The wiring diagram of antennal lobe and mapping a brain circuit that controls chemotaxis behavior in the *Drosophila* larva

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Abstract

Sense of smell enables animals to detect odor cues, which is transformed into behavioral output through neuronal organization called the nervous system. To understand the function of nervous system, it is important to break it down into smaller circuits, where neurons could be identified and studied in isolation for their role in processing a stimulus or while mediating brain command in form of behavioral actions.

Drosophila larvae present unique opportunity for anatomical and functional mapping of their nervous system because of features such as numerical simplicity of neurons its nervous system is composed of, and ability to exhibit quantifiable behaviors such as chemotaxis. Here, we mapped entire antennal lobe of larval *Drosophila* with one of its circuits responsible for controlling sensorimotor transformation in lateral horn (LH) (higher brain) through a single brain descending neuron using electron microscopic 3D reconstruction.

In antennal lobe, we reported a canonical circuit with uniglomerular projection neurons (uPNs), working to relay gain-controlled ORN activity to higher brain centers like Mushroom body and lateral horn. We also found a parallel circuit with multiglomerular projection neurons (mPNs) and hierarchically organized local neurons (LNs) selectively integrating signal from multiple ORNs at the first synapse with LN-LN connectivity putatively implementing gain control mechanism that can potentially switch from computing distinguished odor signals through panglomerular inhibition to allowing system to respond to faint aversive odor in an environment rich with strong appetitive odors.

We also reconstructed and studied one of the olfactory connected circuits in the LH that was found to be influencing chemotaxis behavior in larva through a single brain descending neuron, PVM027. We found that this neuron was responsible in controlling *stop* response of chemotaxis behavior. EM reconstruction revealed its connection with variety of motor systems and SEZ descending neurons in the VNC. Connections were revealed with the peristaltic wave propagation circuit of larva, and PVM027 was found to be implementing *stop* by terminating and ceasing the origin of forward peristaltic waves.

Resumen

El sentido del olfato permite a los animales detectar señales olfativas, las cuales se transforman en comportamiento a través de la organización neuronal conocida como (el) sistema nervioso. Para entender las funciones del sistema nervioso es importante desgranarlo en sistemas más pequeños que nos permitan identificar y estudiar las neuronas aisladamente, según su capacidad para procesar estímulos o mediar órdenes al cerebro para convertirlas en acciones del comportamiento.

Las larvas de *Drosophila* ofrecen una oportunidad única para el mapeo anatómico y funcional de su sistema nervioso debido a propiedades como la simplicidad numérica de neuronas que componen su sistema nervioso y su habilidad de exhibir comportamientos cuantificables como la quimiotaxis. En este estudio hemos mapeado el lóbulo antenal de la larva de *Drosophila* con uno de sus circuitos responsable de controlar la transformación sensorial-motora en el asta lateral (LH) (cerebro superior) a través de una sola neurona descendiente usando la reconstrucción 3D para microscopia electrónica.

Hemos presentado, en el lóbulo antenal, un circuito canónico con proyecciones neuronales uniglomerulares (uPNs) responsables de transmitir aumentos controlados de actividad desde sus ORN* hasta centros superiores del cerebro como el cuerpo fungiforme y el asta lateral del protocerebro. Hemos descubierto también un circuito paralelo formado por neuronas con proyecciones multiglomerulares (mPNs) y neuronas locales (Lns), organizadas jerárquicamente, que integran selectivamente señales desde múltiples ORNs a nivel de primera sinapsis con conectividad LN-LN implementando aparentemente un mecanismo de aumento de control que potencialmente puede intercambiar señales olfativas distintas computacionalmente a través de inhibición panglomerular permitiendo al sistema responder a olores vagamente aversivos en un ambiente rico en fuertes olores apetitosos.

También hemos reconstruido y estudiado uno de los circuitos olfativos que conectan con el LH conocido por influenciar la quimiotaxis de la larva a través de un sola neurona cerebral descendiente, la PVM027. Hemos descubierto que dicha neurona es la responsable de controlar la respuesta *stop* en el comportamiento de quimiotaxis. La reconstrucción por EM revela su conexión con una variedad de sistemas motores así como neuronas

descendientes SEZ en el VNC. Observamos dichas conexiones gracias al circuito de propagación de onda peristáltica de la larva, y descubrimos que la PVM027 implementa la señal de *stop* terminando e interrumpiendo el origen de la onda peristáltica.

Preface

Connectivity is the order of the world. From politics to economics to science, connections have always been considered important for growth and development. The most amusing fact about connections being that it is seeked by everyone and everything from living to non-living world. In living systems, variety of connectivity patterns could be observed shaping from cells to tissues and organs. However, the best of connectivity could be studied in nervous systems where, connecting neurons shape a network, and whose output is often translated into observable behaviors. For decades, various nervous systems have been studied to understand the pattern of connectivity of neurons, encoding for some functions. After the pioneering work of Sydney Brenner and colleagues on connectomics of *C. elegans* in 1986, no other connectome could be made available to study. As the urge to understand the functioning of brain grew exponentially, and with the advancement of tools and techniques, newer mapping strategies are being employed to study the connections underlying variety of functions and probably answer to the core question of neuroscience, "how brain works?"

With the advancement of Electron microscopic techniques, and its integration with the advanced computational tools along with availability of cutting-edge scientific infrastructure, it seems possible that the secret of features such as decision making or control of behaviors could be revealed in a short span of time. Due to its complex function, and unavailability of tools to study it, brain is among the organ we know least about. As the human population on the planet is expanding, the need for better understanding of brain function is growing parallely. This is absolutely important in order to better understand and treatment of a broad spectrum of psychological and neurological diseases, whose numbers are also growing with expanding population. To gain this knowledge on most complex result of evolution, it requires a systematic approach. This is where various animal models come into picture that are being studied to address fundamentals questions of neuroscience.

In this study, we aimed to produce and look into the olfactory connectome of *Drosophila* larva and mapped one of the brain circuits controlling odor-driven navigation behavior.

This would help to understand the mechanisms of olfactory signal processing in the antennal lobe with control of brain on production of behavior.

Contents

Chapter 1: Introduction

Neuroscience is a multifaceted field of study of nervous system of animals but the main aims of neuroscience include structure and function of neurons, both in isolation as well as in structured form by understanding neural circuits (Ohyama, Schneider-Mizell et al. 2015), which ultimately leads towards understanding the most complex organ among all animals, *the brain* . Neurons are connected to one another by various types of connections, called synapses (Foster and Sherrington 1897). They are either connected through chemical synapses or electrical synapses (Phelan et al., 2001, Liu et al., 2016). Chemical synapses act as junctions and provide passage to chemicals such as neurotransmitters from one neuron to another and as a result, the neuron at the receiving end is either activated or inhibited depending on the nature of the neurotransmitter. The neuron that provides neurotransmitter is called presynaptic whereas the neuron at the receiving end is termed as the postsynaptic neuron. Electrical synapse are the other kind of physical connection among neurons where the membranes of pre- and postsynaptic neurons are connected via physical channels that has the ability to pass the electric current through circulation of molecules like ions and cAMP (Phelan et al., 2001, Liu et al., 2016). This leads to change in voltage in the postsynaptic neuron which is dependent on the voltage change in its presynaptic partner. Both of above kinds of connections are used to convey the message further in a neural circuit which gets translated into behavior generation. In this study, we used synapses to reconstruct the connectivity between neurons of some specific centers in central nervous system (CNS) of a *Drosophila* larva using a technique called EM-reconstruction (explained later under Planning and Strategy section).

Modern neuroscience research started more than a century ago but answers to a great range of fundamental questions remain unknown till date. Today, a wide variety of model organisms are used to study specific questions. These organisms range from *C.elegans* to primates and humans. The complexity in the answers to the research questions usually raises with the numerical complexity of the nervous system of the model organism. Therefore, to understand the basics of behavior and function of circuits, it is of great ease to employ the model that exhibits specific set of behavior with manageable numerical simplicity.

Drosophila melanogaster emerged as one of the most powerful model organisms for biological science research almost a century ago, but it was considered for neuroscience research since the emergence of behavioral neurogenetics field (Benzer, 1967). The use of *Drosophila* as a model kept rising ever since due to its short life cycle, small size, non-parasitic nature, ability to be grown in lab in large populations etc. Along with this, availability of genetic tools and resources to manipulate the nervous system, made this model more genetically approachable for research purposes over time (Li et al., 2014). It also displays variety of behaviors displayed in large animals but *Drosophila* achieves these by only 100,000 neurons of which it's brain is composed of. This feature alone makes this animal numerically simpler, in order of magnitudes when compared with humans whose brain is made up of 86 billion neurons, or even mouse, whose brain has around 70 million neurons. This implies to the fact that a fly only has a minimal brain makeup with minimum number of neurons necessary for behavior and hence, the redundancy is cut-down to a great extent contrary to higher animals. Redundancy in nervous system refers to repeatition of similar connections involving more neurons performing similar function. This attributes to additional complexity in the nervous system resulting in more neurons and repeated connections, thus making it more difficult to trace and study.

Life-cycle of *Drosophila* progresses through various stages of development from fertilized egg to emerging adult. While in larval stage, *Drosophila* encounters three stages of metamorphosis viz L1, L2 and L3 also referred as 1st, 2nd and 3rd instar larval stages. The nervous system of larva is composed of 10,000 neurons and it can exhibit variety of behaviors except mating. Some of behaviors exhibited by larvae include foraging, odor-driven navigation or chemotaxis behavior, avoiding dangers(Gomez-Marin et al., 2011, Asahina et al., 2009, Gershow et al., 2012, Fishilevich et al., 2005, Ebrahim et al., 2015). Due to this ability, larval *Drosophila* makes it even simpler of a model organism to be studied for various questions in biology and neuroscience (Python et al., 2002).

1.1 Aims and objectives of this study

1.1.1 To understand circuit principles for signal processing in antennal lobe of *Drosophila melanogaster*

In this study, the entire antennal lobe of first instar larval *Drosophila* was undertaken for reconstruction and the same was attempted for identification and characterization for all the neurons having immediate connectivity with the olfactory receptor neurons (Masuda-Nakagawa et al., 2009, Python et al., 2002). This work contributes to understanding the circuit principles for the processing of various olfactory stimuli at the first sensory neuropil i.e, antennal lobe (AL), and will facilitate the wiring diagram for the larval AL, which is necessary to study the function of circuits (Chou et al., 2010, Python et al., 2002).This study also revealed the existence of all types of neurons of the larval antennal lobe especially most LN types as well as the entire group of multiglomerular projection neurons (mPNs). This would potentially add to the novelty in our findings as most mPNs and LN, types and connections in the larva are still unknown. Some of these LNs were also been characterized for their neurotransmitter profiles that led to a very positive speculation of their important role in processing olfactory stimulus. The main goal of the project was to clarify the processing of various types of olfactory stimuli by networks of various local neuron types and projection neuron types at the first synapse.

In order to understand the brain functions and behavior generation, it is important to break it down to smaller units called neural circuits. A neural circuit is a functional organization of interconnected neurons with ability to self-regulate through feedback loop. These smaller circuits units could be responsible for specific sets of behavior/function or can be helpful for inter- or intraregional processings. One way to achieve this is to study neurons in isolation as well as their immediate connectivity (Ohyama, Schneider-Mizell et al. 2015).

Sensory perception is crucial for the survival of animal life, and starts with interaction of neurons with the external environment by the virtue of specialized neurons known as sensory

neurons. Sensory neurons possess the ability to detect specific external cues. This is called stimulation. Stimulation leads to firing of neurons through a change in electrical properties of a neuron. This is read as neuronal activity or responsiveness and propagates further to the neurons connected with the activated sensory neuron and may lead to generation of behavior. Behavior generation is important for the survival of animals and is presented with features such as foraging, avoiding dangers, opportunity to mate etc. Depending on the nature of cues present in the external environment and their importance, animals have evolved with various types of sensory neurons.

The olfactory system is one of the important sensory modalities across animals. Like any other sensory system, olfaction also has its own underlying circuitry for processing of odor cues or stimulus, which are represented as olfactory signals. This includes stimulus processing, signal filtering and relay to other brain centers, multisensory integration, learning and memory, innate response and sensorimotor organization. (Gomez-Marin et al., 2011, Asahina, Louis et al., 2009, Schulze et al., 2015, Gershow et al., 2012, Gepner et al., 2015). All the above features combine at a functional level to give rise to decision making for the animal. The decision is then manifested in the form of behavior control. Behavior across animals has been shaped, conserved and evolved, and some typical behaviors include sensing danger, food, competitors or mate. Olfaction has been described to be playing a crucial role in the generation of various types of behavioral features, but the principles and mechanisms employed remain largely elusive till date. Here we attempt to dissect the complete antennal lobe circuitry of *Drosophila* larva using EM reconstruction and study various channels for processing specific olfactory information and their affect on entire olfactory system of larva.

Specific aims of this project included a proper reconstruction of antennal lobes of 1st instar larva using 3D EM-reconstruction with identification of neurons and analysis of circuit computations in the larval olfactory system.

1.1.2 To study sensorimotor control of *Drosophila* larval chemotaxis through a single descending neuron

Transformation of sensory information to behavioral control is one of the biggest purpose of nervous system across animals (Asahina et al., 2009, Gomez-Marin et al., 2011). In order to achieve this, animals typically employ cascade of connections among neurons forming a network, sometime chains. In higher animals, the network is usually complex because of more number of neurons and higher redundancy, but in larval fruit fly, some networks controlling specific behavioral actions can be fairly simpler to understand. We investigated one such network downstream of olfactory system of larval *Drosophila* and found a descending neuron PVM027 (Posterior ventro-medial 027), which was found to be influencing odor-driven navigation or chemotaxis behavior in the larva. This was revealed in a loss-of-function chemotaxis screen. We attempted to study the connections of PVM027 descending neuron for a comparatively simpler network of stimulus processing in brain that can process and relay important sensory information while transforming the same into strong behavioral phenotype.

To understand this kind of circuit in a more comprehensive manner, it is of equal importance to unravel the upstream and downstream connectivity of this descending neuron PVM027. Specific aims of this project included reconstruction of presynaptic and postsynaptic circuitry of PVM027 descending neurons, which would present a map of a sensorimotor circuit that influences chemotaxis behavior in the larva with mechanisms of chemotaxis control.
With recent literature, it was found that neurons in similar motor circuits bear similar intrinsic properties, such as electrical properties. Differences in electrical properties is one of the features important for selective recruitment of downstream neurons. Ability to selectively recruit neurons is also called dynamic nature of a neuron or circuit. It was shown that the motor circuits were non-dynamic in nature, and the differences in electrical properties was due to the upstream connectivity.(Zwart et.al. 2016). If this is the case, it will be of great interest to also study this kind of brain command circuit for their role in imparting dynamism to the generation of behavior.

1.2 Planning and strategy

To be able to view the architecture of the neural network of antennal lobe, it was of equal importance to achieve a connectivity diagram downstream of ORNs. The next step followed with the identification of already known neurons and use them as a basis to deepen the understanding of AL network. While some of the neurons in the AL have already been published, some of them were completely unknown. After completing the connectivity downstream, it was important to get some handle on these unknown players for their potential role in relay and processing of olfactory signal to the higher brain.

1.2.1 Electron microscopy reconstruction

High-resolution image data is prerequisite in order to achieve diagrams where hundreds and thousands of components are to be studied for their connectivity and interactions. Also, it is of great importance to acquire and arrange the data in such a way that loss of information could be minimized at the best. EM reconstruction evolved as one of the greatest tool to achieve this kind of mammoth task which enables acquiring large image datasets of biological samples (such as larval brain), which then could be visualized as a 3D volume and annotated with careful review to form a network providing a comprehensive knowledge and basis to speculate on potential functions of distinct circuits of neurons and help envision plans for further experiments (Schneider-Mizell et al., 2016).

All synaptic partners of all 21 ORNs were reconstructed from a serial section Transmission Electron Microscopy (ssTEM) brain-wide (Saalfeld et al., 2009) dataset for both antennal lobes of a 1st instar larva. All the partner neurons were reconstructed with annotation of all the synapses in a single, complete CNS from a 6 hours old wild type *Drosophila* larva imaged at 4.4 x 4.4 x 50 nm resolution. The volume can be found at http://openconnecto.me/catmaid/, titled ”'acardona_0111_8'.

1.2.2 Reconstruction of neurons from EM volume using web-based software

Images generated withthe specifications mentioned in the last section by EM, transforms into a huge dataset. Here, the main challenge is the maneuverability in the dataset. This was made possible by use of a web-based software Collaborative Annotation Toolkit for Massive Amounts of Image Data (CATMAID). (Schneider-Mizell et al., 2016, Saalfeld et al., 2009) This is the main tool for navigation, interaction and annotation of biological image data and in a collaborative manner. The inspiration for the interface was derived from google maps and is based on similar concepts of navigation to explore 3D biological image data. CATMAID possesses unique feature of its partially decentralized architecture (Saalfeld et al., 2009).
More information on CATMAID could be found at http://catmaid.readthedocs.io/en/stable/

As part of a collaboration with Janelia Research Campus to access and work on this image database, we started with reconstruction of ORNs and its downstream circuitry. The reconstructed neurons are carefully reviewed for any errors in reconstruction. Since neurons are reconstructed manually, the data generated is prone to human errors. So,careful reviews are performed on reconstructed neurons to identify and remove human errors. ORN reconstruction was followed by review and identification of ORNs with help from published literature (Masuda-Nakagawa et al., 2009). Further, we selected some of the screened neurons from Larval Olympiad and identified a brain descending neuron. This neuron was named as PVM027. Loss-of-function chemotaxis screen in the lab revealed its role in influencing chemotaxis behavior in larval *Drosophila*. We then identified this neuron in the EM dataset and started with the reconstruction of its upstream and downstream circuitry. Circuit of PVM027 neuron provides a tractable opportunity to study processing of sensory stimulus, multisensory integration and sensorimotor control of larval chemotaxis in *Drosophila*.

1.3 Olfactory system of *Drosophila* larva and it's importance

Animals use external cues in order to interact with the external environment. This interaction has played crucial role in their survival. Chemosensation is one such interaction where animals sense for the presence of chemical cues in their habitat (Gomez-Marin et al., 2011). Upon sensation, animal decides on the nature in which it should respond to specific cue(s). This response could be learned or innate (Gerber and Stocker 2007). Most of these stimuli result in movement of animal, either towards or away from the source. This response is termed as chemotaxis behavior (Gomez-Marin et al., 2011, Asahina, Louis et al., 2009, Gershow et al., 2012, Gao et al, 2015).

A large number of these chemical cues are presented to animals as odors. For this reason, the sense of smell plays a pivotal role in perception and the same is relayed to the olfactory system of animal brain for further processing. Animals decide on type of behavior to be displayed according to the nature of response towards a specific chemical stimulus or mixture of cues (Gomez-Marin et al., 2011, Asahina, Louis et al., 2009, Gershow et al., 2012).

As the study of neurogenetics evolved using *Drosophila* as a model, it is been proven to be an invaluable organism to study olfactory driven behaviors, especially chemotaxis (Gomez-Marin et al., 2011, Asahina et al., 2009, Gershow et al., 2012). There are usually two main features studied in this research field, perception and response. However, numerical simplicity also plays an important role for this qualification (Python et al., 2002, Masuda-Nakagawa et al., 2009). Like adults, larval *Drosophila* also exhibits variety of behavior types and respond to a large number of chemical cues with stereotyped neuronal identities and their behavioral manifestations (Wong et al, 2002). It has been described that odor sampling driving chemotaxis in larva is similar to sniffing in vertebrates (Gomez-Marin et al., 2011, Gershow et al., 2012) The greatest perk of using a larva instead of adult as model is the number of neurons in its brain, which is 10 times low when compared with adult fly. This makes larva a much simpler model to study for neural circuits and network than adult (Python et al., 2002).

Central nervous system of insects are compartmentalized, like higher animals. These compartments are unique in their feature of connectivity, morphological properties, physiological properties resulting in uniqueness in processing signals. Some examples of these compartments in insect brain are olfactory glomeruli in antennal lobe, mushroom body, central complex etc. (Younossi-Hartenstein et al., 2003).

These compartments are also marked as regions with high synaptic density as they are mostly neuropil. And since these compartments are arborized by different types of neurons, they differ in the pattern of signal processing and relay. The regions directly associated with olfactory system are antennal lobe (AL), Sub-oesophageal Zone (SEZ), Mushroom body (MB) and Lateral Horn (LH).

AL is the primary olfactory neuropil which is innervated by axons of olfactory receptor neurons (ORNs) (Vosshall et al., 2000, Gerber and Stocker 2007). Axons of these neurons run through antennal nerve from dorsal organ of the larva. AL is also innervated by the dendrites of olfactory Projection neurons (PNs) along with olfactory local neurons (LNs) and is equivalent to olfactory bulb of vertebrates (Chou et al., 2010, Python et al., 2002). Together, these neurons operate on the olfactory stimuli and initiate processing of odor cues right at the primary olfactory center. The processed olfactory signals are then relayed to higher brain centers for further processing by projection neurons. All AL neurons of the larva are shown to be of embryonic origin (Thum et al., 2011) and similarities have been drawn to the vertebrate system (Prieto-Godino et al., 2012). The structural assembly of an ORN axon within the AL, where they interact with other neurons, is called a glomerulus (Python et al., 2002).

The sub-esophageal Zone (SEZ) is another vast neuropil region that also acts as bridge between brain and VNC of insects. They are shown to be innervated by sensory neurons such as gustatory receptor neurons and neurons of motor circuits like feeding related motor neurons and descending neurons controlling locomotory actions (Pankrutz 2012, Fushiki et al 2016). Recent studies concluded t relationship between motor-system related SEZ neuron and olfactory related neurons along with neurons from other sensory modalities like gustation and thermosensation (Tastekin et al 2015, Python et al., 2002). Since this region seem to be

arborized by both sensory neurons as well as neurons of motor circuits, it will be important to acquire an understanding of processing of sensory signals in this region.

The mushroom body (MB) is a higher brain center in insect brain. This neuropil is innervated mainly by axons of projection neurons from sensory systems (Masuda-Nakagawa et al., 2009, Jefferis et al., 2007, Gerber and Stocker 2007) connecting onto Mushroom body intrinsic (Kenyon Cells) (Masuda-Nakagawa et al., 2009, Luo et al., 2010) and extrinsic neurons. MB is also connected to other brain regions such as LH through some local neurons. This region is associated with learning and memory in insects (de Belle and Heisenberg 1994, Gerber and Stocker 2007) and it has also been shown to be important for motor control in other insects (Campbell et al., 2010). MB has been shown to be important for olfactory behavior and hence is an important region of brain to study olfactory signal processing in higher brain (Python et al., 2002).

The LH is a vast neuropil region in the higher brain of insects. The morphology of LH is not well described but the boundary starts from ventro-lateral position to that of MB calyx (Jefferis et al., 2007) with a conserved topographical structure similar to that of the AL (Wong et al., 2002). A large number of neurons innervating this region and their roles are still heavily understudied. However, the LH has been associated with controlling some innate behaviors, like courtship in insects (de Belle and Heisenberg 1994, Kido and Ito 2002, Fisek and Wilson 2014, Luo et al., 2010). Almost all the olfactory projection neurons, both in adults as well as in larva, extend their axons and project to this region (Jefferis et al., 2007) and present a conserved, stereotyped map of AL glomerular activity in LH (Fisek and Wilson 2014). LH neurons have also been studied to integrate inputs from multiple projection neurons (Tanaka, Awasaki et al. 2004, Jefferis, Potter et al. 2007, Fisek and Wilson 2014). Because this region receives huge amount of olfactory inputs, it will be important to further investigate its role in the generation of behavior.

1.4 Olfactory Receptor Neurons (ORNs)

ORNs are first order neurons that detect odor cues as ligands that bind on specific receptor(s) that are expressed on dendrites of ORNs. Each ORN type expresses unique odorant receptor encoded by OR genes (Olsen et al., 2007, Vosshall et al., 2000, Vosshall and Stocker 2007, Mathew et al., 2013). There are 21 ORNs each side of *Drosophila* larval brain expressing 25 different receptor types (Masuda-Nakagawa et al., 2009, Python et al., 2002, Fishilevich et al., 2005) with known response profiles (Kreher et al., 2008). ORN span from Dorsal organ, which is also known as the larval nose with their axons innervating the antennal lobe in the brain (Gerber and Stocker 2007). Larval ORNs usually express one (occasionally two) olfactory receptor types (Kreher et al., 2008). Along with their unique receptor(s), all ORNs express another ubiquitous co-receptor called Orco, previously known as Or83b (Fishilevich et al., 2005).

Odor cues are detected by ORNs and represented in the antennal lobe in form of ORN activity (Asahina et al., 2009, Schulze et al., 2015), which is then picked up by projection neurons to be relayed to higher brain centers (Masuda-Nakagawa et al., 2009). Transformation of ORN activity stimulated by olfactory signals to behavior could be predicted through quantitative models (Gepner et al., 2015, Schulze et al., 2015, Hernandez-Nunez et al., 2015). Recent literature suggests that, prior to relay, signals from ORN is taken through a network of ORNs-LNs-PNs for primary level of processing right at the first synapse in antennal lobe. Each ORN have stereotyped regional organization in the antennal lobe. LNs along with other PNs, synapse here with that individual ORN, thus forming a glomerular structure. Often, each glomerulus is separated by one another through glial boundaries, growing through various developmental stages (Oland et al., 2008). There are individual glomeruli for 21 different ORNs per antennal lobe (Masuda-Nakagawa et al., 2009). Olfactory driven behavior is the result of processing of odor representation in combination, as the environment could present plethora of odors with or without prominency for single strong chemical cues. However, in larva, the exhibition of behavior has been studied from single or few ORNs being functional (Louis et al., 2008, Kreher et al., 2008, Mathew et al., 2013). This corresponds to the ability of

few select OR types to drive chemotaxis behavior in a single functional state (Asahina, Louis et al, 2009, Schulze et al., 2015, Louis et al., 2008), but with reduced efficiency (Asahina, Louis et al., 2009, Fishilevich et al., 2005).

1.5 Olfactory Projection Neurons (PNs)

Olfactory Projection Neurons (PNs) are the second order neurons relaying processed signals from antennal lobe of the brain to higher brain centers such as mushroom body and lateral horn (Das et al., 2013, Masuda-Nakagawa et al., 2009, Vosshall and Stocker 2007). These neurons have their dendrites in the antennal lobe, where they receive synapses from ORNs and also process the activity of ORNs (Python et al., 2002, Distler and Boeckh, 1996).

Typically, PN activity in adult fly have been described to be reflective of both, ORN activity, which is a readout of stimulus sensing, as well as sensing the increase in stimulus intensity (Kim et al., 2015). It has been shown that PNs integrate the activity from multiple ORNs, evoked by same/similar class of odors, through lateral excitatory connections(Olsen et al., 2007). But for larva, the environment is different with burrowing lifestyle, which is odor rich, and hence, detection of difference in concentration of odor could be the most important signal for PNs to encode (Asahina, Louis et al., 2009). There are two types of projection neurons described in *Drosophila*:

1.5.1 Uniglomerular Projection Neurons (uPNs)

Typically, PNs receive majority of their inputs from single ORN type with their dendritic innervation confined to the region of axonal arborization of connected single ORN type i.e. single glomerulus and targeted by axons of single ORN (Ramaekers et al., 2005). These PNs are termed as uniglomerular projection neurons. Uniglomerular projection neurons are the main types of projection neurons for relaying individual olfactory signal to higher brain. In larval *Drosophila,* these PN types are the most studied and well described ones (Masuda-Nakagawa et al., 2009, Thum et al., 2011). They typically extend their axons to higher brain

centers through the inner-antennocerebral tract (i-ACT) reaching first to MB calyx (Masuda-Nakagawa et al., 2009, Thum et al., 2011, Marin et al., 2002) and further to the LH regions.

They synapse extensively onto mushroom body intrinsic neurons, also known as Kenyon cells. The connectivity of PNs at the LH is understudied as the neurons in LH are mostly unknown.

1.5.2 Multiglomerular Projection Neurons (mPNs)

These projection neurons receive inputs from more than one ORN while innervating more than one glomerulus in antennal lobe while some mPNs also connect to suboesophageal region (Lai et al., 2008, Das et al., 2013). mPNs are described in adult fly, with some as GABAergic and inhibitory (Okada et al., 2009, Liang et al., 2013), but are heavily understudied due to unavailability of genetic reagents to label them. However, PNs with their dendritic processes not confined to single glomerulus have been described in larva, and are termed as wide-field projection neurons (Masuda-Nakagawa et al., 2009, Thum et al., 2011). They seem to take variety of different paths to reach higher brain and also arborize a comparatively bigger region in the higher brain (Marin et al., 2002, Okada et al., 2009, Liang et al., 2013).

1.6 Local neurons of antennal lobe (LNs)

Representation of odor cues in the brain is imparted to larval antennal lobe by ORN activity. This activity is then transmitted to higher brain centers through connecting projection neurons.

Prior to relay of olfactory stimulus to higher brain centers, a network of local neurons (LNs) operate on the signal at the first olfactory processing center, and are thus essential for computations in the AL (Chou et al., 2010, Python et al., 2002). Stimulus processing in the AL has been shown to be essential for reliable and reproducible odor representations encoded in PN activity (Bhandawat et al., 2007). In vertebrates as well as in adult fly, LNs are studied for their morphological diversities and are suggested to play important role in operations such as

decorrelation, spike synchronization and implementing gain control mechanism (Lledo et al., 2008, Chou et al., 2010, Shepherd et al., 2004, Wachowiak et al., 2006, Olsen et al., 2010) that results in broadening dynamic range of PNs (Wilson 2013). Inhibition has been shown to play important role in shaping the PN tuning in AL by dominating interglomerular excitation (Olsen and Wilson 2008). These LNs have been shown to normalize PNs by driving them to saturation scaling with total ORN population activity by the virtue of lateral inhibition (Olsen et al., 2010). There are >1500 individual LNs studied in adult fly and are suggestive of diversity in function of LNs in *Drosophila* AL. And, since the wiring of LNs have been shown to differ among individuals, they have been thought to be responsible for variations in individuals at circuit level (Chou et al., 2010). LNs in general are thought to be inhibitory and GABAergic but there are descriptions on excitatory and cholinergic LNs present (Olsen et al., 2007, Chou et al., 2010, Shang et al., 2007, Das et al., 2008, Olsen et al., 2008). A large number of these LNs are axonless (Wilson and Laurent 2005). Glutamatergic LNs of adult AL are also reported to be inhibitory in nature and constitute one-third of total LNs with the concentration of glutamate release held in interglomerular space, in contrast to GABA release, which is concentrated at intraglomerular space. Glutamatergic LNs inhibit PNs and GABAergic LNs of AL by hyperpolarization through glutamate-gated chloride channel (Liu et al., 2013, Wilson and Laurent 2005). In adult fly, LNs with distinct morphological and physiological diversity exist such as panglomerular, multiglomerular, oligoglomerular, and bilaterally projecting LNs (Chou et al., 2010, Das et al., 2013).

LNs of larval olfactory circuit are poorly studied but they are reported to vary from adults. Gain control mechanisms, similar to that of adult fly (Olsen and Wilson 2008) has been studied in larva influencing dynamic range of PNs (Asahina, Louis et al., 2009).
In the larva, some LNs are also reported to innervate AL as well as SEZ region and are thought to be interacting with feeding related gustatory neurons, connecting circuits of smell and taste (Python et al., 2002, Das et al., 2013).

Exact number and types of these LNs in larva is poorly studied but some reports on their characteristics are very much suggestive of their crucial role of inhibition in olfactory signal

processing as well as presents a great model to study variation in odor-driven behaviors among individuals. (Wilson 2013, Thum 2012, Olsen et al., 2008)

1.7 Neuromodulation in the antennal lobe

Neurons can be affected by processes other than direct synaptic transmission, chemical or electric. Neuromodulation is a phenomenon where a given population of neurons are regulated by chemicals secreted from another neuron. This process does not require physical synaptic connectivity instead the neuromodulators are received by G-protein coupled receptors expressed by the affected neurons. Some important neuromodulators in the CNS include dopamine, serotonin, histamine, acetylcholine. Neuromodulators have been shown to be responsible for variety of functions, for instance, dopamine, in *Drosophila,* can modulate variety of behavior like sleep, learning, locomotion, courtshsip (Van Swinderen et al., 2011), while histamine in honey bees antennal lobe has been shown to be an inhibitory neurotransmitter (Sache et al., 2006). This phenomenon can influence the sensitivity and alter the activity pattern of the neurons in the range of diffused neuromodulators.

In larval antennal lobe, two types of neuromodulatory neurons are described: serotonergic (CSD neuron) (Roy et al., 2007) octopaminergic and tyraminergic (lAL-1 and tdc neurons) (Selcho et al., 2014)

1.7.1 Serotonin-immunoreactive deutocerebral interneurons (CSD neuron)

CSD is a large-field serotonergic neuron described in insects such as moths (Kent et al., 1987), *Drosophila* (Roy et al., 2007, Singh et al., 2013, Dacks et al., 2006). CSD is a contralaterally projecting neuron pair with extensive branching in antennal lobes as well as higher brain centers of both brain hemispheres. In silk moth, this neuron has been described for its responses to mechanosensory stimulation (Hill et al., 2002). Also, serotonin has been described to alter the activity of PNs and LNs of antennal lobe (Kloppenburg et al., 1995) by enhancing

the response of PNs and LNs (Dacks et al., 2009). Together, these are suggestive of thresholding the odor detection by triggering the serotonin release by mechanosensory stimulation such as air currents followed by alteration in state of PNs and LNs by secreted serotonin (Roy et al., 2007). Similar feature has been described in silk moths where the response to female pheromone is modulated (Gatellier et al., 2004).

In *Drosophila* larva, CSD neurons attains basic skeleton structure, but it remodels into a more complex pattern while in pupal stage (Roy et al., 2007, Singh et al., 2013). The importance to study this neuron emerges with the anatomy of this neuron with morphology of its branching patterns and connections as described in published literature. CSD is suggested to bring about some kind of feedback integration in the antennal lobe that can alter the state of antennal lobe activity pattern (Roy et al., 2007)

1.7.2 Octopaminergic and Tyraminergic neurons (lAL-1 and tdc)

Octopamine is a neuromodulator in invertebrates and considered insect equivalent of norepinephrine. Octopamine and Tyramine are described to affect variety of behavioral manifestations like sleep, aggression, egg-laying and brain functions like learning and memory in *Drosophila* (Selcho et al., 2012, 2014). Octopamine is found abundantly in insect nervous system and is also known to function as stress hormone in insects (Verlinden et al., 2010).

Initially, the biological importance of tyramine was unclear and was only understood as a precursor molecule of Octopamine. The significance of Tyramine as neuromodulator came in light with identification of a tyramine receptor in *Drosophila* (Selcho et al., 2012) and was reported to be important for normal locomotion in *Drosophila* larva (Saraswati et al., 2004).

Two neurons, lAL-1 and tdc were reported to be OA and TA neurons respectively, sharing connections with larval antennal lobe (Selcho et al., 2014). Complete knowledge of their role in chemosensory perception is unclear, but role of OA/TA neurons in sweet taste/reward processing through dopaminergic neurons in larval mushroom body has been established

(Selcho et al., 2014). Since neuromodulators play such important roles in affecting and shaping behaviors in insects such as *Drosophila* (Van Swinderen et al., 2011), a proper understanding of functions of these neurons in olfactory circuit is crucial.

Chapter 2: The wiring diagram of a glomerular olfactory system

Berck ME, Khandelwal A, Claus L, Hernandez-Nunez L, Si G, Tabone CJ, et al. The wiring diagram of a glomerular olfactory system. Elife. 2016 May 13;5. DOI: 10.7554/eLife.14859

Chapter 3: Sensorimotor control of *Drosophila* larval chemotaxis through a single brain descending neuron

3.1 Introduction

Animals make decision through expression of various behaviors/responses in their daily lives. The majority of these decisions are based on their interaction with external environment with perception of sensory stimulus being the source point of decision making process, and are crucial for their survival.

Smaller animals are equipped with simpler nervous systems with limited number of neurons while the size of network grows with size and complexity of species (White et al., 1986, Herculano-Houzel et al., 2014). More developed animals have vast neuronal networks that take shape of organs like brain, ventral nerve cord. Neuron numbers in nervous systems can range from around 302 neurons in *C. elegans* (White et al., 1986) to around 257 billion in an African elephant (Herculano-Houzel et al., 2014) with diversification in the role of neurons within the system. Urge to understand functions of this vast and fast network of neurons remains one of the top aims of neuroscience. To achieve this, animals with comparatively simpler and tractable nervous systems with display of specific behaviors are employed as models .

We use larval *Drosophila* as a model system to study sensory-driven behavior. Larva displays variety of behaviors like foraging, sensing danger, also exhibited by higher animals including humans, but with a much simpler CNS comprised of a meagre 10,000 neurons (Gomez-Marin et al., 2011, Asahina et al., 2009, Gershow et al., 2012, Fishilevich et al., 2005, Python et al., 2002)

Most behaviors are generated in response to the sensory information such as olfactory, visual, which is relayed to the brain through multiple channels of processing and multisensory integration (Gomez-Marin et al., 2011, Asahina, Louis et al., 2009, Schulze et al., 2015, Gershow et al., 2012, Gepner et al., 2015). Behavior is usually translated in the form of motor

output that results in one or more types of movements. In *Drosophila* larval brain, similar events occur in a numerically simpler system making it an excellent model system to study the sensorimotor integration and behavior generation (Gershow et al., 2012, Hernandez-Nunez et al., 2015).

In most animals, the majority of sensory stimuli are perceived and processed in the brain and suitable actions are selected and executed by motor systems, upon implementation of command signals from brain through specific set of neurons, called descending neurons (Hsu, Bhandawat 2016, Fushiki et al., 2016, Gomez-Marin, Louis 2014). Descending neurons usually bring down the integrated and processed sensory stimulus from higher brain centers and communicate with the circuits of command execution, usually motor systems (Bouvier et al., 2015, Hsu, Bhandawat 2016) .

Descending neurons offer a great opportunity to understand brain function in a systematic manner while dissecting out various upstream circuits. These circuit could be sensory systems and brain inputs in the upstream and motor systems in the downstream. Variety of descending neurons with diverse functions have been described in insects including *Drosophila* and other vertebrates(Bouvier et al., 2015, Hsu, Bhandawat 2016, Mu et al., 2014, Fushiki et al., 2016).Since it is of great interest for neuroscience research to understand principles of sensory stimulus processing and its transformation to a quantifiable behavior type(s), studying sensorimotor control of olfactory-driven behavior in a tractable model system presents a great opportunity to understand brain function in a nutshell.

Chemotaxis behavior, in simple terms, could be described as movement or locomotory action of an animal in response to a chemical stimulus, mostly odors. Based on the nature of chemical cues or the valence of an odor, animals decide to move towards or away from the stimulus source. Valence here means animal's intrinsic attractiveness (also called positive valence) or intrinsic aversiveness (also called negative valence) towards an odor type. Encoding of these valences could be either learned or innately possessed by animal. *Drosophila* larva exhibits odor-driven chemotaxis behavior for various purposes like foraging, avoiding danger/predator etc.

(Ebrahim et al., 2015, Gershow et al., 2012, Schulze et al., 2015). This based on the quality of chemical/odor cues, whereas other aspect of olfactory-driven behavior is in its responsiveness to intensity of stimulus or concentration. Larvae have been shown to respond to stimulus intensity . For example, they tend to move towards attractive odor source by sensing its intensity and can disrupt their locomotory rhythm and turn upon sensing decrease in concentration. (Asahina, Louis et al., 2009, Schulze et al., 2015).

Larval chemotaxis is determined by three types of actions; runs altered by stops and turns, which is based on olfactory experience (Schulze et al., 2015, Gomez-Marin et al., 2011). Forward locomotion is the default direction of larval movement exhibited by runs. This is interrupted by shorter stop intervals, which is followed by animal's decision on direction of movement through turns upon sampling the environment. However, larva exhibits other actions like lateral head sweeps, also called *head casts* for sampling (Gomez-Marin et al., 2011). Sampling through head casts in larvae is better and faster when compared while in run phase. Yet another feature called *weathervaning* is exhibited by larvae for better chemotaxis where they orient their direction of run suitably without terminating it (Gomez-Marin, Louis 2014). Together, all the above facets of chemotaxis behavior with availability of high resolution imaging dataset and genetic reagents constitute larval *Drosophila* a great model to study the sensorimotor transformation underlying the control of various behavioral subtypes in brain as result of olfactory driven-chemosensation.

Studying behavioral actions with identification of its neural correlates, allows us to enhance our understanding of neural circuits constituting minute steps towards understanding brain functions and mapping. Larval brain, upon processing of the sensory inputs, commands to the motor system in a similar fashion through a number of descending neurons (Fushiki et al, 2016). We identified and attempted to investigate the role of one such descending neuron, PVM027 in larval *Drosophila* in transforming olfactory perception to chemotaxis behavior and its role in transformation of various features of chemotaxis behavior in larva. PVM027 was identified as a candidate for defect in chemotaxis behavior while screening for loss of function phenotype.

3.2 Results

3.2.1 Loss of function chemotaxis screen reveals pair of descending neurons for role in chemotaxis behavior

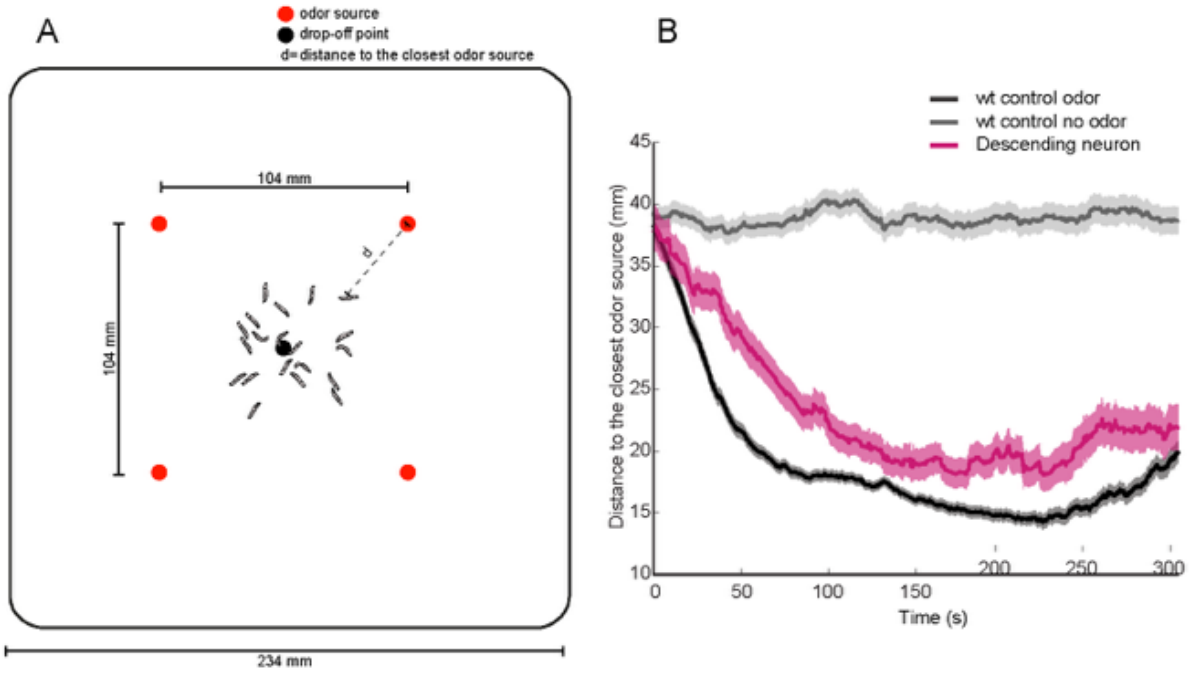


Figure 1: Loss-of-function screen for chemotaxis behavior using Split-Gal4 driver lines. A: Schematic representation of Multi Worm Tracker used for chemotaxis behavior screen. Around 20 larvae were placed at the centre (black dot) of a large petri dish covered with a layer of 4% agar. Four odor droplets of 8 µl (~15mM ETB diluted in paraffin oil) were pipetted on equidistant positions inside the lid of the Petri dish (red dots). Larvae were tracked for 5 min after the lid was closed. The dimensions of the arena and the positions of the odor droplets are mentioned in mm. **B:** Analysis of the chemotaxis screen. Time course of the average distance for positive and negative controls together with line labelling a descending neuron (magenta). Shaded area indicates the standard error of the mean.

A loss-of-function screen for chemotaxis behavior was conducted in the lab to identify candidates for phenotypes. In order to conduct the experiments, multi-worm tracker was used

to study odor-driven navigation behavior in larva (fig. 1A). While screening Split-Gal4 lines for loss-of-function results for chemotaxis behavior, one of the Split-Gal4 lines was observed to have a phenotype for odor-driven chemotaxis behavior. The line labelled a descending neuron pair. Neurons labelled by this line were silenced by targeted expression of UAS-TNT.

Split-Gal4 lines: These are transgenic lines for achieveing cellular specificity for expression of Gal4 protein. Here, the coding region of Gal4 gene is split into Gal4 activating domain and Gal4 DNA binding domain, and are driven by seperate enhancers. The expression of functional Gal4 protein is only possible in cells with both the domains and enhancers (Luan et al., 2006, Pfeiffer et al., 2010).

Multi-worm odor-source tracking experiments from the lab showed that individuals with silenced neurons maintained greater distance to odor source when compared to wild-type controls, and were imprecise in locating odor source. (Fig 1B)

Further, multiple odor-source tracking experiments (Fig. 2A) conducted by Ibrahim Tastekin in the lab showed that animals with silenced neurons labelled by SS01994 maintained longer distance to odor source (Fig. 2B,C) and had normal run speed (Fig. 2D) but turned significantly less when compared to controls (Fig. 2E). This suggested that neurons labelled by SS01994 line had a role in the animal's ability to chemotax precisely. Results from light imaging experiments showed that the line labelled pair of contralaterally projecting descending neurons (Fig. 2F). The neuron pair had posteriorly located soma in the brain lobes with contralateral dendritic processes spanning around pedunculus to LH region. The contralaterally projecting axonal processes from this neuron pair arborize regions from thoracic to abdominal segments up to A4 while descending to the VNC, and with varicosities.

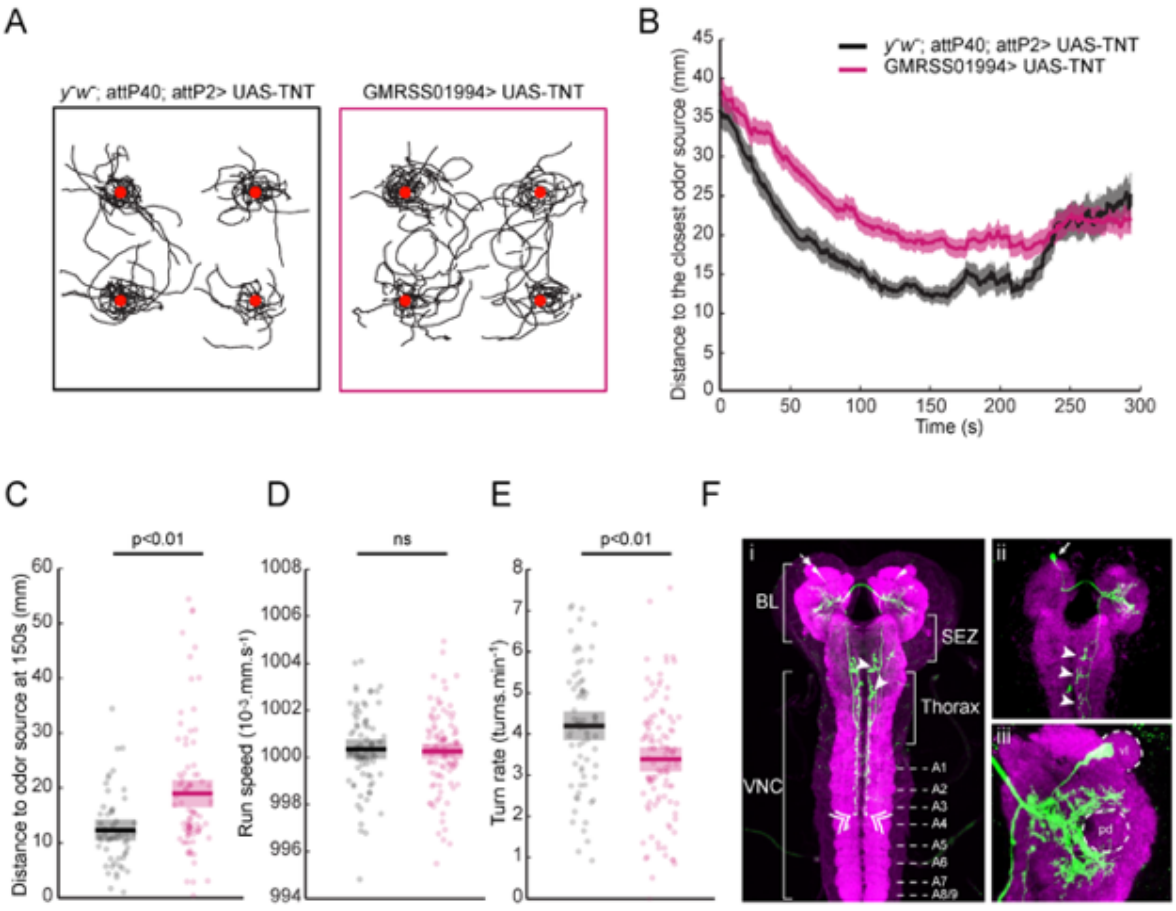


Figure 2: Analysis of its loss-of-function phenotype using Multi Worm Tracker with identification of a descending neuron A: Representative trajectories of the positive parental control (*y w*; attP40; attP2>UAS-TNT) and SS01994>TNT. Red dots indicate the positions of the odor droplets. **B:** Time course of the average distance to the closest odor source for SS01994>TNT (magenta line) and the positive control (black line). Shaded are indicates the standard error of the mean. Here, positive controls that are performed during the same day with SS01994>TNT were plotted in order to avoid the effects of day-to-day variability in chemotaxis. **C:** Quantification of the average distance to the odor source at 150th second at which a plateau was reached. In Multi Worm tracker (MWT), larval identity was lost upon larval collisions. Therefore, MWT contains only trajectories without indicating to which larvae they belong, pooling independent (belonging to different larvae) and dependent (belonging to the same larva) trajectories together. For our statistical analysis we made sure that we included only the independent trajectories, by excluding non-overlapping trajectories from the analysis. SS01994>TNT (magenta) had statistically significant average distance compared to the parental control ($p<0.01$, Wilcoxon signed rank test). Boxes indicate 95% confidence interval. Dots indicate individual data points and horizontal lines indicate mean value. **D:** There is no difference between the run speeds of SS01994>TNT and *y w*; attP40;

attP2>UAS-TNT (Wilcoxon signed rank test). **E:** SS01994>TNT exhibits significantly lower turn rate compared to the parental control ($p<0.01$, Wilcoxon signed rank test). **F:** The anatomy of the descending neuron PVM labeled by SS01994>CsChrimson::mVenus immunostaining with anti-GFP antibodies that recognize mVenus protein. In panel i, the gross anatomy of the PVM neuron is shown. A1-8 indicates the approximate location of each abdominal segment. Arrow indicates the soma location. Arrowheads indicate the axon terminals in the VNC. Note the large varicosities in the initial thoracic segments and near the SEZ. Chevrons indicate the tip of the axonal projection ending around the 4th abdominal segment. In panel ii, a single PVM neuron is labeled using the MCFO method (Nern, Pfeiffer et al. 2015). Note the contralateral locations of the axon terminals (arrowhead) and the dendritic arborization (star) with respect to the soma (arrow). Detailed image of the dendritic arborizations is shown in panel iii. The dendritic tree of the PVM neuron covers a region around the mushroom body peduncle (pd) and the lateral horn. BL: brain lobes, SEZ: subesophageal zone, vl: ventral lobe of the mushroom body, VNC: ventral nerve cord.

3.2.2 Identification and reconstruction of descending neuron pair in EM dataset

Results from light-imaging experiments facilitated structure and important morphological features of labelled descending neuron pair (Fig. 3A,C). This helped the identification and reconstruction of this neuron as PVM027 from ssTEM high-resolution electron microscopy dataset of L1 larval CNS upon collaboration with Janelia Research Campus, USA (Ohyama, Schneider-Mizell et al. 2015, Berck, Khandelwal et al., 2016) (Fig. 3B,D). The identification started with the help of Jim Truman's light imaging dataset from Janelia Research Campus where this neuron was named as PVM027, due to the posterior ventro-medial location of its soma in brain lobes. This was followed by identification of PVM027 in EM dataset and one neuron of the pair was found, which was already reconstructed by Casey Schneider-Mizell from Cardona Lab in Janelia Research Campus. Following this, other neuron from the pair was reconstructed with careful review and annotation of all the important morphological features like synapses. The anatomy of PVM027 from 1st instar EM dataset and 3rd instar light imaging results were compared . Basic skeleton of neuron with soma position looked same from both sources, with slight differences in dendritic and axonal branching.

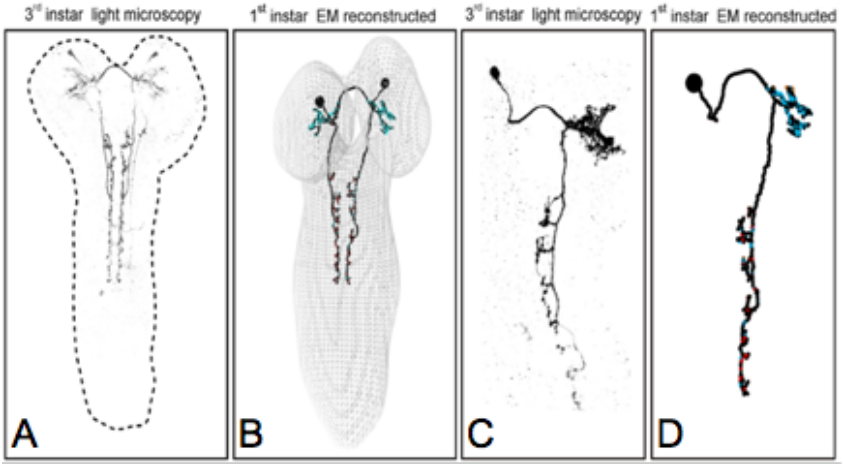


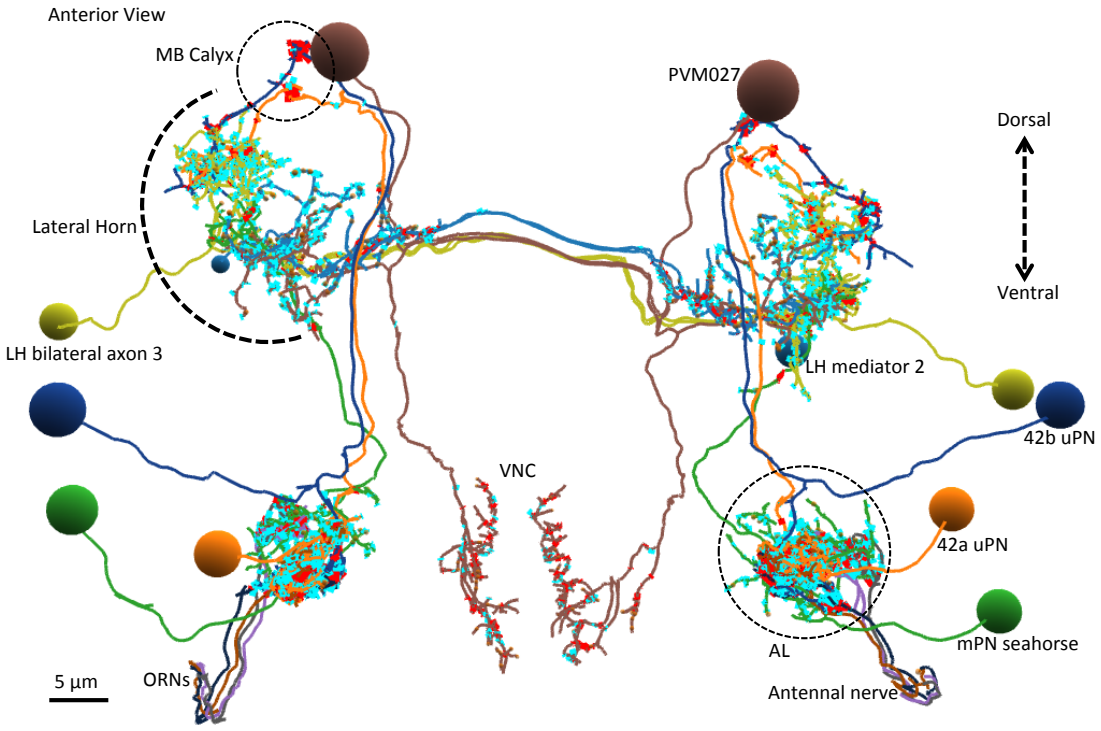
Figure 3: Identification and reconstruction of PVM027 descending neuron pair in the EM dataset. A: Confocal imaging results of SS01994 showing PVM027 neuron pair from 3rd instar larva. **B:** 3D reconstructed PVM027 neuron pair of 1st instar larval brain in EM volume. 1st instar PVM shows innervation pattern for dendrites and axons similar to that of 3rd instar neuron. (Cyan dots represent postsynaptic sites, while red dots on neuron represent presynaptic sites of PVM neuron. **C and D:** Comparision of anatomical features of 3rd and 1st instar PVM neurons from light imaging and EM reconstruction versions respectively. Dendritic branching and axonal innervation patterns were found similar along with soma positions. These features were used as reference points while identification of reconstructed neuron in the L1 EM dataset.

3.2.3 Reconstruction of upstream and downstream circuitry of PVM027

In order to validate our understanding of PVM027's role in influencing larval chemotaxis and to further understand the mechanism of sensorimotor transformation in larval brain, it was important to gain some handle on connectivity map of this neuron, both upstream and downstream by systematic EM reconstruction. This allowed us to identify some connected neurons and their role in PVM027 circuit and helped planning experiments to study it in a comprehensive manner. PVM027 circuit here means upstream and downstream connectivity of PVM027 neuron pair. This provided a great opportunity to map a brain circuit for connection between neurons for sensory perception all the way to motor systems with mediation from higher brain.

3.2.4 PVM027 receives input from sensory systems

PVM027 receives majority of inputs through its dendritic processes innervating mainly the LH region in higher brain. Projection neurons from the AL also innervate various LH regions through their axons (Fig 4A). Taking this into account, all neurons making synapses onto PVM027 were reconstructed along with postsynaptic neurons of group of olfactory projection neurons. We found two bilaterally projecting interneurons in higher brain innervating mainly LH region as connecting neurons from olfactory projection neurons to the PVM027 descending neurons, with a feedforward connectivity motif (Fig 4B). This established the fact that olfactory inputs could be relayed to the PVM027 via LH interneurons. These higher brain LH interneurons were named as LH bilateral axon 3 and LH mediator 2 respectively(Fig 5).



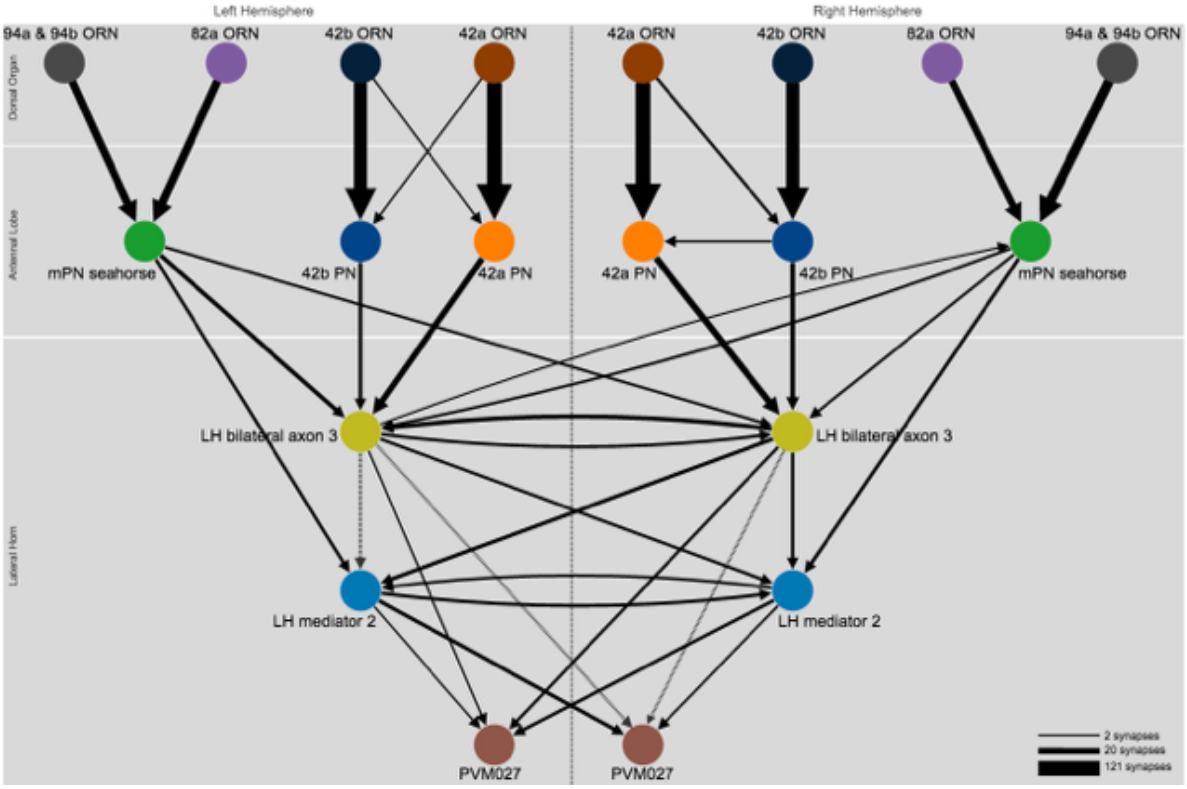


Figure 4: PVM receives olfactory input through LH interneurons. A: Anterior view of EM-reconstructed upstream circuitry of PVM with connection from antennal lobe neurons through LH interneurons such as LH bilateral axon 3 and LH mediator 2 in the LH region. LH interneurons have been shown to pick up and integrate inputs from antennal lobe PNs and making synapses on to PVM. (Cyan dots represent postsynaptic sites, while red dots on neurons represent presynaptic sites). **B:** Connectivity diagram showing EM-reconstructed upstream circuit of PVM as mentioned in panel A. LH interneurons as well as PVM can be seen for making bilateral and reciprocal connections. Thickness of arrows represent number of synaptic connection between two neurons. Dotted arrows represent connections/edges yet to be found. Color of neurons in panel A and B are conserved for better visualization purpose.

LH bilateral axon 3 receives its inputs from a group of uPNs and mPNs from the AL. It also collects input from other sensory systems such as projection neurons of the visual system (Data not shown) and synapses onto its counterpart neuron in the other brain hemisphere connecting reciprocally, operating on multisensory integration with bilateral distribution of sensory information in the higher brain. The main postsynaptic partners of LH bilateral axon 3 include

LH mediator 2 and PVM027 (Fig. 4 A,B). So, LH bilateral axon 3 can potentially relay integrated multisensory signals to the descending circuit forming a feedforward motif with LH mediator 2 and PVM027. Feedforward motif here refers to direct and indirect connection (via LH mediator 2) from LH bilateral axon 3 to PVM027 (Fig. 4B).

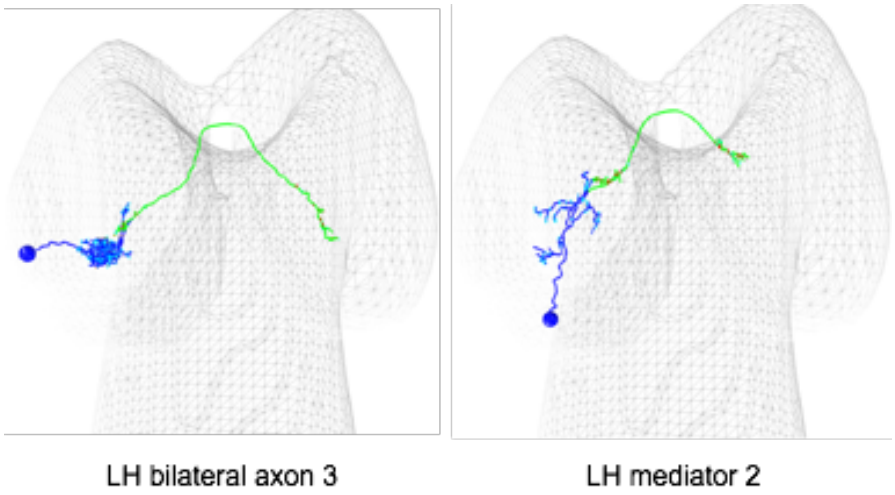


Figure 5: LH interneurons connecting sensory systems to PVM. EM-reconstructed LH interneurons that are presynaptic to PVM. Green colored portion representing bilaterally projecting axonal processes while blue represents soma and ipsilateral dendritic processes of neurons. (Cyan dots represent postsynaptic sites, while red dots on neurons represent presynaptic sites).

LH mediator 2 looks morphologically similar to LH bilateral axon 3, as it also possesses a bilaterally projecting axon (Fig.5), and is one of the main postsynaptic partner of the latter. It also connects to its counterpart neuron on the other brain hemisphere in a fashion similar to LH bilateral axon 3. Interestingly, other presynaptic partner of LH mediator 2 includes mPN seahorse (Berck, Khandelwal et al., 2016), which is one of the mPNs projecting from the AL. mPN seahorse receives its inputs extensively from 82a ORN, which has been shown to mediate aversion in larval *Drosophila* and is sensitive to geranyl acetate, while LH bilateral axon 3 collects olfactory inputs from appetitive ORNs like 42a and 42b through their uPNs(Kreher et al., 2008, Berck, Khandelwal et al., 2016, Schulze et al., 2015). This shows that PVM027 integrates appetitive and aversive olfactory inputs. We found that PVM027 is one of the top postsynaptic

partners of LH mediator 2.

We looked for lines with narrow expression patterns, labelling the two LH interneurons, but were unable to find any. Nevertheless, it will be of great interest to systematically study the role of these important integrators and connectors for a detailed understanding of transformation of sensory signals to behavior in the higher brain of larval *Drosophila*.

3.2.5 Gain of function experiments revealed PVM027 encodes *stop* response in larva

In order to understand role of PVM027 in controlling features of chemotaxis behavior, gain of function studies were conducted in lab by Ibrahim Tastekin through acute optogenetic activation. Light stimulus were provided as flashes on SS01194>CsChrimson (experiment) and y-w-; attP40; attP2 x UAS-CsChrimson (control) larvae, while in movement and selectively activating PVM027 neurons of experiment larvae optogenetically. Results showed that PVM activation significantly and consecutively lowered tail speed of a moving larva and evoked *stops* (Fig. 6).

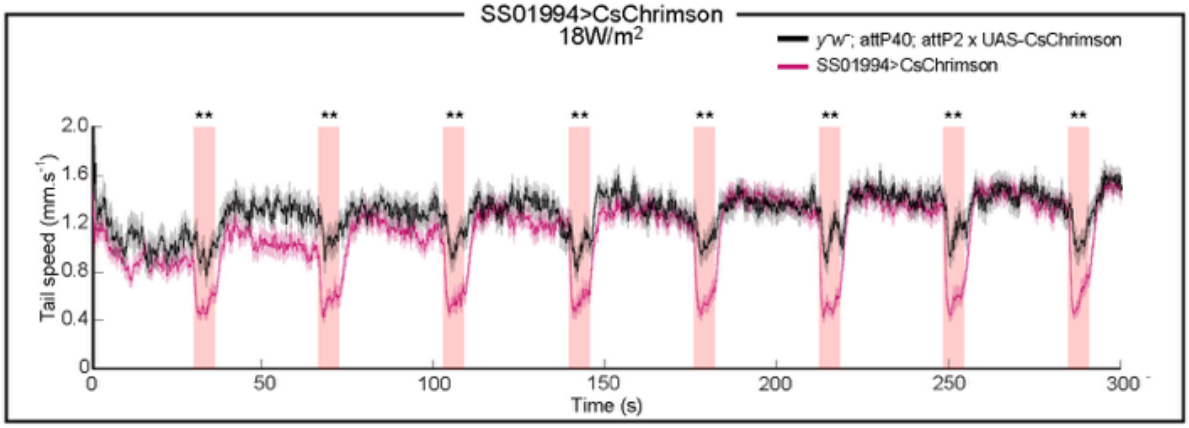


Figure 6: PVM is sufficient to evoke *stops*. Pausing behavior was quantified as a function of tail speed. Average tail speed of the SS01994>CsChrimson (magenta) and the parental control (black) larvae were quantified for a duration of 5 min during which 8 consecutive light flashes that are interspersed by 30 seconds were applied (light

red boxes). For each light flash, the average tail speed was significantly lower for SS01994>CsChrimson compared to the parental control ($p<0.05$, Kolmogorov-Smirnov test). Parental control larvae exhibit a slight decrease in the tail speed due to the startle response. Shaded area indicates standard error of the mean.

The same was not observed among control animals. This led to the conclusion that PVM027 was responsible for controlling *stop* response in larva.

3.2.6 Single PVM027 is sufficient to control *stop* response in larva

Since the PVM027 seemed to be collecting bilateral inputs from the same sensory-system

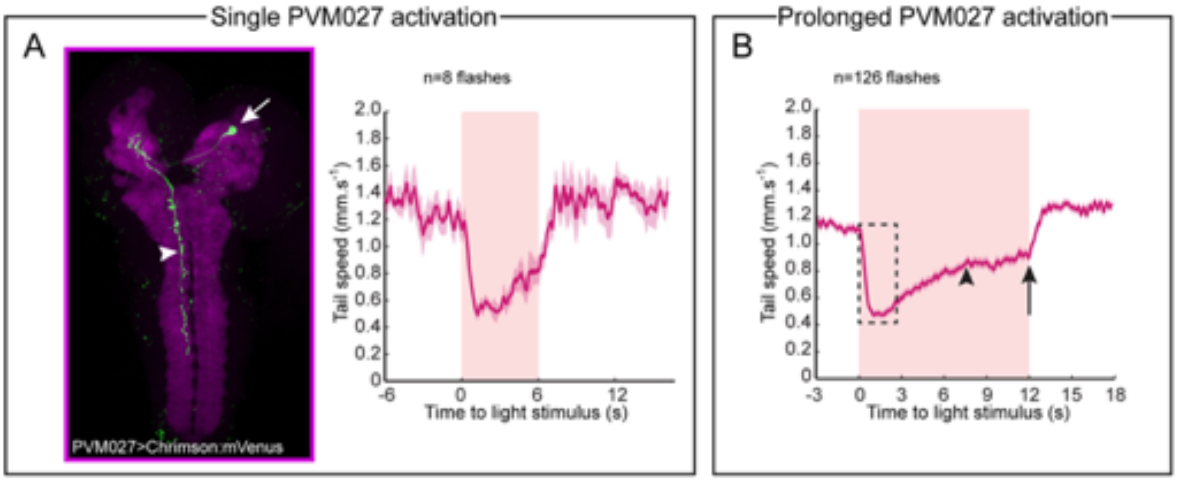


Figure 7: High-resolution analysis of PVM>CsChrimson larvae upon acute optogenetic activation A: Unilateral activation of PVM by stochastically expressing CsChrimson::mVenus in a single PVM neuron using the 'Flip-out' technique. Immunostaining against mVenus protein confirms that CsChrimson::mVenus is expressed unilaterally (left panel). Acute optogenetic activation of a single PVM neuron is sufficient to evoke pauses quantified as average tail speed (right panel). Shaded area indicates SEM. **B:** Prolonged activation of the PVM neuron led to a transient pause (dashed box) followed by slower locomotion that plateaus around the 7th second of optogenetic activation (arrow head). Upon light offset, larvae immediately switch back to normal crawling speed (arrow).

connectors in brain, it was interesting to ask for the role of single PVM027 activity in the *stop*

response. Flip-out clones were made with stochastically labelling single PVM027 neuron from one brain hemisphere for optogenetic activation experiments and results were selected from larvae with single PVM027 labelling upon confirmation from light imaging experiments. (Fig. 7 A). Interestingly, optogenetic activation of single PVM027 led to similar drop in tail speed and *stop* response as observed from gain of function experiments explained above, concluding that a single descending neuron is sufficient to control *stops* in moving larvae (Fig. 7A, right panel). Acute optogenetic activation of single PVM027 neuron led to steep drop in tail speed of moving larvae within one second of flash, and dropped further with increase in light intensity with bringing animals to a complete halt for up to 3 seconds, after which, the larvae gradually regained the movement as *stop* is only a temporary event in larval locomotion (Fig. 7 B).

3.2.7 PVM027 is an excitatory descending neuron pair

Neurotransmitter profiling is an important aspect to determine role of neurons in a circuit as neurotransmitters are indicator of nature of neuronal activity and their influence. Descending neurons expressing variety of neurotransmitters such as acetylcholine, GABA, glutamate, serotonin, dopamine and octopamine have been reported in adult *Drosophila* (Hsu et al., 2016). PVM027 was imaged in the lab by Ibrahim Tastekin, from SS01994 line upon staining with GABA, Glutamate and ChAT antibodies separately. No colocalization was observed with GABA or Glutamate staining, but was observed with ChAT, indicating that PVM027 is cholinergic (Fig. 8A). To validate above light imaging antibody staining results, optogenetic activation experiment of PVM027 was conducted with silencing ChAT in labelled SS01994 line by ChAT-RNAi. Here, PVM027 silenced by ChAT-RNAi showed drop in larval tail speed similar to y-w-; attP40; attP2 x UAS-CsChrimson; ChAT-RNAi (negative control) and was different from SS01994>CsChrimson (positive control) activity results (Fig. 8B). This confirmed that acetylcholine was required for optimal activity of PVM027 and hence the neuron is cholinergic. Since acetylcholine is an excitatory neurotransmitter in *Drosophila* (Liu and Wilson 2013), PVM027 was confirmed to be excitatory in nature.

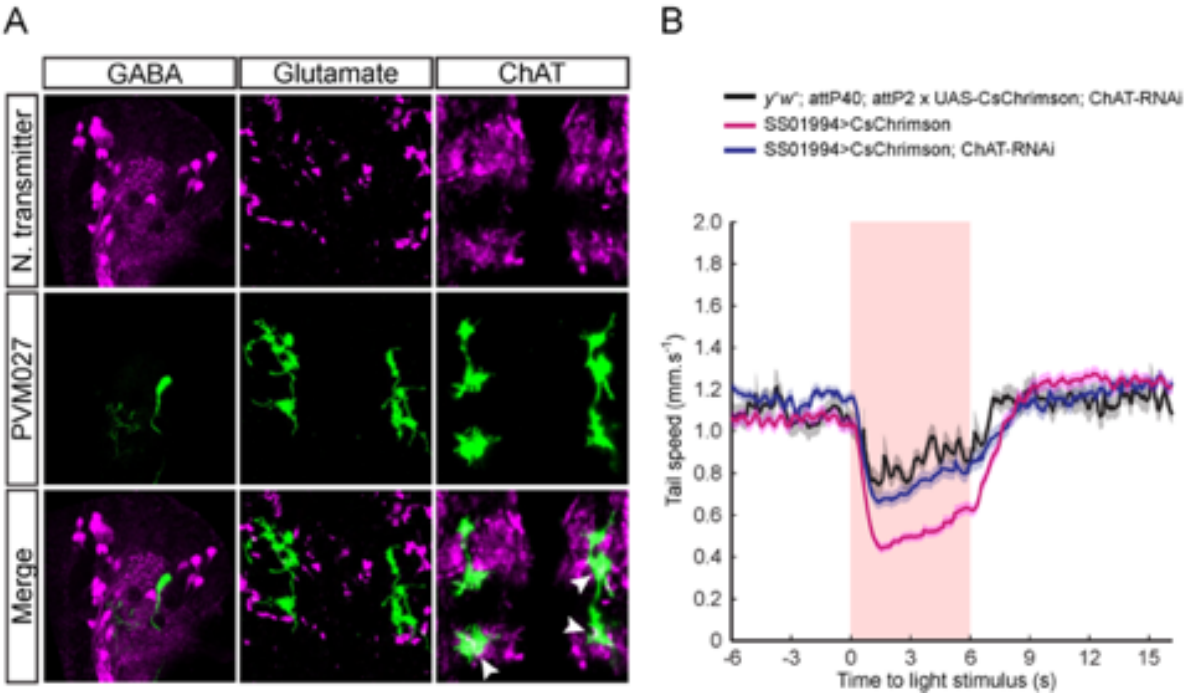


Figure 8: Neurotransmitter profiling of the PVM neuron A: SS01994>CsChrimson::mVenus larvae were immunostained against the mVenus protein (green) together with immunostaining against each of the three main neurotransmitters of the larval nervous system (magenta): Acetylcholine, gamma-aminobutyric acid (GABA) and glutamate. GABA is clearly visible in neuronal cell bodies in the larval nervous system. However, there is no colocalization of GABA signals in the soma of the PVM neuron (left column, MERGE). Glutamate was not colocalized in the PVM axon terminals (middle column, MERGE) while it is clearly visible as magenta puncta for other neurons (upper row). PVM labeling colocalize with choline-acetyltranferase immunostaining shown as white puncta (bottom row, MERGE), suggesting that PVM neuron is cholinergic. **B:** RNAi knockdown of acetylcholine (ChaT) abolishes the pausing behavior triggered by optogenetic PVM activation. CsChrimson::mVenus is co-expressed with choline-acetyltranferase RNAi (SS01994>CsChrimson, ChAT-RNAi, blue line) and response of the larvae to red light flashes was tested as a function of tail speed. Shaded areas indicate standard error of the mean.

3.2.8 PVM027 descending neuron circuit connects to peristaltic wave propagation circuit of larva

Although the role of PVM027 neuron in controlling locomotor action in larva was confirmed by above experimentations, it was of equal importance to look for mechanisms for control of chemotaxis behavior. This would not only help us comprehend the control of motor pattern generation by brain, but also lay out a complete map from sensory to motor transformations for specific behavioral subtype.

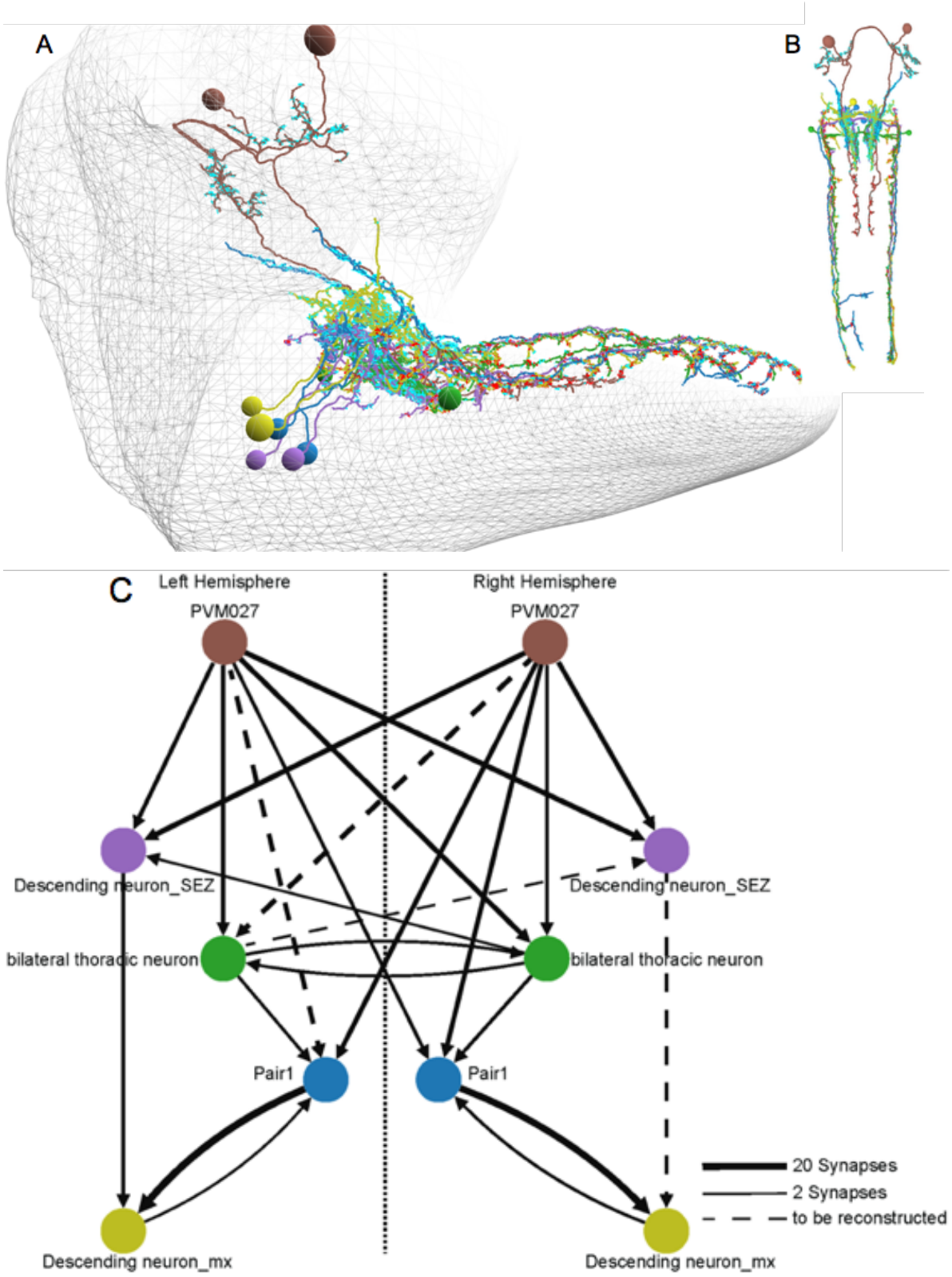
Larval locomotion is the result of origin and propagation of peristaltic waves shaped by sequential contraction and relaxation of muscles in different segments (Heckscher et al., 2012).

These muscles receive inputs from motor system in the VNC with segmentally repeated arrangement with intersegmental interactions. Waves originating from posterior abdominal segments propagating towards anterior result in forward locomotion whereas waves running from anterior to posterior body segments give rise to backward locomotion (Fushiki et al., 2016). In order for larvae to stop, it is mandatory to interrupt either origin or propagation of these peristaltic waves or both.

With the help of recently published findings on mechanism of wave propagation circuit in larva (Fushiki et al., 2016), we planned for systematic reconstruction of downstream circuitry of PVM027 to study the mechanism of control of locomotory circuit. This study described a circuit mechanism responsible for peristaltic wave propagation in larva with segmentally repeated interaction of an inhibitory premotor interneuron, GDL and a bilaterally projecting excitatory premotor interneuron, A27h, connected with each other forming a chain throughout abdominal and late thoracic segments. It also showed that inhibitory GDL neurons were necessary for both, forward and backward locomotion, while A27h was only necessary for forward locomotion receiving inputs from stretch receptors and SEZ descending neurons (Fushiki et al., 2016). This led us to investigate for any link between PVM027 circuit and the one responsible for wave propagation.

A. PVM027 organizes microcircuits with SEZ descending neurons in VNC

Immediate downstream reconstruction of PVM027 neuron pair revealed its connectivity with a number of SEZ descending neurons arborizing through later abdominal segments with dendritic innervation ranging from SEZ to thoracic segments (Fig. 9A,B). Description of SEZ descending neurons being one of the major input providers to the wave propagation circuit (Fushiki et al., 2016) led us to investigate their connectivity further downstream. These descending neurons were listed among top postsynaptic partners of PVM027 and synapse mostly on either other SEZ descending neurons (Fig. 9C) connecting hierarchically or onto premotor neurons, with few SEZ descending neurons synapsing directly on distinct motor neuron populations. Similar premotor descending neurons have been described in dipterans such as *Sarcophaga bullata* with sensory stimulus driven activity pattern, and axonal branches of descending neurons spanning thoracic neuropil that contains dendrites of flight related motor neurons Gronenberg et al., 1992). This indicated that PVM027 may influence motor circuit for larval locomotion through recruitment of SEZ descending neurons. Some of the strongly connected descending neurons were names as Descending neuron_SEZ, Bilateral thoracic neuron, Pair 1 neuron and Descending neurons_mx and all of them differed in their birth order (Fig. 9D).


A
B
C
Left Hemisphere
Right Hemisphere
PVM027
PVM027
Descending neuron_SEZ
Descending neuron_SEZ
bilateral thoracic neuron
bilateral thoracic neuron
Pair1
Pair1
20 Synapses
2 Synapses
to be reconstructed
Descending neuron_mx
Descending neuron_mx

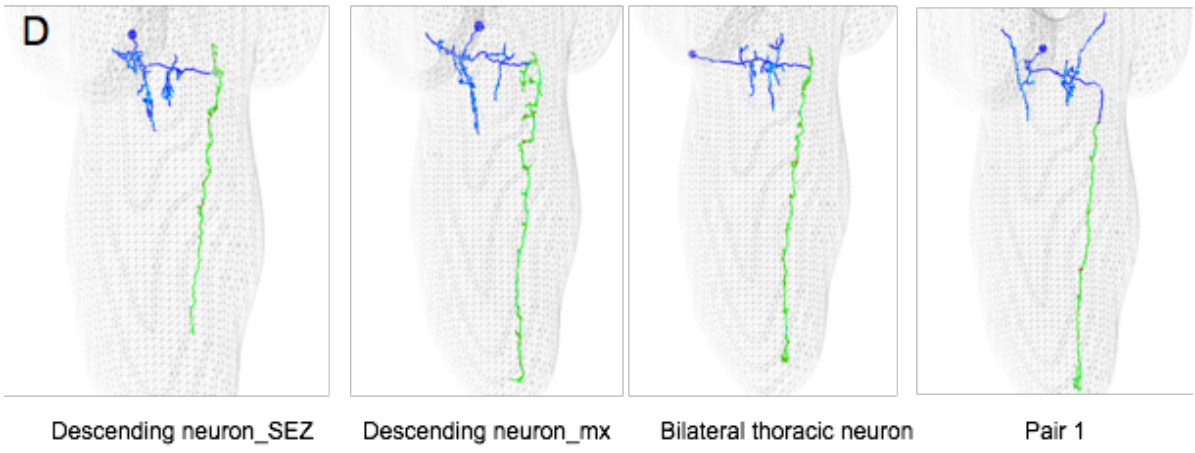
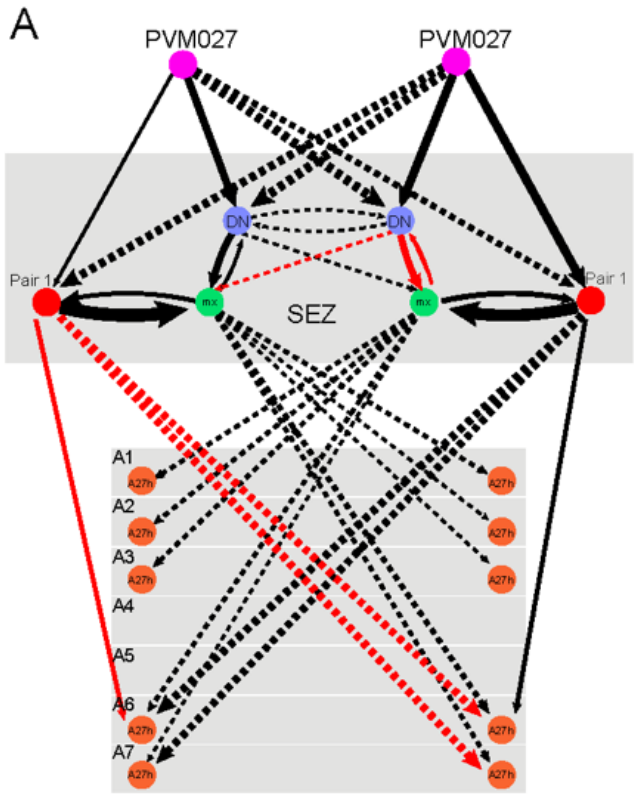


Figure 9: Descending neuron microcircuit in larval VNC. A and B: Lateral and dorsal view of EM-reconstructed downstream circuitry of PVM with SEZ descending neurons in the VNC respectively. These SEZ descending neurons originate from posterior SEZ with their dendritic processes spanning upto thoracic segments, while their axons innervate all the way to posterior abdominal segments. PVM synapses directly on these SEZ descending neurons except Descending neuron_mx forming a descending neuron microcircuit in the VNC. (Cyan dots represent postsynaptic sites, while red dots on neurons represent presynaptic sites). **C:** Connectivity diagram showing EM-reconstructed downstream circuit of PVM with descending neurons as mentioned in panel A and B. The diagram shows multilayered connectivity withing descending circuit within VNC before interaction with premotor or motor neurons. Dotted arrows represent connections that are yet to be found. Color of neurons in panel A, B and C are conserved for better visualization purpose. **D:** EM-reconstructed images of single SEZ descending neurons downstream of PVM. Blue portions of neurons represent soma and bilateral dendritic processes, while green portion representing contralateral axonal innervation pattern. Soma location for each of these SEZ descending neurons was found to be different refering to differences in their lineages.

B. PVM027 connects to wave propagation circuit in larva through SEZ descending neurons

Upon careful reconstruction and review of these descending neurons with annotation of majority of morphological synapses, we found that Pair1 and Descending neuron_mx were synapsing directly on A27h excitatory premotor neuron (Fig. 10A,B), which is necessary for forward larval locomotion (Fushiki et al., 2016).


A
PVM027
PVM027
DN
DN
Pair 1
Pair 1
mx
mx
SEZ
A1
A2
A3
A4
A5
A6
A7
A27h

B

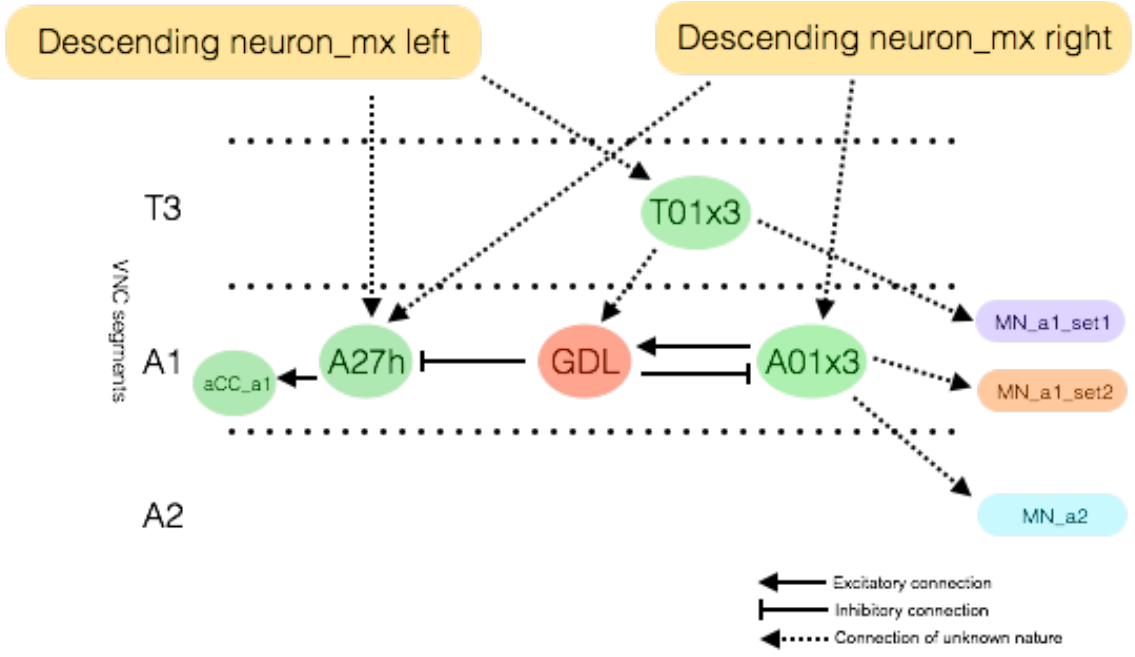

Descending neuron_mx left
Descending neuron_mx right
T3
T01x3
VNC segments
A1
A27h
GDL
A01x3
aCC_a1
MN_a1_set1
MN_a1_set2
A2
MN_a2
Excitatory connection
Inhibitory connection
Connection of unknown nature

Figure 10: PVM circuit is presynaptic to peristaltic wave propagation circuit of larva. A: EM-reconstruction based schematic representation of connection of downstream circuit of PVM with the forward peristalsis wave propagation circuit of the larva. Here, SEZ descendning neurons like Pair 1 and Descending neurons_mx are shown to be providing differential inputs to excitatory premotor neurons A27h, which has been described to be necessary for propagation of forward peristalsis waves in larva (Fushiki et al., 2016). Inputs from SEZ descending neurons to A27h neurons were found to be non-uniform and were restricted only to initial and late abdominal segments. Black solid arrows represent ipsilateral synaptic connection and black dotted arrows represent contralateral synaptic connections. Red arrows, both solid and dotted ones represent ipsilateral and contralateral connections that are yet to be found respectively. **B:** EM-reconstruction based schematic representation of descending neuron_mx connectivity with candidates for forward and backward peristaltic wave propagation in late thoracic to initial abdominal segments of VNC. Here, Descending neuron_mx, which is one of the downstream neurons of PVM circuit is shown to be providing inputs to A27h as well as T01x3 and A01x3 neurons, and the connection between the two wave propagation circuit is mediated by the GDL inhibitory neuron. T01x3 and A01x3 are thoracic and abdominal homologs and are described to be potential candidate neurons for propagation of backward peristalsis waves in larva by Fushiki et al., 2016 and are presynaptic to a number of different motor neuron types. Black dotted arrows here represent actual synaptic connections of unknown nature.

We also found asymmetric connectivity between descending neuron_mx and candidate premotor neurons for backward wave propagation described by Fushiki et al., 2016 as T01x3 and A01x3 neurons (Fig. 10B). T01x3 and A01x3 are two premotor neurons indicated for their role in backward locomotion in larval *Drosophila* and are thoracic and abdominal counterparts. Since there are no genetic reagents available to study them, the mechanism by which they achieve the described functions remain elusive.

Interestingly, the connectivity between SEZ descending neurons and forward wave propagation circuit was not uniform and did not recur segmentally. The connections were only found restricted to late thoracic to initial abdominal segments and then in the later abdominal segments A6 and A7 (Fig. 10A).

C. PVM027 circuit shares connection with distinct motor neuron types

Tracing further to the motor neuron connectivity from PVM027 circuit led us to the SEZ descending neurons connected to various motor neurons types. Some of these motor neurons include RP motor neurons that innervate longitudinal muscles in larva, various segmental and intersegmental-nerve motor neurons (Landgraf et al., 1997) and larval feeding circuit related motor neurons like Pro-thoracic Motor neurons (PaN MNs) (Fig. 11 A,B) (Hückesfeld et al., 2015). It thus requires a systematic investigation of the role of PVM027 inputs to the SEZ descending neurons that seem to control distinct motor circuits.

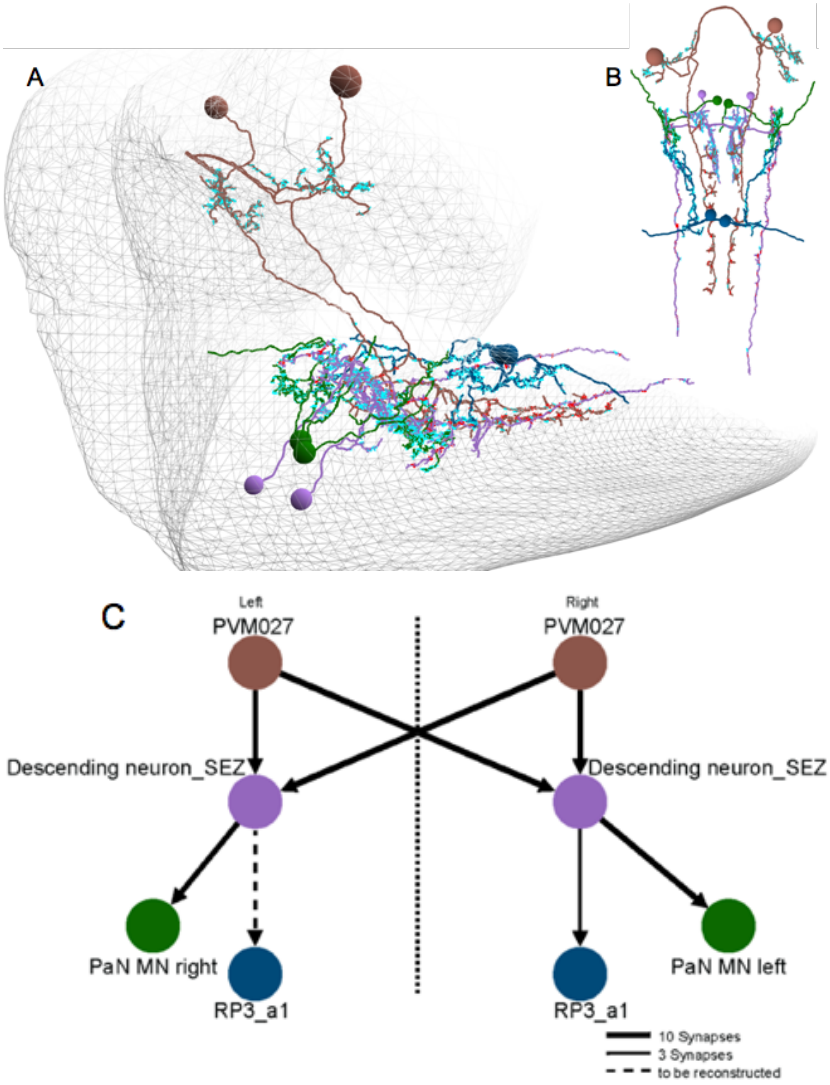


Figure 11: PVM connects to various motor systems in larval VNC. A and B: Lateral and dorsal view of EM-reconstructed downstream circuitry of PVM to other motor neurons through Descending neuron_SEZ in the VNC. Here, Descending neuron_SEZ has been shown as a premotor neuron as it is seen to be synapting directly on different motor neuron types such as PaN motor neurons (feeding related motor neuron) and RP3 motor neurons. These connections range from posterior SEZ to abdominal segments of VNC. (Cyan dots represent postsynaptic sites, while red dots on neurons represent presynaptic sites). **C:** Connectivity diagram showing EM-reconstructed downstream circuit of PVM with different motor neurons through Descending neuron_SEZ as mentioned in panel A and B. Dotted arrows represent connections that are yet to be found. Color of neurons in panel A, B and C are conserved for better visualization purpose.

3.2.9 Effect of PVM027 activity on forward peristaltic waves in larva

Once the anatomical connections between PVM027 and wave propagation circuit were revealed, we then asked how PVM027 activity affects forward peristaltic waves. Forward waves originate in the most posterior segments and proceed towards anterior body by sequential contraction and relaxations of muscles (Fushiki et al., 2016). This led us to look into influence of PVM activity during various phases of peristaltic wave propagation starting from posterior to anterior segments (Fig. 12B).

Skeleton length and tail speed are two features, linked with larval locomotion and are readouts of ideal wave propagation. Tail speed has been observed to increase at the start of a wave, while skeleton length reaches maximum marking end of the wave. These two features are exhibited in an alternate manner in a moving larvae and mark for coordination between contraction and relaxation resulting in movement of larva in forward direction. This gives them a wave like shape described in Fig. 12A.

Ibrahim Tastekin in the lab studied these features of wave propagation mechanism and found that, while the forward wave is in its initial phase of propagation, PVM stimulation can lead to sharp decline in the tail speed to its minimum with simultaneous and steep increase in skeleton length of larva within a very short period of activation (Fig. 12C), and the same was not

observed in parental controls (Fig. 12D). This showed that PVM activity is most effective during initial phase of wave propagation and affects peristaltic movement by influencing the forward wave propagation mechanism in larvae.

Ibrahim furthered the study while asking for pattern for this influence. Optogenetic experiments conducted in lab by Ibrahim on pinned larvae (Fig. 13A) indicated that PVM027 activation during the initial phase of wave propagation is more probable to terminate the current wave without completion. Wave termination occurs only if the current wave is in A6 or A7 segments while PVM027 is activated (Fig. 13B). Upon PVM027 activation, the current wave usually gets terminated in A5 or A4 segment (Fig. 13C). If the wave has already passed A6 segment before PVM027 activation, it is more likely that the current wave will be completed, but a new wave will not originate.

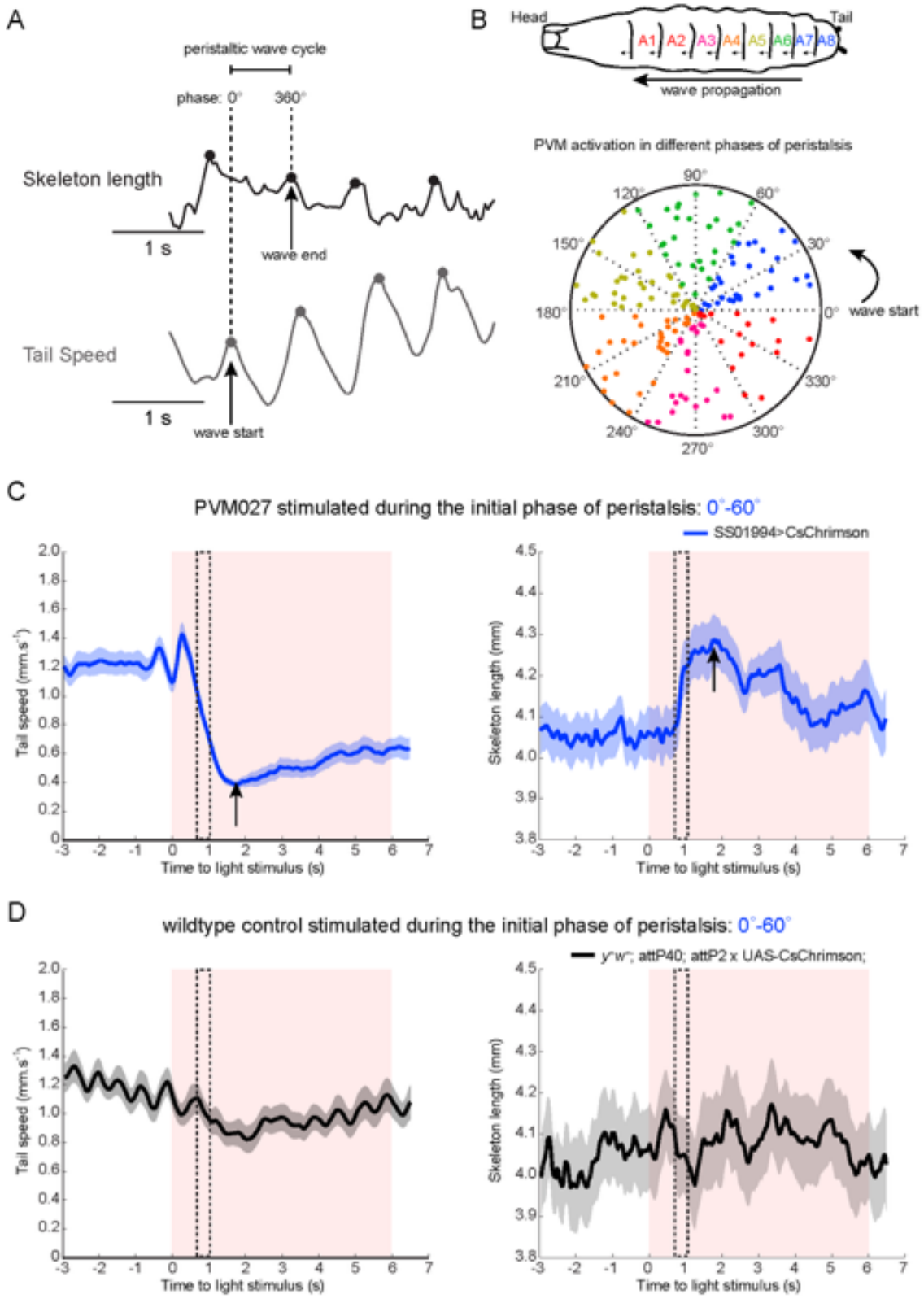

A
peristaltic wave cycle
phase: 0°
360°
Skeleton length
1 s
wave end
Tail Speed
1 s
wave start
B
Head
Tail
A1
A2
A3
A4
A5
A6
A7
A8
wave propagation
PVM activation in different phases of peristalsis
90°
120°
60°
150°
30°
180°
0°
wave start
210°
330°
240°
300°
270°
C
PVM027 stimulated during the initial phase of peristalsis: 0°-60°
SS01994>CsChrimson
Tail speed (mm.s⁻¹)
Time to light stimulus (s)
Skeleton length (mm)
Time to light stimulus (s)
D
wildtype control stimulated during the initial phase of peristalsis: 0°-60°
y¹w¹; attP40; attP2 x UAS-CsChrimson;
Tail speed (mm.s⁻¹)
Time to light stimulus (s)
Skeleton length (mm)
Time to light stimulus (s)

Figure 12: Effect of the PVM activation on the peristaltic waves A: Skeleton length (upper trace) and tail speed (bottom trace) exhibit wave-like pattern during forward locomotion. At the beginning of a peristaltic wave tail speed (gray dots) is maximized followed by the extension of the larval body to its maximum length at the end of the wave (black dots). The peristaltic wave cycle was defined as the period between consecutive tail speed and skeleton length maxima. **B:** The wave cycle was divided into six equal phase bins. Upper panel indicates the abdominal segments color-coded according to phase bin they contract (blue: 0 °- 60°, green: 60 °- 120°, yellow: 120 °- 180°, orange: 180 °- 240°, magenta: 240 °- 300° and red 300 °- 360°). A1-A8 indicates the abdominal segments. Upon post-hoc analysis of PVM activation, the corresponding phases of the onsets of PVM activation were shown on a phase plot (bottom panel). Identical color-codes were used for both panels. **C:** Tail speed (left panel and skeleton length (right panel) were quantified for the PVM activation during the initial phase of the peristaltic wave (0 °- 60°, blue). In the left panel, larvae experience a sharp decrease in the tail speed shortly after the onset of the light flashes (left panel, dashed rectangular box) and the tail speed quickly reaches its minimum value (left panel, arrow). During the same period the skeleton length increases steeply (right panel, dashed rectangular box) reaching a maximum around the same time the tail speed is minimized (right panel, arrow). **D:** Same as C for the parental control. The skeleton length was not maximized (right panel) upon light stimulation although there is a slight decrease in the tail speed (left panel). For comparison, the dashed rectangular boxes were placed at the same time point they were placed in C.

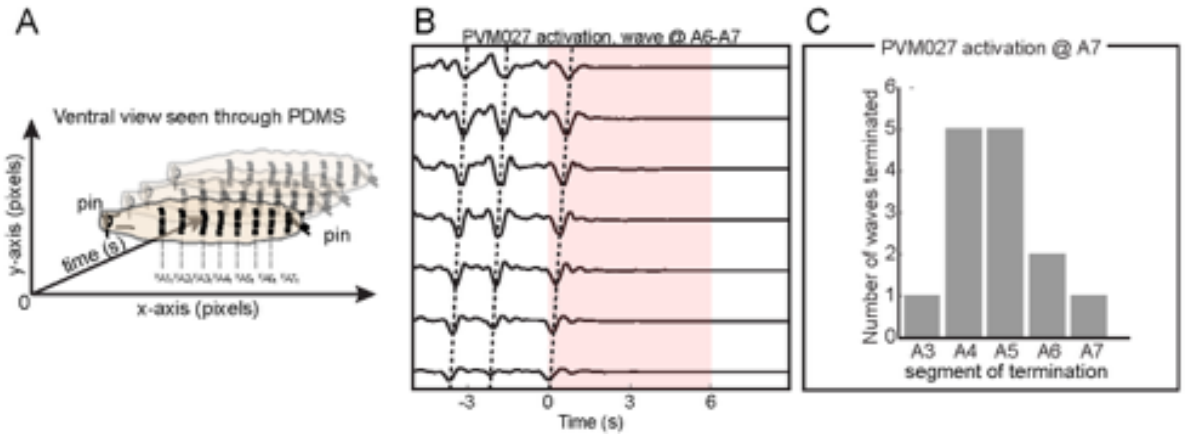


Figure 13: Characterization of the PVM activation phenotype at the level of muscle contractions A: Schematic for the experimental setup. 3rd instar larva was pinned down on a PVMS slab ventral side touching the PVMS surface. The contractions of the abdominal segments could be quantified by visualizing the denticle bands on the ventral side of the body (each abdominal segment has a denticle band). **B**: The contractions of the abdominal segments upon PVM activation were quantified as a function of denticle band displacement for A1-A7.

Each horizontal dashed line indicates a complete peristaltic wave such that wave starts with the contraction of A7 and ends with A1. Onset of PVM activation was between the end of a wave and start of a new wave (A7-A1). Here, Onset of PVM activation is between the contractions of A6 and A7. A wave that was already started could not be ceased immediately by PVM activation although a new wave could not be initiated at least until the offset of PVM activation. **C:** When onset of PVM activation was coincides with the contraction of A7, the wave was immaturely ceased at different segments ranging from A3 to A7. The wave was immaturely terminated often in the 4th and the 5th segments (10/14 cases. ~70% of all cases).

3.2.10 PVM027 activation terminated forward waves by suppressing and transforming them into backward waves

Evidence for termination of forward waves by PVM027 activity furthered us to look into the mechanism through which it is shaped. Because these waves are result of motor neuron activity, which are sequentially recruited, inhibited and excited for relaxation followed by contraction of muscles, the motor neuron activity pattern helped us dig into the mechanism. Calcium activity in motor neurons was imaged (Fig. 14A) by Ibrahim in the lab using VGLUT-LexA>lexAOP-GCaMP6f construct upon optogenetic activation of PVM027. Light stimulus was flashed when the wave was in A6 or A7 abdominal segments with quantification of motor neurons activity (Fig. 14B). It was found that in most cases, upon activation of PVM027, motor neuron activity corresponding to forward wave in segments anterior to A6/A7 got suppressed ultimately converting to backward waves. It also showed that the amplitude of the waves even in the initial phase of propagation is affected, making it incapable to be completed and as a result of this inability through suppression, wave is terminated prematurily before completion(Fig. 14C).

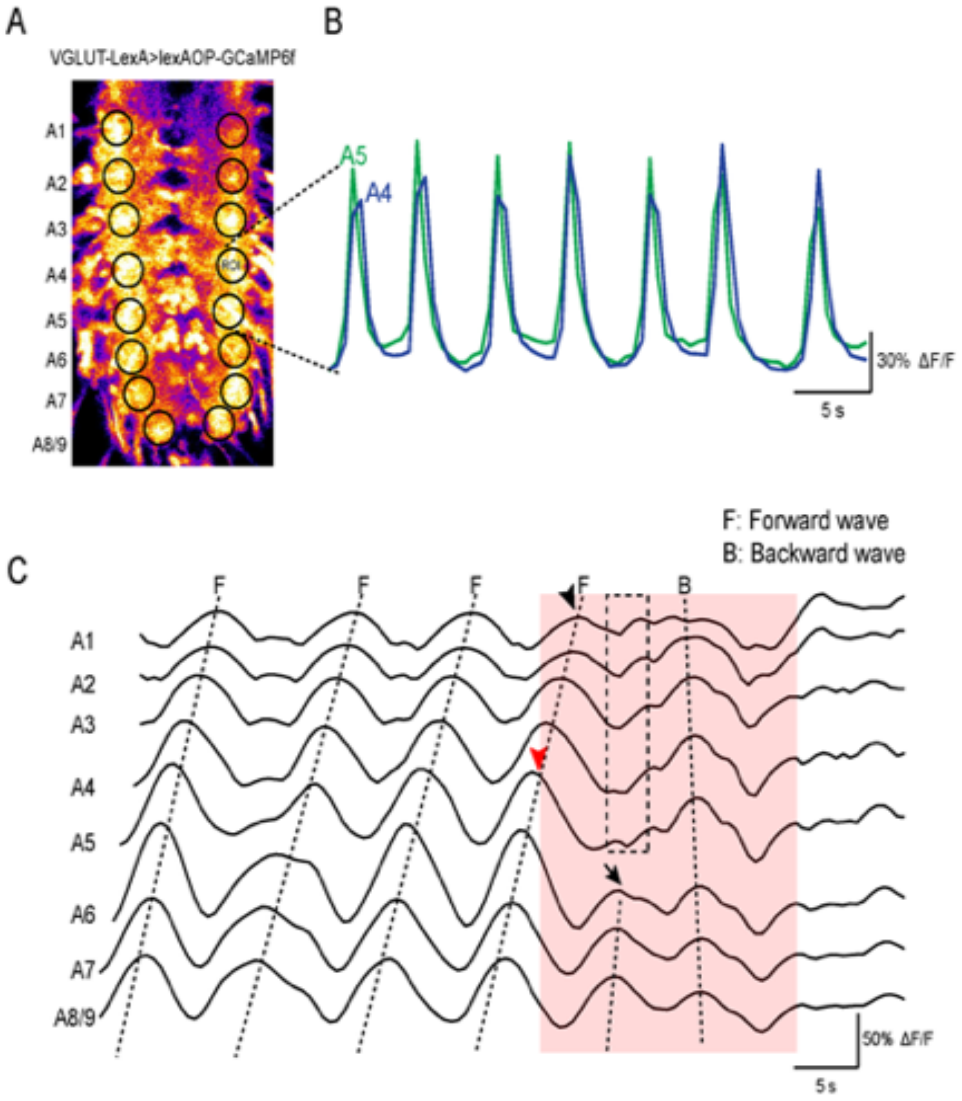


Figure 14: The effect of PVM activation at the level of motor neurons A: In order to measure neural activity, motor neurons were labeled by expressing GCamP6f under the control of VGLUT-LexA driver line. For each abdominal segment, isometric regions of interest (ROIs) were drawn on where motor neuron axons from the same segment bundle together (circles). A1-A8/9 indicates the segment locations. **B:** Motor neuron activity in an isolated CNS was quantified from ROIs as a function of normalized change in fluorescence intensity (ΔF/F). In an isolated CNS covered with saline, motor neurons formed wave-like activity pattern from posterior to anterior resembling the peristaltic waves of muscle contractions (fictive locomotion). **C:** Fictive locomotion shown as waves of motor neuron activity. Each horizontal dashed line indicates a single fictive peristaltic wave beginning at A8/9 and ending at A1. After a sequence of forward waves (F), PVM neuron was optogenetically activated (SS01994>CsChrimson::mVenus) with an onset coinciding with increase in motor neuron activity at A4 (red arrowhead). The wave was successfully finalized at A1 (black arrowhead). Then, although a weak wave was

reinitiated at A8/9 it was immaturely terminated at A6 (arrow) without observable waves of motor neuron activity in from A5 to A1 (dashed box). Eventually a backward wave (B) was generated. Red box indicates red light flash to activate the PVM neuron.

3.3 Discussion

Circuit mechanisms for sensory perception and for generation of movements have been studied in some detail (Bhatt and Neckameyer 2013, Fushiki et al., 2016). This includes studies on various sensory systems like, olfaction, vision, gustation etc. with circuits for locomotion through works on various motor systems (Bader et al, 2007, Keene and Waddell 2007, Melcher and Pankratz 2005, Olsen and Wilson 2008). We possess some understanding on how animals perceive a sensory stimulus or how motor neurons coordinate as a circuit and interact with specific muscle types shaping locomotory action in larva (Landgraf et al, 1997, Fushiki et al, 2016, Zwart et al., 2016). The next question, which is significantly understudied is the processing mechanics through which a stimulus, or a set of stimuli go through, in higher brain and get transformed to one or more behavior type, which is then implemented through circuits that take the brain command to the points of execution i.e., to motor pools. Such studies are of vital nature and are steps towards understanding the core of behavior generation in an inclusive manner with dynamics of responsiveness of brain, through which neurons from motor circuits are recruited selectively for command execution. Bridging this gap is extremely important in order to acquire adequate and inclusive understanding circuit functions shaping behaviors.

We investigated one such brain circuit for neural mechanisms controlling chemotaxis behavior. Using *Drosophila* larva, we showed the potential connections by which sensorimotor transformations may take place in higher brain with understanding the mechanism of execution of command on the motor system responsible for peristaltic wave propagation in larva shaping forward crawling. This is done by a single, excitatory brain descending neuron, PVM027. We also show the connectivity pattern for different types of descending neurons in the CNS and the diversity in their connection with various motor pools, some of which are previously studied to perform variety of motor functions. Our results show that brain executes its processed inputs to shape behavioral actions by recruitment of different neuron types. Among

them, descending neurons are one of the important players in connecting brain output to the circuit for command execution, which is usually located in VNC in larva.

Our results also show that brain descending neurons receive majority of their inputs through their dendritic processes innervating higher brain regions from variety of different neuron types. Some of these brain neurons are downstream of various sensory systems. Our anatomical mapping of upstream circuit of PVM027 descending neuron revealed that sensory signals can be relayed to command circuit in as short as two to three synapses. These connections were found to be mediated by just two interneurons in the LH. We named them LH bilateral axon 3 and LH mediator 2. There were no reports found mentioning these neuron types in the literature, but they might be important as they just don't relay sensory signals, but also integrate inputs from various projection neurons, both uPNs as well as mPNs from olfactory system along with strong inputs from other sensory systems, and may act as centers for multisensory integration in higher brain. Another remarkable feature of higher order connectivity and relay that we observed was that the circuit structure for processing sensory inputs, integration and relay of processed brain signals could be confined to mediation by two to three neurons in higher brain. LH bilateral axon 3 and LH mediator 2 indeed have interactions with various other neuron types in brain, and studying them with availability of genetic tools could lead to a greater understanding of mechanisms of signal processing in higher brain.

In the VNC of larva, the descending neuron PVM027 was found to be presynaptic to a pool of SEZ descending neurons. Most of these descending neurons were among the top postsynaptic partners of PVM027 in the VNC. Further reconstruction revealed that these SEZ descending neurons are organized in an interconnected circuit structure, while providing inputs to a variety of different motor pools. Some of these motor systems, downstream of these SEZ descending neurons are studied for their functions while many of these motor systems are still understudied. Majority of these SEZ descending neurons provided inputs to premotor neurons while some shared direct inputs to motor neurons. This clarifies that descending neurons can recruit motor neurons either by interaction with premotor circuit or through direct connections, or both. Current unavailability of genetic tools to study these descending neuron

pools is one of the main barriers in mapping perception to response circuits in nervous system.

Based on the experimental evidences from our lab, that showed PVM027 activation can lead to *stop* in a moving larva, motivated us to dig further into the circuit to understand the mechanism of its interference with the larval locomotory circuit. One of the recently published study by Fushiki et al., 2016 described the mechanism of propagation of forward peristaltic waves in larva while providing hints at potential candidate neurons responsible for backward wave propagation. They also reported that SEZ descending neurons are one of the major input providers to the wave propagation circuit. SEZ has previously been shown to have premotor links that is connected to various sensory systems (Tastekin et al., 2015). Since forward movement is the default mode of larval locomotion, and PVM027 was found to be interruptive to this upon activation, we attempted to investigate the connection between PVM027 and forward peristaltic wave propagation circuit. We showed that PVM027 connects to this motor pool through some of its postsynaptic and downstream SEZ descending neurons. These SEZ descending neurons were found to be presynaptic to a group of excitatory premotor neurons A27h. A27h premotor neurons drive aCC motor neurons in larvae and have been shown to be responsible for propagation of forward peristaltic waves. Two of the SEZ descending neurons of PVM027 circuit were found presynaptic to A27h premotor neurons, but these connections were restricted to anterior and late abdominal segments only while A27h is a segmentally repeated neuron forming chain across segments with another inhibitory neuron of circuit GDL neurons. This led us to investigate this restricted nature of connectivity between PVM027 circuit and forward wave propagation circuit and drove us to look for mechanism by which PVM027 might be able to interrupt forward locomotion in larva. Pinning experiments (Fig. 13A) from our lab, conducted by Ibrahim suggested that PVM027 activation can terminate forward waves more efficiently while it is still in the initial stage of propagation i.e., in later abdominal segments. This was suggestive of importance of segmentally restricted connections between PVM027 and forward wave propagation circuit.

Another aspect to our finding is the nature of SEZ descending neurons themselves. Since PVM027 as well as A27h premotor neurons, both are excitatory, and in order to interrupt the

wave propagation, it is essential to inhibit A27h premotor neurons. Therefore, it is certain that one or both SEZ descending neurons connecting PVM027 to A27h could be inhibitory in nature. This states that inhibition plays a very important role in shaping the sensory driven behavior while executing the brain command in the VNC. It also emphasises that the origin of this kind of inhibition is not necessarily originated in brain, but could be employed on local basis.

Experiments on motor neuron activity while PVM027 activation took us closer to the mechanism of this control of chemotaxis behavior. Calcium imaging data from our lab suggested that PVM027 activation in initial stages of forward wave propagation is more efficient and likely to terminate the current wave. Motor neuron activity pattern showed that forward wave while in initial stage of propagation get suppressed while passing through subsequent anterior segments and eventually get transformed into backward waves (Fig. 14C). This brings connection between PVM027 connected SEZ descending neurons and candidates for backward wave propagation (as described in Fushiki et al., 2016) into picture. Since there are no transgenic lines labelling these candidates available at the moment, it would be interesting to study the role of these connections for conversion of a forward wave to a backward one in future.

Another recent study (Zwart et al., 2016) showed that the neurons of motor systems for command execution in larval VNC have similar intrinsic properties. Shaping of output of a neural network is based on intrinsic properties of its neurons. This leads to sequential recruitment of neurons, which is attributed by features such as difference in electrical properties of neurons. Since the neurons of motor systems are shown to have similar electrical properties, the sequential recruitment of motor neurons is because of the differences in synaptic inputs they receive (Zwart et al., 2016). This codes for dynamism in a neural network, which is the ability to sequentially recruit postsynaptic neurons in a circuit. This is important for transient nature of behavior and is proportional to the command to the motor network. Since, the motor neurons for wave propagation circuit are shown to be receiving synaptic inputs from SEZ descending neurons of PVM027 circuit, they might also be responsible for encoding dynamism

in this circuit for control of chemotaxis.

Author Contributions:

The study described in this chapter was performed as a collaborative project between me and Ibrahim Tastekin, CRG, Barcelona. All the experiments for functional studies were performed by Ibrahim Tastekin, whereas I contributed for anatomical mapping using EM-reconstruction. Figure 1, 2, 3, 6, 7, 8, 11, 12, 13 and 14 show experimental results conducted by Ibrahim Tastekin and were generated by him with legends. I also acknowledge Cardona lab, Janelia Research Campus and their collaborators for contribution in EM reconstruction.

Chapter 4: General discussion and future directions

Understanding neural circuits remains one of the highly wandered areas of neuroscience as it provides the map for brain for a given set of functions. Circuits for sensory perception and behavior generation in brain are most widely studied across animals. Animals, like *Drosophila* larvae present a great model to study for circuit-function analysis due to their ability to perform complex behavior with a numerically much simpler CNS (Asahina et al., 2009, Schulze et al., 2015). Olfactory system is one of the best described and studied systems in *Drosophila* larva, and is the point of origin for shaping odor-driven navigation behavior or chemotaxis behavior. Larvae react to odor cues by display of behavioral features that involves variety of movements like stops, turns, casting of head and locomotion (Gomez-Marin et al., 2011). Since, the origin for this is olfactory perception, we looked at the primary center for olfactory processing in larva i.e., antennal lobe circuit and mapped it using EM-reconstruction technique. This provided us with the wiring diagram of antennal lobe with neuronal connectivity at the resolution of single synapses. Various studies have already reported number of neurons for their potential role in stimulus processing. However, to understand the function of a system, it is imperative to gain some understanding of connection between all the neurons of the system. We analysed the AL wiring diagram that resulted in a greater understanding of larval olfactory system, as a whole and helped us speculate on functions of various circuits.

We found that the canonical circuit with uniglomerular projection neurons (uPNs) and panglomerular LNs was relaying gain-controlled ORN response to higher brain centers. Three main types of LNs were found to be controlling all the stimulus processing in the antennal lobe, Panglomerular Broad LNs, Oligoglomerular Picky LNs and a pair of multiglomerular and bilateral LN called Keystone. These LNs were shown to be playing important role in maintaining the state of activity of the AL in either homogenous or heterogeneous state, depending on the activity of each LN type. Homogeneous and heterogenous are two operational states of inhibition of the antennal lobe, implemented by homogenous (panglomerular) or heterogenous (selective) inhibition respectively. Switch from one to another state is dependent on activity of Picky LN 0, which promotes homogenous state by inhibiting

Keystone and thereby disinhibiting Broad LN trio (Fig. 5b, chapter 2 of this thesis). We showed that odor saliency is computed by panglomerular inhibition from Broad LN trio whereas Keystone LN was found to be driving AL towards heterogeneous state enabling the system to respond to odors that could be important for survival of animal, such as pheromone signals from predator wasps (Ebrahim et al., 2015).

While we elaborated on functioning of various circuits that we found in the AL in second chapter of this thesis, some points still remain to be discussed, and are discussed below:

Role of multiglomerular projection neurons (mPNs) in processing and relay of olfactory signals

We also found a parallel circuit that we speculated to be operating on channel-mixing system for sensory processing in the AL. Channel-mixing corresponds to multiglomerular nature of mPNs connectivity, by which they integrate inputs from multiple ORNs. This was constituted by hierarchically connected Picky LNs along with mPNs-that innervated more than one glomerulus of antennal lobe. Each mPN type seems to have a stereotyped pattern of connectivity, which is evident from the two ALs we reconstructed. In higher brain, mPNs innervate mostly LH and its surrounding regions, with few exceptions innervating MB. In the LH, they innervate a region far wider compared to that of uPNs, and follow variety of tracks to reach higher brain.

mPNs are among the least studied neurons of larval olfactory system, but have been studied for their functions in adult *Drosophila*. Some studies showed that mPNs in adult flies could be GABAergic and inhibitory in nature. The mPNs we found in the larva did not match any of the mPNs described in the adults. We tried to look for genetic driver lines labelling some of the mPNs with interesting connections, but were unable to find any of them. Some mPNs also innervated regions of the SEZ and were found to be interacting with SEZ neurons, potentially with gustatory circuit in the SEZ, and had been previously described by Das et al., 2013. This could be another aspect of multisensory integration in brain, taking place right at the primary

sensory centers. Further studies could reveal their role in processing olfactory stimulus upon integration with inputs from other sensory centers.

Role of LNs in multisensory integration with SEZ

LNs are among the best studied neurons of adult *Drosophila*, and have been described to be both, excitatory as well as inhibitory. In larva, we studied all the LNs except two, and found that all the studied LNs were either GABAergic or Glutamatergic. We could not image a couple of LNs due to unavailability of driver lines to label them. Apart from processing ORN inputs in the AL, some LNs were found to be sharing strong connections with neurons in the SEZ.We also found 6 pairs of oligoglomerular neurons projecting to SEZ from AL. We could not find these neurons described anywhere in the literature along with genetic tools that label them. The fact that some mPNs also share similar connectivity with SEZ neurons, it can be speculated that there exist a circuit that operates on integrated olfactory and gustatory inputs and processes the integrated multisensory information before it is relayed to the higher brain centers.

Effect of neuromodulatory neurons on stimulus processing in the AL

Two types of neuromodulatory neurons innervate AL, serotonergic (CSD) and octopaminergic/tyraminergic (lAL-1 and tdc neurons). These neuromodulators have been described to influence various systems and functions of larval brain from aggression to learning and memory. According to general conception, neuromodulatory neurons do not rely on classical synaptic transmission for influencing other neurons. This is due to the fact that neuromodulators released by neuromodulatory neurons are absorbed by G-protein coupled receptors of the affected neurons, and so, they do not require ligand-gated ion channels used for classical chemical transmission between neurons. We found that these neuromodulatory neurons connect to other neurons in the system through actual chemical synapses. This was indicative of these neuromodulatory neurons expressing more than one neurotransmitter.

A recently published study showed that embryonic born larval CSD neuron expresses acetylcholine along with serotonin (Zhang and Gaudry, 2016). It is reported that CSD neuron is inhibited by all odors, which is supported from our finding that CSD receives a number of synapses from almost all the LNs of the AL, while synapsing extensively on few selected LNs.

Feedback from brain to the AL

We found a descending neuron pair providing feedback to the selected mPNs and LNs in the AL from higher brain. Because this neuron targets the two mPNs (mPN A3 and B3) that we postulated to be aversive, and receives axo-axonic inputs from an aversive ORN 45a (Hernandez-Nunez et al., 2015), we postulated this neuron to be involved in processing of aversive stimuli in the AL. Feedback to the sensory centers could also be result of the synapses received on the axons of mPNs in the LH region.

Bibliography:

Asahina, K., et al. (2009). "A circuit supporting concentration-invariant odor perception in Drosophila." Journal of Biology 8(1): 1-19.

Bader, R., et al. (2007). "Genetic dissection of neural circuit anatomy underlying feeding behavior in Drosophila: Distinct classes of hugin-expressing neurons." The Journal of Comparative Neurology **502**(5): 848-856.

Benzer, S. (1967). Behavioral mutants of Drosophila isolated by countercurrent distribution. Proc. Natl. Acad. Sci. U.S.A. **58**: 1112--1119.

Bhandawat, V., et al. (2007). "Sensory processing in the Drosophila antennal lobe increases reliability and separability of ensemble odor representations." Nat Neurosci 10(11): 1474-1482.

Bhatt, P. K., Neckameyer, W. S. Functional Analysis of the Larval Feeding Circuit in *Drosophila*. *J. Vis. Exp.* (81), e51062, doi:10.3791/51062 (2013).

Bouvier, J., et al. "Descending Command Neurons in the Brainstem that Halt Locomotion." Cell **163**(5): 1191-1203.

Campbell, R. A. A. and G. C. Turner (2010). "The mushroom body." Current Biology **20**(1): R11-R12.

Chou, Y.-H., et al. (2010). "Diversity and wiring variability of olfactory local interneurons in the Drosophila antennal lobe." Nat Neurosci 13(4): 439-449.

Dacks, A. M., et al. (2006). "Phylogeny of a serotonin-immunoreactive neuron in the primary olfactory center of the insect brain." J Comp Neurol 498.

Dacks, A. M., et al. (2009). "Serotonin Modulates Olfactory Processing in the Antennal Lobe of Drosophila." Journal of Neurogenetics 23(4): 366-377.

Das, A., et al. (2008). "Drosophila olfactory local interneurons and projection neurons derive from a common neuroblast lineage specified by the empty spiracles gene." Neural Development 3(1): 1-17.

Das, A., et al. (2013). "Neuroblast lineage-specific origin of the neurons of the Drosophila larval olfactory system." Developmental Biology 373(2): 322-337.

de Belle, J. S. and M. Heisenberg (1994). "Associative odor learning in Drosophila abolished by chemical ablation of mushroom bodies." Science 263(5147): 692-695.

Distler, P. G. and J. Boeckh (1996). "Synaptic connection between olfactory receptor cells and uniglomerular projection neurons in the antennal lobe of the American cockroach, Periplaneta americana." The Journal of Comparative Neurology 370(1): 35-46.

Ebrahim, S. A. M., et al. (2015). "Drosophila Avoids Parasitoids by Sensing Their Semiochemicals via a Dedicated Olfactory Circuit." PLoS Biol **13**(12): e1002318.

Farooqui T. 2007. Octopamine-mediated neuromodulation of insect senses. Neurochem Res 32:1511–1529.

Fisek, M. and R. I. Wilson (2014). "Stereotyped connectivity and computations in higher-order olfactory neurons." Nat Neurosci 17(2): 280-288.

Fishilevich, E., et al. "Chemotaxis Behavior Mediated by Single Larval Olfactory Neurons in Drosophila." Current Biology 15(23): 2086-2096.

Foster, M.; Sherrington, C.S. (1897). *Textbook of Physiology, volume 3* (7th ed.). London: Macmillan. p. 929.

Fushiki, A., et al. (2016). "A circuit mechanism for the propagation of waves of muscle contraction in Drosophila." eLife **5**: e13253.

Gao, X. J., et al. (2015). "Extremely Sparse Olfactory Inputs Are Sufficient to Mediate Innate Aversion in Drosophila." PLoS ONE 10(4): e0125986.

Gatellier, L., et al. (2004). "Serotonin modifies the sensitivity of the male silkmoth to pheromone." J Exp Biol 207.

Gepner, R., et al. (2015). "Computations underlying Drosophila photo-taxis, odor-taxis, and multi-sensory integration." eLife 4: e06229.

Gerber, B. and R. F. Stocker (2007). "The Drosophila Larva as a Model for Studying Chemosensation and Chemosensory Learning: A Review." Chemical Senses 32(1): 65-89.

Gershow, M., et al. (2012). "Controlling airborne cues to study small animal navigation." Nat Meth 9(3): 290-296.

Gomez-Marin, A. and M. Louis (2014). "Multilevel control of run orientation in Drosophila larval chemotaxis." Front Behav Neurosci **8**: 38.

Gomez-Marin, A., et al. (2011). "Active sampling and decision making in Drosophila chemotaxis." Nature Communications 2: 441.

Gronenberg, W. and N. J. Strausfeld (1992). "Premotor descending neurons responding selectively to local visual stimuli in flies." The Journal of Comparative Neurology **316**(1): 87-103.

Heckscher, E. S., et al. (2012). "Characterization of Drosophila Larval Crawling at the Level of Organism, Segment, and Somatic Body Wall Musculature." The Journal of Neuroscience **32**(36): 12460-12471.

Herculano-Houzel, S., et al. (2014). "The elephant brain in numbers." Front Neuroanat **8**: 46.

Hernandez-Nunez, L., et al. (2015). "Reverse-correlation analysis of navigation dynamics in Drosophila larva using optogenetics." eLife 4: e06225.

Hill, E. S., et al. (2002). "Morphology and physiology of the serotonin-immunoreactive putative antennal lobe feedback neuron in the male silkmoth Bombyx mori." Chem Senses 27.

Homberg, U. and J. G. Hildebrand (1989). "Serotonin immunoreactivity in the optic lobes of the sphinx moth Manduca sexta and colocalization with FMRFamide and SCPB immunoreactivity." J Comp Neurol 288.

Hsu, C. T. and V. Bhandawat (2016). "Organization of descending neurons in Drosophila melanogaster." Scientific Reports **6**: 20259.

Hückesfeld, S., et al. (2015). "Localization of Motor Neurons and Central Pattern Generators for Motor Patterns Underlying Feeding Behavior in Drosophila Larvae." PLoS ONE 10(8): e0135011.

Jefferis, G. S. X. E., et al. "Comprehensive Maps of Drosophila Higher Olfactory Centers: Spatially Segregated Fruit and Pheromone Representation." Cell 128(6): 1187-1203.

Keene, A. C. and S. Waddell (2007). "Drosophila olfactory memory: single genes to complex neural circuits." Nat Rev Neurosci **8**(5): 341-354.

Kido, A. and K. Ito (2002). "Mushroom bodies are not required for courtship behavior by normal and sexually mosaic Drosophila." J Neurobiol 52(4): 302-311.

Kim, A. J., et al. (2015). "Projection neurons in Drosophila antennal lobes signal the acceleration of odor concentrations." eLife 4: e06651.

Kloppenburg, P. and J. G. Hildebrand (1995). "Neuromodulation by 5-hydroxytryptamine in the antennal lobe of the sphinx moth Manduca sexta." J Exp Biol 198.

Kreher, S. A., et al. "Translation of Sensory Input into Behavioral Output via an Olfactory System." Neuron **59**(1): 110-124.

Lai, S.-L., et al. (2008). "Clonal analysis of Drosophila antennal lobe neurons: diverse neuronal architectures in the lateral neuroblast lineage." Development 135(17): 2883-2893.

Landgraf, M., et al. (1997). "The origin, location, and projections of the embryonic abdominal motorneurons of Drosophila." J Neurosci **17**(24): 9642-9655.

Li, H.-H., et al. "A GAL4 Driver Resource for Developmental and Behavioral Studies on the Larval CNS of Drosophila." Cell Reports 8(3): 897-908.

Liang, L., et al. (2013). "GABAergic Projection Neurons Route Selective Olfactory Inputs to Specific Higher-Order Neurons." Neuron 79(5): 917-931.

Liu, Q., et al. (2016). "Gap junction networks in mushroom bodies participate in visual learning and memory in Drosophila." eLife 5: e13238.

Liu, W. W. and R. I. Wilson (2013). "Glutamate is an inhibitory neurotransmitter in the Drosophila olfactory system." Proceedings of the National Academy of Sciences 110(25): 10294-10299.

Lledo, P.-M., et al. (2008). "Origin and function of olfactory bulb interneuron diversity." Trends in Neurosciences 31(8): 392-400.

Louis, M., et al. (2008). "Bilateral olfactory sensory input enhances chemotaxis behavior." Nat Neurosci 11(2): 187-199.

Luan, H., et al. (2006). "Refined spatial manipulation of neuronal function by combinatorial restriction of transgene expression." Neuron **52**(3): 425-436.

Luo, S. X., et al. (2010). "Generating sparse and selective third-order responses in the olfactory system of the fly." Proceedings of the National Academy of Sciences 107(23): 10713-10718.

Marin, E. C., et al. "Representation of the Glomerular Olfactory Map in the Drosophila Brain." Cell 109(2): 243-255.

Masuda-Nakagawa, L. M., et al. (2009). "Localized olfactory representation in mushroom bodies of Drosophila larvae." Proceedings of the National Academy of Sciences 106(25): 10314-10319.

Mathew, D., et al. (2013). "Functional diversity among sensory receptors in a Drosophila olfactory circuit." Proceedings of the National Academy of Sciences 110(23): E2134-E2143.

Melcher, C. and M. J. Pankratz (2005). "Candidate Gustatory Interneurons Modulating Feeding Behavior in the Drosophila Brain." PLoS Biol **3**(9): e305.

Mu, L., et al. (2014). "Responses of Drosophila giant descending neurons to visual and mechanical stimuli." The Journal of Experimental Biology **217**(12): 2121-2129.

Nern, A., B. D. Pfeiffer and G. M. Rubin (2015). "Optimized tools for multicolor stochastic labeling reveal diverse stereotyped cell arrangements in the fly visual system." Proc Natl Acad Sci U S A **112**(22): E2967-2976.

Ohyama, T., et al. (2015). "A multilevel multimodal circuit enhances action selection in Drosophila." Nature 520(7549): 633-639.

Okada, R., et al. (2009). "Gamma-aminobutyric acid (GABA)-mediated neural connections in the Drosophila antennal lobe." The Journal of Comparative Neurology 514(1): 74-91.

Oland, L. A., et al. (2008). "Glial investment of the adult and developing antennal lobe of Drosophila." The Journal of Comparative Neurology 509(5): 526-550.

Olsen, S. R. and R. I. Wilson "Cracking neural circuits in a tiny brain: new approaches for understanding the neural circuitry of Drosophila." Trends in Neurosciences **31**(10): 512-520.

Olsen, S. R. and R. I. Wilson (2008). "Lateral presynaptic inhibition mediates gain control in an olfactory circuit." Nature 452(7190): 956-960.

Olsen, S. R., et al. "Divisive Normalization in Olfactory Population Codes." Neuron 66(2): 287-299.

Olsen, S. R., et al. "Excitatory Interactions between Olfactory Processing Channels in the Drosophila Antennal Lobe." Neuron 54(1): 89-103.

Pfeiffer, B. D., et al. (2010). "Refinement of tools for targeted gene expression in Drosophila." Genetics **186**(2): 735-755.

Phelan, P. and T. A. Starich (2001). "Innexins get into the gap." BioEssays 23(5): 388-396.

Prieto-Godino, L. L., et al. (2012). "Embryonic Origin of Olfactory Circuitry in Drosophila: Contact and Activity-Mediated Interactions Pattern Connectivity in the Antennal Lobe." PLoS Biol 10(10): e1001400.

Python, F. and R. F. Stocker (2002). "Adult-like complexity of the larval antennal lobe of D. melanogaster despite markedly low numbers of odorant receptor neurons." The Journal of Comparative Neurology 445(4): 374-387.

Ramaekers, A., et al. "Glomerular Maps without Cellular Redundancy at Successive Levels of the Drosophila Larval Olfactory Circuit." Current Biology 15(11): 982-992.

Roy, B., et al. (2007). "Metamorphosis of an identified serotonergic neuron in the Drosophila olfactory system." Neural Development 2(1): 1-17.

Saalfeld, S., et al. (2009). "CATMAID: collaborative annotation toolkit for massive amounts of image data." Bioinformatics 25(15): 1984-1986.

Sachse, S., et al. (2006). "Role of histamine as a putative inhibitory transmitter in the honeybee antennal lobe." Frontiers in Zoology **3**(1): 1-7.

Saraswati, S., et al. (2004). "Tyramine and octopamine have opposite effects on the locomotion of Drosophila larvae." Journal of Neurobiology 58(4): 425-441.

Schneider-Mizell, C. M., et al. (2016). "Quantitative neuroanatomy for connectomics in Drosophila." eLife 5: e12059.

Schulze, A., et al. (2015). "Dynamical feature extraction at the sensory periphery guides chemotaxis." eLife **4**: e06694.

Selcho, M., et al. (2012). "The Role of octopamine and tyramine in Drosophila larval locomotion." The Journal of Comparative Neurology 520(16): 3764-3785.

Selcho, M., et al. (2014). "Characterization of the octopaminergic and tyraminergic neurons in the central brain of Drosophila larvae." Journal of Comparative Neurology 522(15): 3485-3500.

Shang, Y., et al. (2007). "Excitatory Local Circuits and Their Implications for Olfactory Processing in the Fly Antennal Lobe." Cell 128(3): 601-612.

Shepherd, G.M., Chen, W.R. & Greer, C.A. Olfactory Bulb. in *The synaptic Organization of the Brain* (ed. Shepherd, G.M.) (Oxford University Press, Oxford, 2004).

Singh, A. P., et al. (2013). "Sensory Neuron-Derived Eph Regulates Glomerular Arbors and Modulatory Function of a Central Serotonergic Neuron." PLoS Genet 9(4): e1003452.

Tastekin, I., et al. "Role of the Subesophageal Zone in Sensorimotor Control of Orientation in Drosophila Larva." Current Biology **25**(11): 1448-1460.

Thum, A. S., et al. (2011). "Diversity, variability, and suboesophageal connectivity of antennal lobe neurons in D. melanogaster larvae." The Journal of Comparative Neurology 519(17): 3415-3432.

Van Swinderen, B. and R. Andretic (2011). "Dopamine in Drosophila: setting arousal thresholds in a miniature brain." Proc Biol Sci **278**(1707): 906-913.

Verlinden, H., et al. (2010). "The role of octopamine in locusts and other arthropods." Journal of Insect Physiology 56(8): 854-867.

Vosshall, L. B. and R. F. Stocker (2007). "Molecular Architecture of Smell and Taste in Drosophila." Annual Review of Neuroscience 30(1): 505-533.

Vosshall, L. B., et al. "An Olfactory Sensory Map in the Fly Brain." Cell 102(2): 147-159.

Wachowiak, M. and M. T. Shipley (2006). "Coding and synaptic processing of sensory information in the glomerular layer of the olfactory bulb." Seminars in Cell & Developmental Biology 17(4): 411-423.

White, J. G., et al. (1986). "The Structure of the Nervous System of the Nematode Caenorhabditis elegans." Philosophical Transactions of the Royal Society of London. B, Biological Sciences **314**(1165): 1-340.

Wilson, R. I. and G. Laurent (2005). "Role of GABAergic Inhibition in Shaping Odor-Evoked Spatiotemporal Patterns in the Drosophila Antennal Lobe." The Journal of Neuroscience 25(40): 9069-9079.

Wong, A. M., et al. (2002). "Spatial Representation of the Glomerular Map in the Drosophila Protocerebrum." Cell 109(2): 229-241.

Younossi-Hartenstein, A., et al. (2003). "Early development of the Drosophila brain: IV. Larval neuropile compartments defined by glial septa." The Journal of Comparative Neurology 455(4): 435-450.

Zhang, X. and Q. Gaudry (2016). "Functional integration of a serotonergic neuron in the Drosophila antennal lobe." eLife **5**: e16836.

Zwart, Maarten F., et al. "Selective Inhibition Mediates the Sequential Recruitment of Motor Pools." Neuron **91**(3): 615-628.