts of lineages, and DN-cadherin is expressed in synapses, thereby visualizing the neuropil. The rationale of my study is to assign the GFP-positive axons to BP104-positive lineages, allowing me to state whether the corresponding Gal4 line is expressed in primary neurons or secondary neurons and (if the pattern is reasonably sparse) in which lineage it is expressed. In some cases I also analyzed early (L1) larval brains, which have only primary lineages and primary axon tracts.

Methods

Fly Stocks and crosses

The following fly lines were used in this study: TRH-Gal4, TH(ple)-Gal4, Tdc2-Gal4, npf-Gal4, V-Glut-Gal4, Cha-Gal4, Gad-Gal4

Origin of lines (Bloomington)

To obtain experimental flies TRH-Gal4, TH(ple)-Gal4, Tdc2-Gal4, npf-Gal4, V-Glut-Gal4, Cha-Gal4, Gad-Gal4 males were crossed to UAS-mcd8-GFP females.

Prior to immunohistochemistry, I generated crosses for each of the 8 lines in my study. UAS-mcd8-GFP virgin females were collected and crossed to males from each of the lines and separate crosses were generated. F1 progeny were collected at different stages (L1, L3 and adult for our study). Larvae was collected ~20 hours post cross and brains were dissected for the L3 stage. Then crosses were allowed to propagate until adults were collected several days later ~7-10 days. Same procedure was followed for adult brain dissection.

Dissection of brains

Brains were dissected in PBS at room temperature. Then fixed for 25 minutes in 4% formaldehyde in PBS. Tissues were then washed three times in PBS for 30 minutes. Then brains were blocked with NGS in PBT (900ul PBT in 100ul NGS) for 30 minutes. Then primary and secondary antibodies were prepared for staining.

Staining of L3 brains

Incubate in primary antibody overnight shaking at 4 degrees celcius. Primary antibody (BP106 anti-neurotactin , DX-EX #8S, Hybridoma Bank). 1 ml 0.1%PBT/NGS was prepared

(900ul 0.1%PBT and 100ul NGS). 150ul was removed out of solution. 100ul BP106 and 50ul Ncad was added. Anti-DNcadherin will be used to label neuropile, thus visualizing landmarks used for orientation Next day, incubate in conjugated secondary antibody for 2 nights (mouse-Alexa 546 and rat-Alexa 647, Invitrogen, Carlsbad, CA). 1ml PBT/NGS was prepared. To this mixture, 2ul anti mouse Alexa546 (1:500) and 10ul Alexa647 (1:100) was added.

Staining of adult brains

Incubate in primary antibody overnight shaking at 4 degrees celcius. Primary antibody (BP104 anti-neurotactin, DX-EX #8S, Hybridoma Bank). 1 ml 0.3%PBT/NGS was prepared (900ul 0.3%PBT and 100ul NGS). 83ul was removed out of solution. 33ul BP104 (1:30) and 50ul Ncad was added. Anti-DNcadherin will be used to label neuropile, thus visualizing landmarks used for orientation Next day, incubate in conjugated secondary antibody for 2 nights (mouse-Alexa 546 and rat-Alexa 647). 1ml PBT/NGS was prepared. To this mixture, 2ul anti mouse Alexa546 (1:500) and 10ul Alexa647 (1:100) was added.

Mounting and visualization:

Wash tissues in 0.1% PBT for 1 hour, PBT (1X phosphate-buffered saline [PBS] with 0.1% Tween-20). Place samples on shaker in between washes. Remove as much PBT as possible and a drop of vectashield mounting medium (Vector, Burlingame, CA, #H1000) was added to the brains. Prepare slides for mounting. Spacers were used on each side of the slide to ensure samples were place in a localized area within the slide (typically center). Samples of about 6-10 were retrieved and placed on the slide with as little liquid as possible. Samples were then positioned anteriorly and mounted in rows. Then the cover slip was placed on the top and additional vectashield mounting medium was applied to ensure that the

samples were immersed prior to sealing. White nail polish was used to seal off the edges of the slide for permanent storage. At this point, samples are ready for visualization under confocal microscopy.

Confocal microscopy and ImageJ

Sample preparations were initially located on 10X magnification and positioned appropriately. Then using a drop of oil, I moved to the 40X magnification to focus the prep. Settings were adjusted accordingly. I zoomed such that one brain hemisphere fits the region on the screen. All three channels (red, blue and green) were adjusted with color and contrast to ensure we are able to visualize different areas of the brain well. Sections were recorded for an average of 2-3 minutes depending on the thickness of the stacks, which ranged between 65 to 80 sections. Two brains were visualized per line and several stacked were recorded. This technique allows visualization of optical sections of material labeled with fluorescence or reflecting probes. By excluding light from planes above and below the plane of focus it is possible to obtain sharp images of objects deep within sections. Sections can be combined to construct three-dimensional images. Confocal microscope used is LSM700. Z projections images that are used in the figures within this paper were generated using ImageJ software. The program allows one to generate Z projections of desired thickness. We generated Z projections that were five consecutive frontal confocal sections of L3 and adult brain hemisphere, we generated anterior, posterior and central levels Z-projections to illustrate the various lineages identified along with primary and secondary neuron clusters.

Results and Discussion

Overview

My Results part is organized in sub-sections that correspond to the 7 driver lines studied.

Each section is structured consistently, starting with a summary of the known functions of the transmitter, then data regarding expression patterns at different stages. We

investigated two different stages: L3 and Adult. At both stages, lineages are visible by their secondary tracts, which are positive for BP106 (optimal at larval stage) or BP104 (optimal at adult stage). Therefore, for each line, we prepared 5-10 stacks at the L3 and Adult stage.

For selected lines, I also looked at L1. Subsequently, figures were generated using ImageJ software. Z-projections were generated from 5 consecutive sections that were merged in Image J. We performed this for three different levels along the anterior posterior (AP) axis. The left column of each figure corresponds to the L3 stage; right column corresponds to the adult stage; rows correspond to AP levels: upper row anterior; middle row central part of brain; bottom row posterior.

Lineages of the fly brain and ventral nerve cord were mapped in a number of studies, which are used here as reference. These studies made it possible to identify the lineages in my material. Lineages of the ventral nerve cord are described in Truman et al. (2004); those of the brain in Păreanu and Hartenstein (2006), Cardona et al. (2010), and Lovick et al. (2013). Other studies that were used to distinguish relevant details in my preparations were the description of the relationship between primary and secondary lineages (Larsen et al., 2009), and papers that identified the compartmental organization of the brain (Younossi-Hartenstein et al., 2003, for the larva; Păreanu et al., 2010, for the adult).

I subdivided the Gal4 line expression patterns, corresponding to distinct transmitters, into two groups. Group 1 contains lines/transmitters expressed in a sparse pattern, corresponding to individual neurons belonging to one or a few lineages. This group includes: TRH-Gal4, TH (ple)-Gal4, Tdc2-Gal4, npf-Gal4.

Groups 2 has lines/transmitters with widespread expression pattern; essentially, subsets of neurons belonging to every lineage are positive. This group includes: VGlut-Gal4, Cha-Gal4, and Gad-Gal4.

Group 1 lines

1.TRH-Gal4: tryptophanhydroxylase expressed in serotonergic neurons

Serotonin function in the Drosophila brain:

Aggression for resources and primarily survival is seen in essentially all species of animals (Alekseyenko et al., 2010). Dopamine (DA) and serotonin (5HT) are reported to have critical roles in aggression in a wide variety of animals including humans. The Drosophila system offers the advantage of having powerful genetic methods already available that can be used to unravel the roles of amine neurons in behavior of aggression (Coleman et al., 2005, de Almeida et al., 2005). The rate-limiting enzymes in the biosynthesis of the amines in Drosophila are tyrosine hydroxylase (TH) for DA and tryptophanhydroxylase (dTPH and dTRH) for 5HT (Livingstone et al., 1983 and Hirsh et al., 1981. The final common step in the biosynthesis of both amines involves the enzyme dopadecarboxylase (DDC) (Hirsh et al., 1981). Flies deficient for the gene DDC and DA null mutants die during development. The use of the binary Gal4/UAS system allows for both acute or chronic manipulation of the function of amine neurons at different times during development and in adults. Both DDC-Gal4 and TH-Gal4 transgenic lines have been generated for this purpose. A line that drives

expression in serotonergic neurons has been mentioned before but not characterized (Alekseyenko et al., 2010). To date, putative roles for 5HT neurons have been suggested in sleep, aggression and memory using pharmacology and genetic tools containing DDC and TH regulatory elements. Previous investigations involving pharmacological manipulations of 5HT function in adult *Drosophila* behavior target both DA and 5HT neurons. Previous investigators have generated transgenic line that allows selective, direct manipulation of serotonergic neurons and asked whether DA and 5HT have separable effects on aggression. Based on quantitative morphological examination, they showed that tryptophanhydroxylase (TRH)-Gal4 driver line was highly selective for 5HT-containing neurons. This line was used in conjunction with already available Gal4 driver lines that target DA or both DA and 5HT neurons to acutely alter the function of aminergic systems (Alekseyenko et al., 2010, Coleman et al., 2005, de Almeida et al., 2005). First, the investigators showed that acute localized impairment of DA and 5HT neurotransmission using expression of a temperature sensitive form of dynamin completely abolished mid- and high-level aggression. These flies did not escalate fights beyond brief low-intensity interactions and therefore did not yield dominance relationships. They showed next that manipulation of either 5HT or DA neurotransmission failed to duplicate this phenotype. Selective disruption of 5HT neurotransmission yielded flies that fought, but with reduced ability to escalate fights, leading to fewer dominance relationships. Acute activation of 5HT neurons using temperature sensitive channel expression, in contrast, resulted in flies that escalated fights faster and that fought at higher intensities. Finally, acute disruption of DA neurotransmission produced hyperactive flies that moved faster than controls, and rarely engaged in any social interactions. By separately manipulating 5HT- and DA- neuron

systems, the researchers collected evidence demonstrating a direct role for 5HT in the escalation of aggression in *Drosophila* (Alekseyenko et al., 2010). Thus dopamine (DA) and serotonin (5HT) are known to play crucial roles in aggression in a wide range of animals. The enzyme dopadecarboxylase (DDC) is shown to be expressed in both serotonergic and dopaminergic neurons in the fruit fly nervous system (Hirsh et al., 1981). Previous research has shown that *Drosophila* was used as a model system to explore separately the roles of 5-HT and DA in aggression in flies. TRH-Gal4 driver lines were generated to direct expression of some effector genes selectively in 5HT-containing neurons. The behavioral consequences of impaired serotonergic neuron function were compared and contrasted with similar targeted disruption of dopaminergic neurotransmission using a TH-Gal4 driver, or by interference with neurotransmission of both amines using a DDC-Gal4 driver. Although Alekseyenko and his colleagues focused on the two amines, DDC also has been reported to be expressed in neurons containing corazonin and possibly other unknown substances (Alekseyenko et al., 2010 and Coleman et al., 2005).

Thus, by generating genetic tools that allowed previous researchers to selectively manipulate serotonergic neuron function without changing dopaminergic neurons. They were able to collect evidence for a specific role of 5HT in aggressive behavior in fruit flies. Serotonin is not required to initiate fights, but is necessary for the proper escalation of aggression to higher intensity levels and thereby to facilitate the establishment of hierarchical relationships. Further studies will be required to identify the subsets of 5-HT neurons involved in these processes and to begin to unravel the circuitry involved (Alekseyenko et al., 2010). TRH Gal4 line was generated to provide information on the role of potential serotonergic function. Furthermore, using this line, we could potentially

visualize the 5HT expression distribution in the CNS of drosophila. We along with previous investigators have shown that this line focuses on the identification of small cluster of serotonergic neurons based on localization and presence of specific arborizations in the CNS of flies.

Expression of TRH-Gal4 in L3

TRH Gal4 is expressed most strongly in a group of 4 or 5 neurons located in the subesophageal ganglion (SOG). Due to their spatial closeness to each other, we interpret these neurons as a lineage. The neurons innervate parts of the SOG that's known to represent one of the gustatory centers called PONPa, which is a sensory center that integrates information about taste and gut motility and tension. Neurons located in the mouth and gut send their projections to the PONPa. Neurons in the mouth may possibly play a role in feeding behavior while in the mouth cavity reside sensory neurons that are involved in gustatory information. In addition to the SOG lineage, TRH-Gal4 appears in 4-5 primary neurons of the supraesophageal ganglion (SPG; the "brain proper" of the larva; Figure 2 panel a, yellow arrow). Using TRH-Gal4 as an example, I will explain in the following in detail what conclusions can be drawn regarding the type of neuron/lineage that expresses this line. Similar reasoning will apply to the analysis of other group 1 lines. My first conclusion is that the cluster of TRH-Gal4-positive neurons in the SOG most likely belong to one primary lineage. The evidence is that neuronal cell bodies form a tight cluster and project their axons through a common bundle (the PAT): that argues for them being a lineage (Figure 2, yellow arrow, panel a). Furthermore, the neurons are differentiated, forming dense processes in the SOG (Figure 2, white arrow, panel b). Only primary neurons

are differentiated at the larval stage, demonstrating that the TRH-Gal4-positive cells of the SOG are primary.

Secondly, the question of lineage identity arises: what lineage does the SOG TRH-Gal4-positive cluster belong to? Lineages were mapped with respect to their secondary axon tracts, which are the structures that can be most easily visualized and described at the late larval stage. For the SOG, the relevant descriptions are two recent publications, Kuert et al. (2012; 2014), where the secondary lineages of the SOG were mapped. The surprising conclusion of these papers was that in the SOG, as opposed to the brain (or thoracic neuromeres) only a small fraction of neuroblasts that made primary lineages reactivate in the larva to produce secondary lineages. In all, for the entire SOG which consists of 4 neuromeres, called labium, maxilla, mandible, and tritocerebrum, 18 paired secondary lineages exist, whereas there are about 100 primary lineages. In other words, when mapping primary SOG lineages with respect to secondary axon tracts, only some will correspond to one of these, whereas most won't. The latter is true for the TRH-Gal4-positive primary lineage, which enters the anterior SOG (presumably, the tritocerebrum, without an associated secondary axon tract (Figure 2, white arrow, panel b).

The situation is different with respect to the TRH-Gal4-positive neurons of the SPG (brain). As stated above, and shown in (Figure 2, yellow arrow, panel a), there are 4-5 cells, which are spread out over the entire brain hemisphere, implying that they each belong to a different lineage. Like the SOG neurons, these cells are primary neurons, since they have differentiated arbors (Figure 2, panel b). In brain, each primary lineage is accompanied by a secondary one. We used BP106 to visualize the secondary tracts, and identify the corresponding lineages. Based on the maps published by Perea and Hartenstein (2006)

and Cardona et al. (2010) I conclude that the dorsal-most cell of the TRH group belongs to a paired lineage called DPMpl1/2 (Figure 2, panel b). Two neurons further ventrally, but near the posterior pole, belong to CM1 and CM4, respectively (Figure 2, panel b). A neuron located dorso-laterally belongs to the BLD cluster, a group of 6 lineages; the most likely candidate is BLD1 (Figure 2 panel b, white arrow). Finally, in the anterior region of the brain, we are able to identify a pair of neurons that belong to the paired DALv2/3 lineages. This pair, according to recent findings from the Hartenstein Lab (Lovick et al., in preparation) represents the only exception to the rule that secondary and primary axons project together. Primary axons of DALv2/3 enter at a more ventro-posterior level, at the lateral surface of a compartment called lateral accessory lobe (LAL), than the corresponding secondary axons. The point of entry and trajectory of the TRH-Gal4-positive neurons clearly corresponds to primary DALv2/3 (Figure 2; magenta arrow, panel c).

2) TH (ple)-Gal4: tyrosine hydroxylase expressed in dopaminergic neurons

Role of DA in adult fly behavior

Dopaminergic neurons have been identified in the larval and adult central nervous system (CNS) in *Drosophila* and several insects (Antonov et al., 2003). Aside from its transmitter function, DA is required in insects for cuticle development and pigmentation. Previous studies have shown that DA activates and modulates locomotion behavior in insects (Kandel, 2001). In mammals, DA is an essential modulator of motor commands based on genetic studies. In humans, studies have shown that DA deficiency in the basal ganglia is the cause of Parkinson's disease. In addition, DA deficiency is also involved in attention-deficit hyperactivity disorder (ADHD) (McBride et al., 1999). Finally, an increasing body of evidence demonstrates that DA is involved in learning and memory; specific clusters of DA

neurons spatially closely associated with the mushroom body are separately involved in negative and positive reinforcement (Friggi-Grelín et al., 2003).

In *Drosophila* as in vertebrates, the rate-limiting step in DA biosynthesis is catalyzed by the enzyme tyrosine hydroxylase (TH). The *Drosophila* TH gene (DTH) is specifically expressed in all dopaminergic cells and the corresponding mutant, pale (ple), is embryonic lethal (Mery and Kawecki, 2002 and Kandel, 2001). The transgene, TH-GAL4, contains regulatory sequences from the DTH 5' flanking and downstream coding regions. TH-GAL4 specifically expresses in dopaminergic cells in embryos, larval CNS, and adult brain when introduced into the *Drosophila* genome (Yin and Tully, 1996). The pattern closely resembles the known pattern that can be visualized by using an antibody against TH (Tempel et al., 1983). As a first application of this driver, it was observed that in vivo inhibition of DA release induces a hyperexcitability in adult flies (Schwaerzel et al., 2003).

Expression of DA in L3

TH (ple) Gal4 is expressed in several discrete clusters that represent primary lineages about 5 or 6 lineages. To determine the specific lineage identity of the dopaminergic (DA) clusters, we adapted mapping technique from (Cruz et al., 2013), we mapped the trajectory of their primary axon tracts relative to the secondary tracts in the late larval brain (L3), for which a complete lineage atlas has been established. The axon tracts of secondary lineages fasciculate with or is within close proximity to the axon tracts formed by primary neurons. To visualize the secondary axon tracts (SATs), brains were labeled with anti-Neurotactin. It is apparent that most of the GFP-positive axons of DA neurons fasciculate with specific SATs, which we then took as the criterion to assign a given DA neuron to the corresponding lineage (Cruz et al., 2013).

We are able to identify all differentiated primary neurons, one group of primary neurons identified by (Figure 3, yellow arrow, panel a). Of those several lineages identified, a subset of those neurons are dopaminergic and produce dopamine (Figure 3). A network of bundled arborizations are shown in (Figure 3, panel b), outlined in yellow. Fasciculated with these network of branches are primary and secondary neurons (Figure 3, panel b, white arrows and yellow arrow).

Expression of DA in Adult

In the pupae and adult stages, there are more primary neurons present (Figure 3, panels d and e, yellow arrows). These neurons are present in the larvae but don't produce dopamine. They are differentiated neurons that are destined to produce dopamine but at this stage they don't they come on until much later, which is in the pupae and adult stages. In addition, there is one secondary lineage where there are secondary neurons that express dopamine (Figure 3).

3)Tdc-Gal4: tyrosine carboxylase expressed in octopaminergic neurons

Genetics and function of octopamine in the fly brain and ventral cord

Tyrosine decarboxylase 2 (Tdc2) is an enzyme that catalyses the reaction of tyrosine to tyramine. Tyramine and octopamine are found in most organisms, including bacteria, fungi, and plants, and in animals ranging in complexity from nematodes to mammals (Cole et al., 2005). Despite their wide range layout, the biological roles of these bioamines are still poorly understood. In vertebrates, traces of these bioamines are present in low levels. There are currently no data to suggest that there are specific synapses for trace amines in the vertebrate brain. However G-protein-coupled receptors were discovered that are

activated by trace amines, and this suggests that these agents may function independently of classical neurotransmitters. Several papers also indicate a role for tyramine in several psychiatric disorders (Cole et al., 2005).

Octopamine in invertebrates is often considered to be the functional analog of vertebrate norepinephrin and is involved in a diverse range of physiological processes, including *Drosophila* female fertility. Flies that lack a functional octopamine receptor or cannot synthesize octopamine due to a null allele of Tyramine alpha-hydroxylase (*Tαh*) show a complete egg retention phenotype. To further understand the roles of tyramine and its metabolite octopamine, two TDC genes were cloned from flies, *dTdc1* and *dTdc2*. It has been shown that both gene products have TDC activity in vivo, *dTdc1* is expressed non-neurally, whereas *dTdc2* is expressed neurally. Flies with a mutation in *dTdc2* lack neural tyramine and octopamine and are female sterile due to egg retention.

Expression of Tdc-Gal4 in L3

Tdc-gal 4 is expressed in two distinct populations of neurons. First, one can observe the presence of a median cluster of several large primary neurons in each segment of the SOG and ventral nerve cord (Figure 4 panel a, yellow arrows), which possess high intensity signal. This metameric group of cells is composed of three to four cells that, based on the line of argument outlined above for *TRH-Gal4* (see section 1) belong to a single lineage. According to the maps of Truman et al. (2004) and Kuert et al. (2014), this lineage corresponds to the lineage 0 (Figure 4 panel b, white arrow) in the ventral nerve cord (VNC), which is located in the midline and is unpaired. Again, one has to take into account the peculiar development of the SOG, in regard to the fact that only a minority of neuroblasts, after forming primary lineages, go ahead and generate secondary lineages (see

section 1 above). Thus, for lineage 0, only the most posterior SOG segment (labium) has a secondary lineage 0. Here we see a Tdc-Gal4-positive PAT accompanying the BP106-positive lineage 0 tract into the neuropile (refer to panel). The other 3 SOG segments have no secondary lineage 0; however, primary, Tdc-Gal4 positive PATs can be recognized entering the SOG. These then correspond to the primary lineage) of the SOG segments. I conclude that Tdc-Gal4 is a highly useful marker for primary lineage 0, which so far has not been detected by any other reagent.

Expression of Tdc-Gal4 in the Adult

The primary Tdc-Gal4-positive neurons survive in the adult. This is typical; most primary lineages will survive, following a more or less severe remodeling during metamorphosis, into the adult. The subesophageal segments, labium, maxilla, mandible, and tritocerebrum, which in the larva formed the anterior part of the ventral nerve cord, become incorporated into the brain, where they form the fused SOG (Figure 4). One can still identify the Tdc-Gal4-positive metameric lineages 0 of the four segments in the adult brain (Figure 4). Neurons are large and have wide reaching neurite arbors (Figure 4 panel e, white arrow). In addition to these primary neurons one observes additional, smaller secondary neurons for each of the lineages, which then must have differentiated during metamorphosis (Figure 4, panel, panel d).

4) Npf-Gal4: neuropeptide F expressed in peptidergic neurons

The role of NPF in fly behavior

Drosophila Neuropeptide F (NPF), a human NPY homolog, coordinates larval behavioral changes related to food intake during development. The vertebrate neuropeptide Y (NPY), a conserved 36 amino acid neuromodulator, is present in the hypothalamus that is

responsible for the central regulation of feeding in vertebrates (Wen et al., 2005; Bannion et al., 2000). Pharmacological studies have implicated hypothalamic NPY as a prominent stimulator for appetitive behavior. Chronic administration of NPY in the paraventricular nucleus induces uncontrolled food intake in rats, which subsequently develop severe obesity (Beck, 2001; Stanley, 1986).

Neuropeptide F (NPF) is the only member of the NPY family in the flies' genome. Its action is mediated via a G-protein coupled 7-transmembrane receptor mechanism. Previous studies suggest that the npf system integrated neural sensory information, as well as, modulates motor output information (Beck, 2001; Wu et al., 2003). Furthermore, it has been shown that the npf neural network is involved in acute modulation of alcohol sensitivity in adult flies (Wen et al., 2005). In insects, the expression of npf in the brain is high in larvae that are attracted to food, whereas its down-regulation coincides with the onset of behaviors of older larvae, including food aversion and excessive mobility. The neuropeptide F receptor (NPFR1) has also been characterized, and has been found to be expressed in a pair of dorsolateral neurons in the central brain and a small number of neurons in the subesophagealganglion (Garczynski, 2002; Wen et al., 2005). The Npf system provides a new paradigm for studying the central control of cooperative behavior (Wu, 2003 and Wen et al., 2005).

It has been postulated that mammalian neuropeptide Y (NPY) might play a motivational role in foraging behavior. Genetic and pharmacological studies have provided consistent evidence for a role of NPY in suppressing anxiety, fear, and responsiveness to aversive/stress stimuli (Wu et al., 2003). For example, NPY knockout mice exhibited less center activity in an open-field test and increased startle response to an acoustic stimulus

(Bannon et al., 2000), whereas mice over-expressing NPY showed increased tolerance to stress and lack of fear suppression of behavior (Wu et al., 2003). Interestingly, mice injected with NPY are more willing to work for food and display increased tolerance to the aversive taste of quinine-adulterated milk. These properties of NPY appear to bode well for its speculated role in promoting food searching and acquisition, especially under adverse conditions (Bannon et al., 2000).

Drosophila might be a simpler genetic model for studying the physiological role of the vertebrate NPY system. The *Drosophila* genome contains a single coding sequence for the NPY homolog (Bannon et al., 2000; Wen et al., 2005). The Npy neuronal network has been characterized in the CNS of *Drosophila* larvae (Wu et al., 2003). The Npf neural system comprises four to six Npf neurons located in the brain and subesophageal ganglia. In response to chemosensory stimulation by sugar, the Npf neuronal circuit undergoes long-term, dose-dependent modifications through npf activation and an increase in the number of Npf-positive varicosities (. These properties of the Npf neurons support its potential role in the regulation of food-related behaviors. Although an NPY homolog has not been identified in *C. elegans*, a genetic study has implicated a conserved NPY-like signaling system in regulating food-conditioned foraging behavior in the worm (Beck, 2001).

Expression of Npf-Gal4 in L3

Npf-Gal4 is expressed in a small set of primary neurons (Figure 5 panel a, yellow arrow). We are able to assign these discrete neurons into lineages, because the GFP-labeled axons are associated with defined BP106-positive SATs (Figure 5 panel b, white arrow). The dorsomedial primary neurons belong to a lineage called DPMm2 (Figure 5 panel b). It has a peculiar tract. The axon continues to the midline and crosses in the other side forming a

chiasm and terminates in the ventral nerve cord. The second lineage belongs to a group called BLD 1-4 (Figure 5 panel c, yellow arrow).

Expression of Npf-Gal4 in Adult

Here we see many primary neurons that cover the anterior portion of the brain compared to the few we see in the larva. We see the difference in size between the primary and secondary neurons (Figure 5 panel d, yellow arrow). We have DPMm2 located medially and are able to see arborizations, which branches laterally and medially and follow a tract, which then converges towards the midline. Thus, the same chiasmatic tract seen in the larva is also evident in the adult. Dorsolaterally one sees the adult version of the BLD neuron. The cells in the posterior brain are secondary neurons based on their size and morphology (Figure 5 panel f, yellow arrow), these secondary neurons are known to innervate the fan-shaped body and ellipsoid body.

Group 2 lines

1) V-Glut-Gal4: vesicular glutamate transporter expressed in glutamatergic neurons

Glutamate as a transmitter

Glutamate is the predominant excitatory neurotransmitter in the mammalian central nervous system (CNS). In addition, its also the major neurotransmitter at the drosophila neuromuscular junction (Janand Jan, 1976). Glutamate, as the case with many of the other neurotransmitters, is stored in synaptic vesicles and released upon stimulation (Daniels et al., 2008).

The homeostasis of the glutamatergic system is maintained by a set of transporters present in membrane and in the membrane of synaptic vesicles. The family of vesicular glutamate transporters in mammals is comprised of three highly homologous proteins: VGLUT1-

3. VGLUTs are regulated developmentally and determine functionally distinct populations of glutamatergic neurons. Controlling the activity of these proteins could potentially modulate the efficiency of excitatory neurotransmission (Daniels et al., 2008). VGLUT1 is localized mainly in the neocortex (layers I–III), entorhinal and piriform cortex, hippocampus, amygdala and subiculum. VGLUT2 can be observed in olfactory bulb, layer IV of the cerebral cortex, granular layer of the dentate gyrus, thalamus, hypothalamus and in the brain stem. In the cerebellum, VGLUT1 is expressed in parallel fibers and VGLUT2 in climbing fibers and in Purkinje cell dendrites. Such a complementary distribution has inspired scientists to make an attempt to functionally categorize glutamatergic neurons on the basis of the VGLUT isoform expressed. They have proposed that VGLUT1 is present at synapses with low release probability, which are known to exhibit long-term potentiation (LTP), whereas VGLUT2 is expressed in synapses that exhibit high release probability and long-term depression (LTD). It was in line with the data that had revealed the highest level of VGLUT2 detected early in the development (Jan et al., 1976; Daniels et al., 2008).

In addition, the vesicular glutamate transporter (dVGluT) is widely expressed throughout the *Drosophila* nervous system. Thus, glutamate is an important neurotransmitter in flies. Many putative Glutamate receptor (GluR) subunits are expressed in neurons. However, most of these neuronal glutamate receptors remain uncharacterized, and some of the ones that are turn out to be inhibitory (Mahr and Aberle, 2006; Daniels et al., 2008).

In the larval CNS, previous studies found synaptic dVGluT in all brain lobe compartments except in the mushroom body. Similarly, in the adult CNS, glutamatergic synapses are abundant throughout all major brain structures except the mushroom body. In addition, prior researchers have identified that the larval ventral nerve cord neuropil compartment

is rich in glutamatergic synapses. The glutamatergic system in *Drosophila* highlights the prevalence of glutamatergic neurons in the CNS in flies and presents tools for future study and manipulation of glutamatergic transmission mechanisms (Mahr and Aberle, 2006).

Expression of V-Glut in L3

V-Glut Gal4 appears in a large population of larval primary neurons (Figure 6 panel a, yellow arrow). They are large, close to the neuropile and have differentiated axon tracts and terminal branches (Figure 6 panel b, white arrows). These are terminal branches, which are differentiated (Figure 6 panel b, yellow arrows). Based on my data, the majority of lineages of the brain and ventral nerve cord possess V-Glut-positive neurons. In each lineage, roughly 2-5 neurons (that is, about 25-50%) of neurons are positive. Expression levels vary. One lineage, identified as TRdm, stands out because it has more than 10 neurons and is brightly labeled (Figure 6, white arrow, panel c). The branching of this lineage is at the tip of the ventral nerve cord, corresponding to part of the gustatory center (Figure 6, white arrow, panel c).

Expression of V-Glut in Adult

As in the larva, most, if not all lineages contain V-Glut-positive neurons. Based on cell size, we are able to differentiate between primary and secondary neurons (Figure 6 panel e, white arrow). The larger set of neurons are primary neurons and the smaller ones based on relative size are secondary neurons.

I noted that the expression of V-Glut-Gal4 is peculiar, in that expression was faint in most of the neurites forming the neuropil, and stronger in the cell bodies. This may be an attribute of this particular driver line. As an exception, on secondary lineage, DALv2, and the compartment it forms, the ellipsoid body, are labeled strongly by V-Glut-Gal4 (Figure 6

panel d, yellow arrows). Also, neurons of the antennal lobe lineages stand out by a moderately stronger expression than most other neurons. These lineages are centered around the antennal lobe and send their dendritic projections to the antennal lobe, and their axons towards ALT (Figure 6, white arrow, panel e), which then projects towards the calyx (a higher olfactory center). In particular, lineages BAlc and BAmv3, shows stronger than average labeling (Figure 6, panel f).

2) Cha-Gal4: cholineacetyltransferase expressed in cholinergic neurons

Drosophila melanogaster offers a favorable and interesting biological system for studying acetylcholine (ACh) metabolism in animals (Salvaterra and Kitamoto, 2001). ACh is thought to be the primary neurotransmitter in arthropods and significant levels of the biosynthetic enzyme cholineacetyltransferase (ChAT) are present in most central neurons of the insect nervous system. The gene for ChAT has been identified and mapped in *Drosophila* (Salvaterra and Kitamoto, 2001). The levels of ChAT activity have been manipulated in intact flies via altering the temperature, and the effects of these manipulations resulted in physiological and behavioral changes including paralysis, and altered courtship patterns (Salvaterra et al., 1987).

The relationship of ChAT activity to ACh levels in the nervous system is complex and controversial. The enzyme reaction involves acetylation of choline using acetyl-CoA. Choline can also be utilized in other metabolic pathways, mainly in phospholipid synthesis, regulation of ChAT activity could serve as an attractive and selective target for the *in vivo* control of ACh synthesis. Indeed, ChAT activity has been implicated as a regulatory step for ACh production in a number of early studies (Salvaterra and Kitamoto, 2001). Others have

argued against ChAT being a regulatory and/or rate-limiting step in Ach production on theoretical as well as practical grounds and have attempted to further try to investigate alternative hypotheses for the biochemical control point(s) regulating Ach production (Salvaterra, 1985).

Expression of Cha-Gal4 in L3

My data confirm that Cha-Gal4 is expressed in most neurons of the majority of lineages (Figure 7 panel a, white arrows). According to the literature, it is known that more than 80% of neurons are Cha-positive (Figure 7 panel c, magenta arrows). In the larval brain, one observes expression in differentiated primary neurons (Figure 7 panel b, white arrows). As in the case of V-Glut, Cha-gal4 is strongly expressed in cell bodies, but also labels extensive network of neurite arborizations (Figure 7 panel c, yellow arrows). Cell bodies or axon tracts of secondary neurons don't express Cha (nor V-Glut), because these cells are not yet differentiated. No particular lineage stands out with respect to the intensity of Cha expression (Figure 7).

Expression of Cha-Gal4 in the Adult

As in the larva, the entire neuropile is labeled. In the cortex one can distinguish the large cell bodies of primary neurons (Figure 7 panel f, magenta arrow) from the smaller, more numerous secondary neurons. Cha expression is strong in the lineages of the mushroom body: these neurons, forming the calyx, peduncle and bifurcated lobes stand out from the rest of the neuropil (Figure 7). Furthermore, parts of the central complex, including the ellipsoid body and two layers of the fan-shaped body stand out by higher expression levels of Cha-Gal4.

3) GAD-Gal4: glutamic acid decarboxylase1 expressed in glutamatergic neurons

Inhibitory role of GABA in the insect brain

As in vertebrates, GABA is the main inhibitory neurotransmitter in the insect brain. GABA function in the context of local inhibition has been studied in the antennal lobe (Jackson et al., 1990). Three classes of neurons form synapses in the antennal lobe of flies, the insect counterpart of the vertebrate olfactory bulb: olfactory receptor neurons, projection neurons, and inhibitory local interneurons (Ng et al., 2002). Previous research has used a genetically encoded optical reporter of synaptic transmission targeted to each of these classes of neurons, and visualized population responses to natural odors. The activation of an odor- specific ensemble of olfactory receptor neurons leads to the activation of a symmetric ensemble of projection neurons across the glomerular synaptic relay. Virtually all excited glomeruli receive inhibitory input from local interneurons firing. These circuits may serve as foundation to generate higher order features from afferent activity patterns (Jackson et al., 1990).

GABA is a major inhibitory neurotransmitter in *Drosophila* and other insects and distributed in large numbers of neurons. We confirmed some previous reports for *Drosophila*: for example, the presence of GABA in the centrifugal neurons C2 and C3 identified by Gal4-driven GFP (Liu and Davis, 2008). GABAergic neurotransmitter systems are critical for many cognitive processes such as learning and memory. Previous studies have identified a single neuron in each hemisphere of the *Drosophila* brain as a GABAergic neuron that broadly innervated the mushroom bodies (Jackson et al., 1990; Liu and Davis, 2008).

Expression of Gad-Gal4 in L3

GAD- gal4 is expressed in many neurons (Figure 8 panel a, yellow arrows), including a large fraction of the Kenyon cells of the mushroom body (Figure 8 panel b, white arrow). The vertical and medial lobes and the posterior brain surfaces are shown and their neurons express Gad-gal4. Likewise, the antennal lobe is highly enriched in GABAergic terminal arborization (Figure 8 panel c, blue arrow, yellow arrows). The emitted signal is stronger than its adjacent compartments, but not as intense as seen in the mushroom body. As expected, no secondary neurons are expressed labeled in the larva (Figure 8).

Expression of Gad-Gal4 in the Adult

As in the L3 larva, GAD-Gal 4 is expressed in a large number of neurons, belonging to virtually every lineage, at the adult stage (Figure 8 panel d). Both primary and secondary neurons are labeled (Figure 8 panels e and f, white arrows). The mushroom body neurons express this driver (Figure 8).

I have to conclude that the expression of this GAD-Gal4 line does not faithfully reflect the pattern of GABA-ergic neurons. Thus, using antibodies against GAD (Ng et al., 2002; Liu and Davis et al., 2008) show a much sparser pattern of labeled neurons than GAD-Gal4.

Moreover, the neurons of the mushroom body, which are GAD-Gal4-positive, are not known to be GABA-ergic.

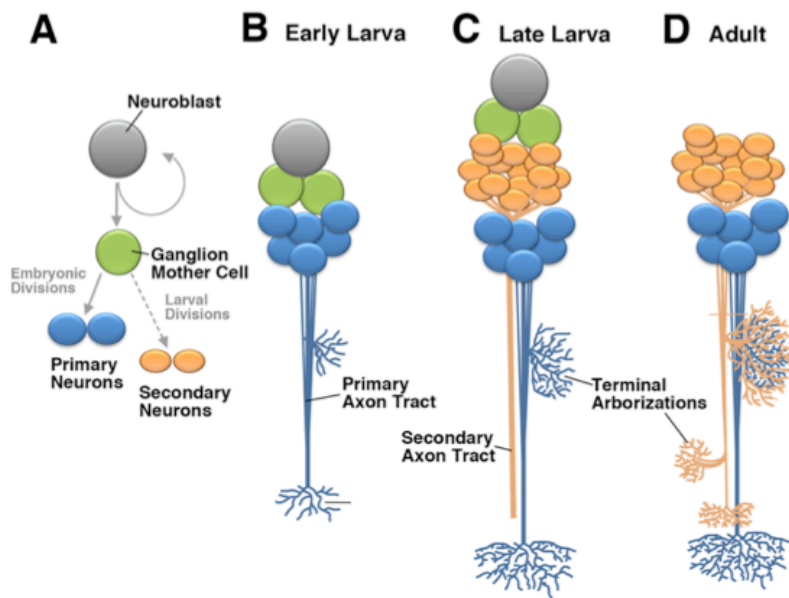


Figure 1: The evolution of lineages of Drosophila neuroanatomy, neuroblasts are born in the embryo which then give rise to primary and secondary neurons through an invariant of several asymmetric divisions (A). Primary neurons differentiate in the late embryo and form primary axon tracts (PAT) and terminal arborizations in the larval brain neuropile (B). During later larval stages (C) neuroblasts produce secondary neurons, which form secondary axon tracts (SATs) along their primary counterparts. Secondary neurons, along with the majority of primary neurons, differentiate during metamorphosis to form the adult brain (D). Subsequent figures are structured as explained here. This Figure is adapted from Cruz et al., 2013

Group 1:

- 1) TRH-Gal4: tryptophanhydroxylase expressed in serotonergic neurons

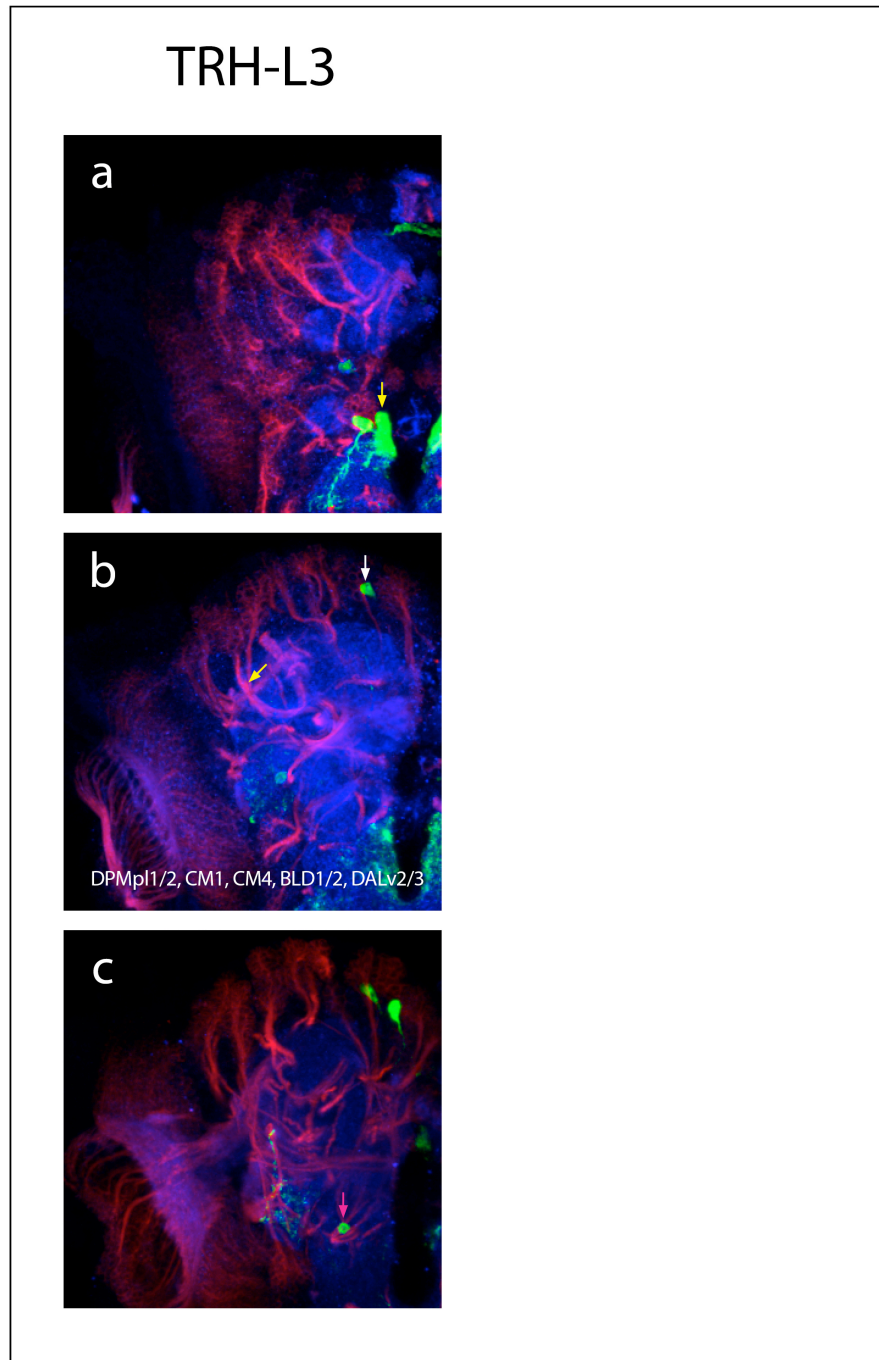


Figure 2: Lineage identity of larval TRH neurons. Lineage identity of these neurons, established in this paper, is generated from Z-projections that were generated from five consecutive frontal confocal sections of L3 and adult brain hemisphere (B: posterior level, C: central level. Dorsal is up, medial to the left. TRH neurons are labeled by TH- Gal4 > UAS-GFP (green); brain neuropile is labeled by anti-DN-cadherin (blue) along with secondary lineages. Dorsal is up, medial to the left. Each lineage forms a cluster of neuronal cell bodies at the periphery of the brain (examples shown by white arrowheads in B) from which a tract of axons (secondary axon tract, SAT) projects radially into the neuropile.

2) TH (ple)-Gal4: tyrosine hydroxylase expressed in dopaminergic neurons

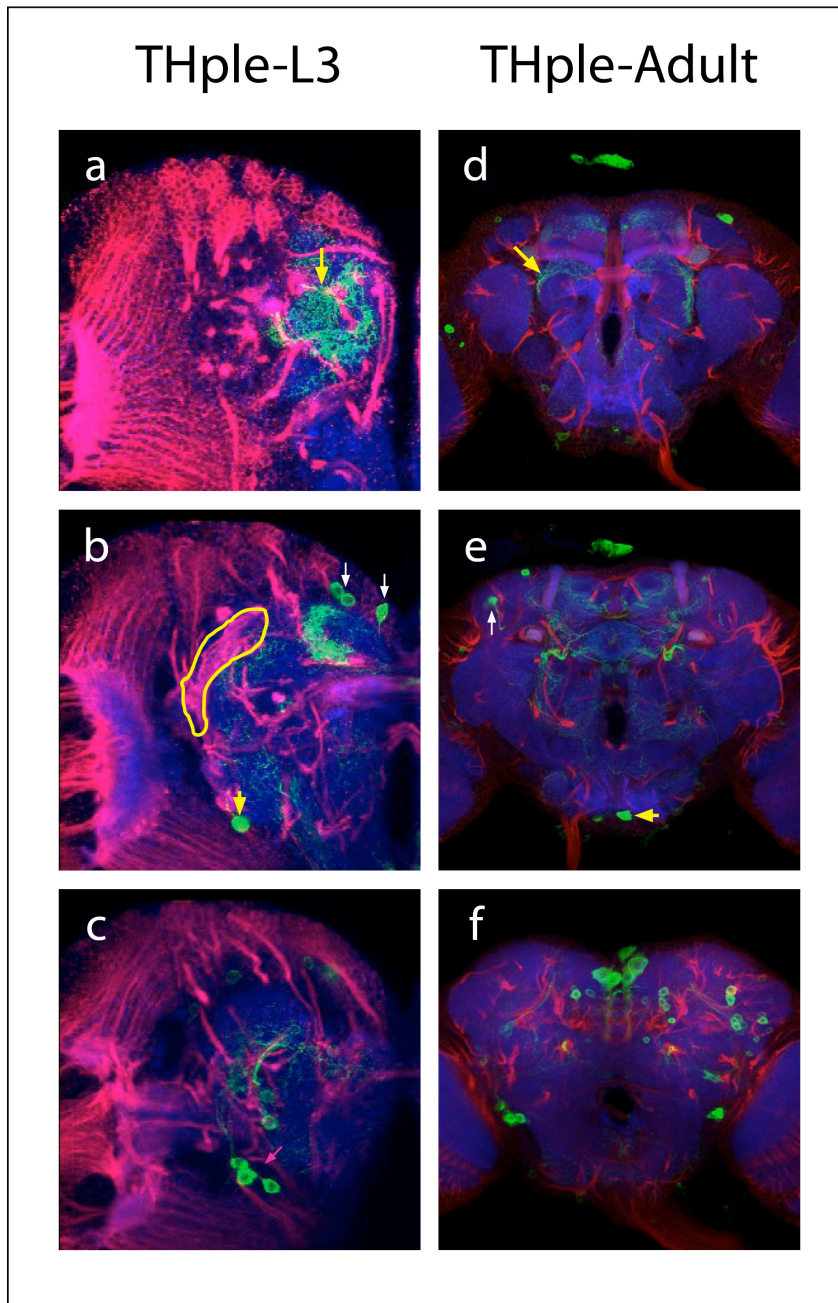


Figure 3 Lineage identity of larval TH (ple) gal4 neurons. Panels were generated as described in Figure 2. Primary neurons are shown (yellow arrows), secondary neurons (white arrows). Network arborizations are outlined in yellow.

3) Tdc-Gal4: tyrosine carboxylase

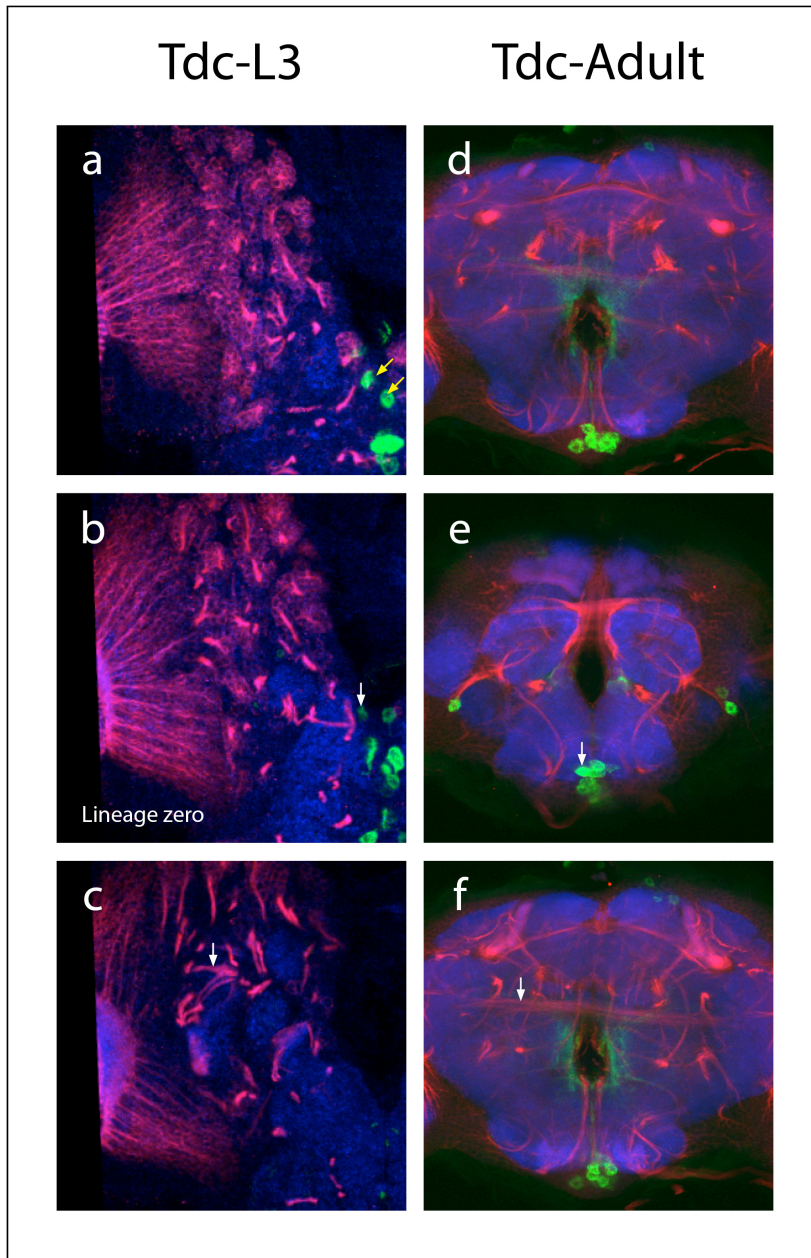


Figure 4 Lineage identity of larval Tdc gal4 neurons. Panels were generated as described in Figure 2. Primary neurons are shown (yellow arrows), secondary neurons (white arrows). Network arborizations are in red. One lineage identified, lineage zero.

4) Npf-Gal4: neuropeptide F expressed in peptidergic neurons

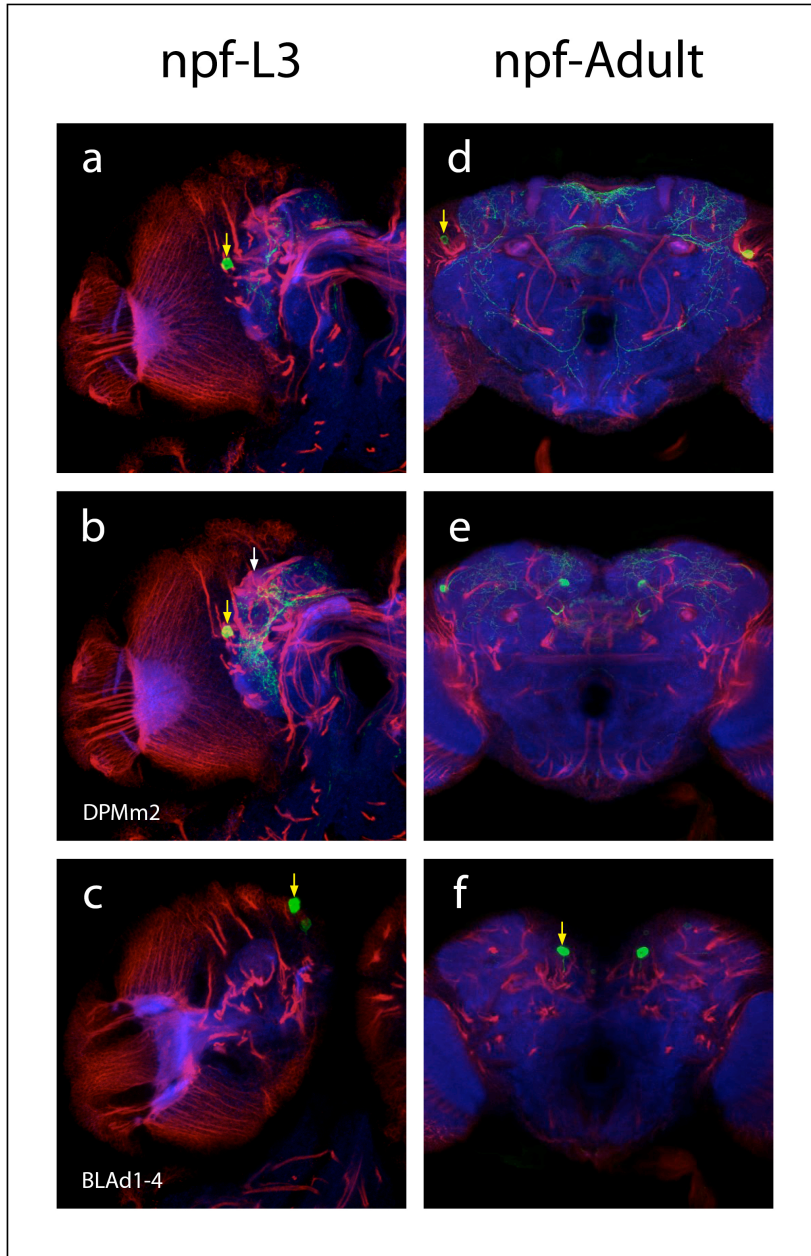


Figure 5: Lineage identity of larval Npf Gal4 neurons. Panels were generated as described in Figure 2. Primary neurons are shown (yellow arrows), secondary neurons (white arrows). Network arborizations are in red. Two lineages identified DPMm2 and BLAd1-4

Group 2:

1) V-Glut-Gal4: vesicular glutamate transporter expressed in glutamatergic neurons

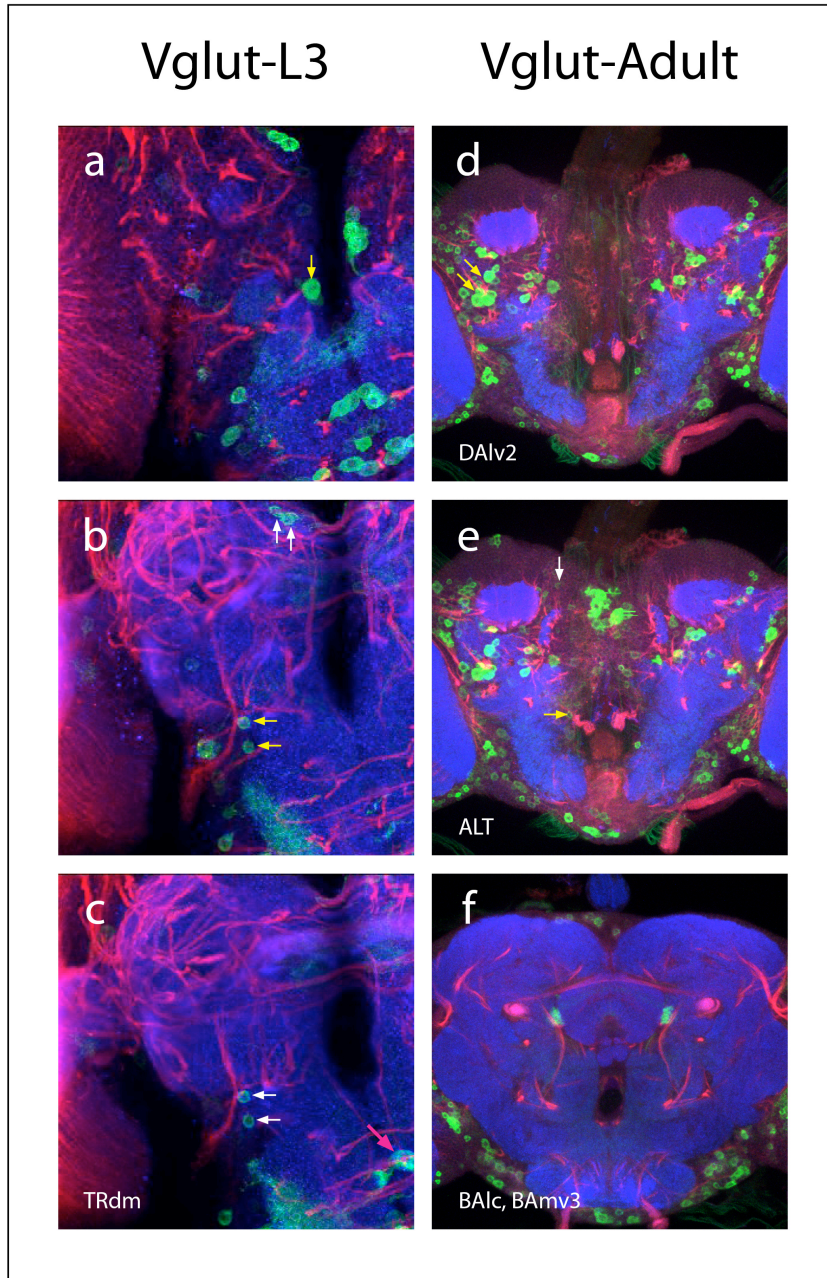


Figure 6: Lineage identity of larval V-Glut Gal4 neurons. Panels were generated as described in Figure 2. Primary neurons are shown (yellow arrows), secondary neurons (white arrows). Network arborizations are in red.

2) Cha-Gal4:Cholineacetyltransferase expressed in cholinergic neurons

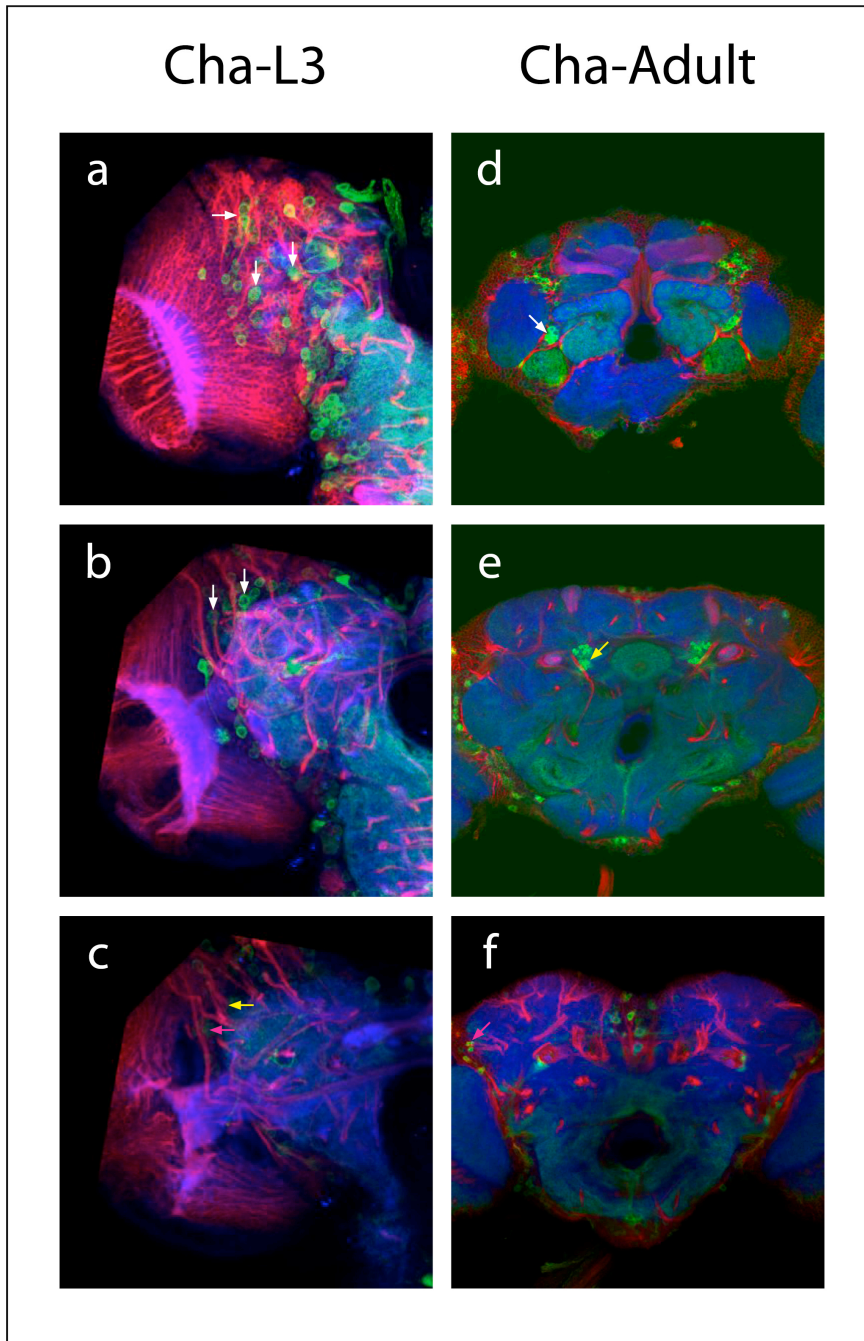


Figure 7: Lineage identity of larval Cha gal4 neurons. Panels were generated as described in Figure 2. Primary neurons are shown (white arrows), secondary neurons (yellow arrows). Network arborizations are in red (magenta arrows).

3) GAD-Gal4: glutamic acid decarboxylase1 expressed in glutamatergic neurons

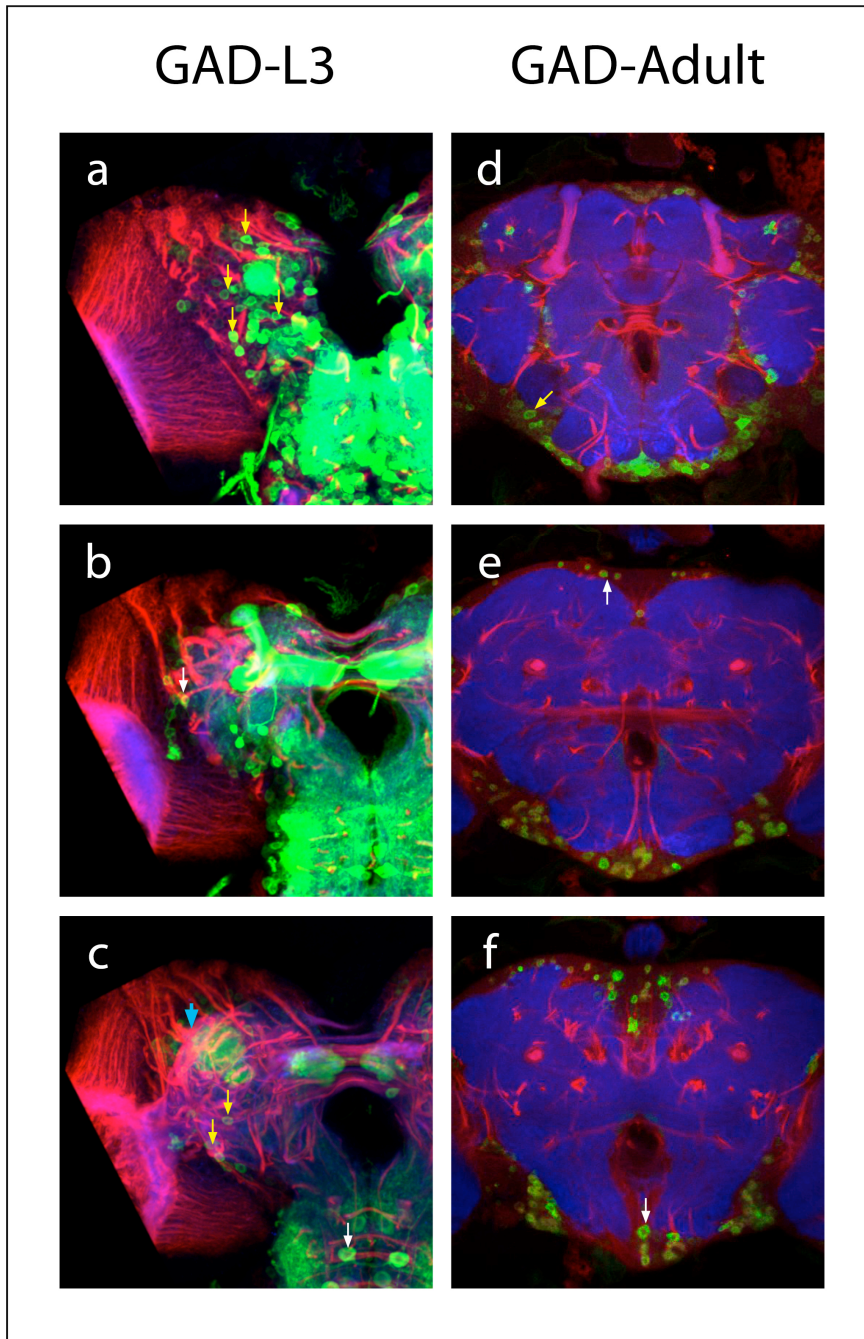


Figure 8: Lineage identity of larval GAD gal4 neurons. Panels were generated as described in Figure 2. Primary neurons are shown (yellow arrows), secondary neurons (white arrows). Network arborizations are in red (large blue arrowhead).

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