ABSTRACT

Moths detect CO_2 on labial palps and axons from Labial palps project into a single glomerulus, the Labial palp pit organ glomerulus (LPOG). Mass-staining of the LPOG visualized the secondary pathway in the brain. These data was used for 3D reconstruction of neurons in that particular pathway. Based on the preparations from Ingrid Moe Dahl we performed intensification, immunostaining and rescanned for better visualization. After the visualization, brain neuropils and the projection neurons of each of the brain preparation were constructed manually using the computer software AMIRA 5.3. The projection neurons from the LPOG were found to project to three main tracts: the lateral antennal lobe tract, the mediolateral antennal lobe tract and the second mediolateral antennal lobe tract. In addition, we also observed projection neurons in the dorsomedial antennal lobe tract. The main termination areas of the LPOG projection neurons are the superior protocerebrum, and the ipsi- and contralateral lateral protocerebrum. In contrast to the previous finding, we observed LPOG projection neurons in the mushroom body calyces, indicating some modulation by experience. In the antennal lobe, interneurons from the LPOG projecting to other glomeruli were seen, i.e. indicating communication between the LPOG and the other glomeruli. The presence of LPOG neurons projecting toward antennae indicates the possibility of LPOG sensory neurons on the base of antennae. No sexual dimorphism was observed between male and female LPOG projection pathways. Thus, the reconstructions yielded new information relevant for understanding the putative CO_2 pathway.

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Trondheim

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Abbreviations

LPOG	Labial pit organ glomerulus
SBA	Standard Brain Atlas
LPO	Labial Pit Organ
ORN	olfactory receptor neuron
MGC	macro glomerular complex
AL	Antennal lobe
ALT	Antennal lobe tract
l-	lateral
ml-	medio-lateral
m-	medial
dm-	dorsomedial
AST	antenno-subesophageal tract
SEG	sub- esophageal ganglion
MB	Mushroom body
LH	lateral Horn
LNs	local interneurons
GABA	γ-aminobutyric acid

1 Introduction

The nervous system is highly complex. To understand it, various methods are employed, ranging from lesioning, gene expressions, electrophysiology to imaging data and combinations of it. Despite many years of research, we still do not fully understand even the simplest nervous system or can build artificial sensors as complex and small as nature manages. In the ongoing work to decipher nature's mystery, mapping of neurons into standard frameworks is a versatile tool. Combined with the large knowledge about olfaction, this thesis will present a project tracing the secondary pathway of putative CO_2 responsive neurons of the model species *H. virescens* to higher brain areas.

1.1 Standard Brain Atlases (SBA)

Standard Brain Atlases are very important tools to assemble and integrate rapidly growing data of neural structures and networks. Such standard brain atlases allow data to be displayed and analyzed in a common reference framework. In insects, three-dimensional digital brain atlases have been generated for five species: the fruit fly *Drosophila melanogaster (K.* Rein, Zockler, Mader, Grubel, *&* Heisenberg, 2002*)*, the honey bee *Apis mellifera (*Brandt et *al., 2005)* the desert locust *Schistocerca gregaria (*Kurylas, Rohlfing, Krofczik, Jenett, *&* Homberg, 2008*)*, the tobacco hornworm *Manduca sexta (El* Jundi, Huetteroth, Kurylas, *& Schachtner, 2009)* and the tobacco budworm *Heliothis virescens (*Kvello, Lofaldli, Rybak, Menzel, *&* Mustaparta, 2009*).*

Two approaches are used for the construction of a SBA. The first one is called "iterative shape averaging" (ISA), and has been developed for studies on honeybees. This approach reduces anatomical variability (Brandt et al., 2005; El Jundi et al., 2009). The second approach is the so-called Virtual Insect Brain (VIB), and has been developed by researchers working with *Drosophila melanogaster*. This method preserves the anatomical variability (El Jundi et al., 2009; Jenett, Schindelin, & Heisenberg, 2006).

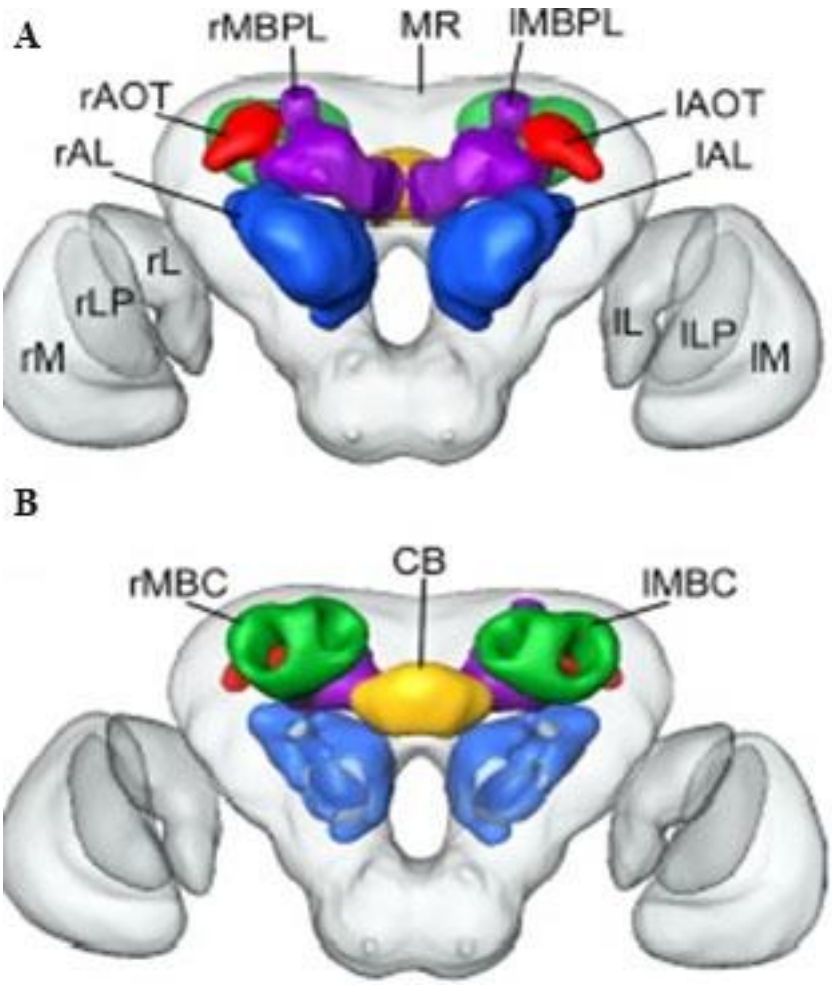


Figure 1. 1: The average standard brain of *H. virescens*.(a) Anterior view. (b) Posterior view. MR = Midbrain region, rMBC = Right mushroom body calyces, rMBPL = right mushroom body peduncle and lobes, CB = central body, rAOT = right anterior optic tubercle rAOT,rAL = right antennal lobe, rM = right medulla, rLP = right lobula plate, rL=right lobula, lMBC=left mushroom body calyces, lMBPL=left mushroom body peduncle and lobes, lAOT=left andterior optic tubercle, lAL=left antennal lobe, LM=left medulla,lLP=left lobula plate, lL=left lobula(Kvello et al., 2009).

Standard brain atlases can serve as a tool for organizing and analyzing data that has been substantial in many species. Data from different anatomical structures can be incorporated into standard brain atlas for better understanding and interpretation. In this paper we reconstructed the labial palp sensory neuron pathway from mass-staining experiments done by Ingrid Moe Dahl, a previous master student in the lab (graduated 2013). More details are given in the method section. The Master project presented here provides the reconstructions that can be used for implementing the pathway into the standard atlas of *H. virescens.* This

should provide a better understanding of the complexity in the labial palp sensory pathway, which may play a role, and hence be different from the other odorants, in CO_2 processing.

1.2 Aims and objectives:

The main objective of this study was to acquire knowledge on the projection pattern of the LPOG pathway. This study attempted to identify the higher brain centers of the LPOG pathway, to reconstruct it, and to give it a correct 3D visualization.

- Which brain regions are innervated by LPOG projection neurons?
- Do the target regions of the LPOG pathway overlap with those of ordinary olfactory neurons?
- What characterizes the LPOG pathways in a three-dimensional brain?

I will briefly review the olfactory system and CO_2 processing before showing reconstructions and how this work compliments the experimental work from I. Moe Dahl.

1.3 Perception of an odour

Olfactory cues have been one of the most important factors for the survival of an animal. Olfaction is involved in induction of various behaviors including feeding, navigation, mating, communication and ovipositioning. The detection of olfactory stimuli or odorants occurs via olfactory receptor neurons (ORN). The information about the odor molecule from these ORN are sent via the axon to the primary olfactory center; the antennal lobe in insects (see Figure 1). The multidimensional odor information is processed as a complex activity pattern in the neuronal network of glomeruli in the primary olfactory centre (Lledo, Gheusi, & Vincent, 2005). This information is transferred to higher centers through parallel pathways from the primary olfactory center for further processing and integration (Galizia & Rossler, 2010). In insects, the main target areas of the primary olfactory center are the mushroom bodies (associated with learning and memory), the superior protocerebrum, and the lateral protocerebrum, which also contains the premotor area (Gerber, Tanimoto, & Heisenberg, 2004). This resembles the mammalian olfactory pathway. Here, the olfactory neurons project

from the olfactory bulb to the olfactory cortex (piriform cortex) and the limbic system (hippocampal formation associated with several types of memories, hypothalamus and amygdala associated with emotional responses) (Lledo et al., 2005). In insects, the main target areas of primary olfactory center are mushroom bodies (associated with learning and memory), superior protocerebrum, and lateral protocerebrum (also contains premotor area) (Gerber et al., 2004)

1.3.1 Primary olfactory processing

In insects, the antennae is the main olfactory organ consisting of numerous ORN housed within a sensillum. In addition, many species have olfactory sensors in the mouthparts such as the labial palp in moth and the maxillary palp in mosquitoes (Distler & Boeckh, 1997; Zhao et al., 2013). The dendrites of ORN remain surrounded in lymph within a sensillum. Olfactory action potential from the antennae is sent directly to the antennal lobe, which is the primary olfactory centre of the insect brain (Masse, Turner, & Jefferis, 2009). The antennal lobe is made up of neuropils, called glomeruli. The number of ordinary glomeruli varies among species, 43 in case of Drosophila (Wong, Wang, & Axel, 2002), 62 in case of *H*. virescens (Berg, Galizia, Brandt, & Mustaparta, 2002), 63 in case of *Manduca sexta* (Rospars & Hildebrand, 2000), and approximately 160 in *Apis mellifera* (Flanagan & Mercer, 1989*)*. The size and shape of all the glomeruli are more or less similar. However in *Heliothine virescens* male moths, some glomeruli are enlarged and make up the macro glomerular complex (MGC) (Berg et al., 2002). The MGC detects the long-range sex pheromones from females. In *H. vir.*, the MGC comprise four enlarged glomeruli located at the entrance of the antennal nerve in the AL (Berg et al., 2002; Vickers, Christensen, & Hildebrand, 1998) . This shows a specialized arrangement for important odorants, here pheromones. As we will see further, CO_2 is also an important odorant and processed in a dedicated glomerulus. Plant volatiles seem to be processed across glomeruli (Skiri, Galizia, & Mustaparta, 2004). This arrangement is more flexible.

The AL mainly contains three types of neurons: the receptor neurons, local inhibitory neurons and the uni- and multiglomerular projection neurons (Stocker, Lienhard, Borst, & Fischbach, 1990). In addition to these three main types of neurons, modulatory centrifugal neurons are found. They have been detected by their neurotransmitter, e.g. the serotonergic neuron found across moth species (Kloppenburg & Mercer, 2008; Zhao & Berg, 2009) or the octapominergic neuron in the honey bee (J. Rein, Mustard, Strauch, Smith, & Galizia, 2013). Most, if not all of these centrifugal neurons innervate all glomeruli (Zhao et al., 2013) .

1.3.2 The antennal lobe tracts

From the antennal lobe, the olfactory information is sent to higher centers through different antennal lobe tracts. There are mainly three antennal lobe tracts: i) the lateral antennal lobe tract (l-ALT), ii) the medio-lateral antennal lobe tract (ml-ALT), and iii) the medial antennal lobe tract (m-ALT) (Homberg, Montague, & Hildebrand, 1988). Each of these tracts consists of numerous projection neurons. The projection neurons can originate from only one glomerulus (uniglomerular) or have arborization in multiple glomeruli, i.e. they are multiglomerular (Homberg et al., 1988; Ro, Muller, & Mustaparta, 2007). Similarly, apart from these three tracts, two other tracts, the dorsomedial antennal-lobe tract (dm-ALT) and the antenno-subesophageal tract (AST) have been described in moths (Homberg et al., 1988). The dorsomedial tract contains only uniglomerular projection neurons. This is unlike the other tracts which contain both uniglomerular and multiglomerular projection neurons (Homberg et al., 1988). Also a neuron with an unpaired median soma in the subesophageal ganglion was observed in *H. virescens (*Ro *et* al., 2007*).*

The medial ALT, which is the most prominent tract, leaves the AL dorsomedially. It runs posteriorly in the protocerebrum, passing closely to the lateral edge of the central body. It turns laterally near the protocerebral bridge and continues to the anterior surface of the ipsilateral calyces, where it sends branches and innervates the neuropil. The m-ALT continues to the lateral parts of the median protocerebrum from the calyces (Homberg et al., 1988; Ro et al., 2007).

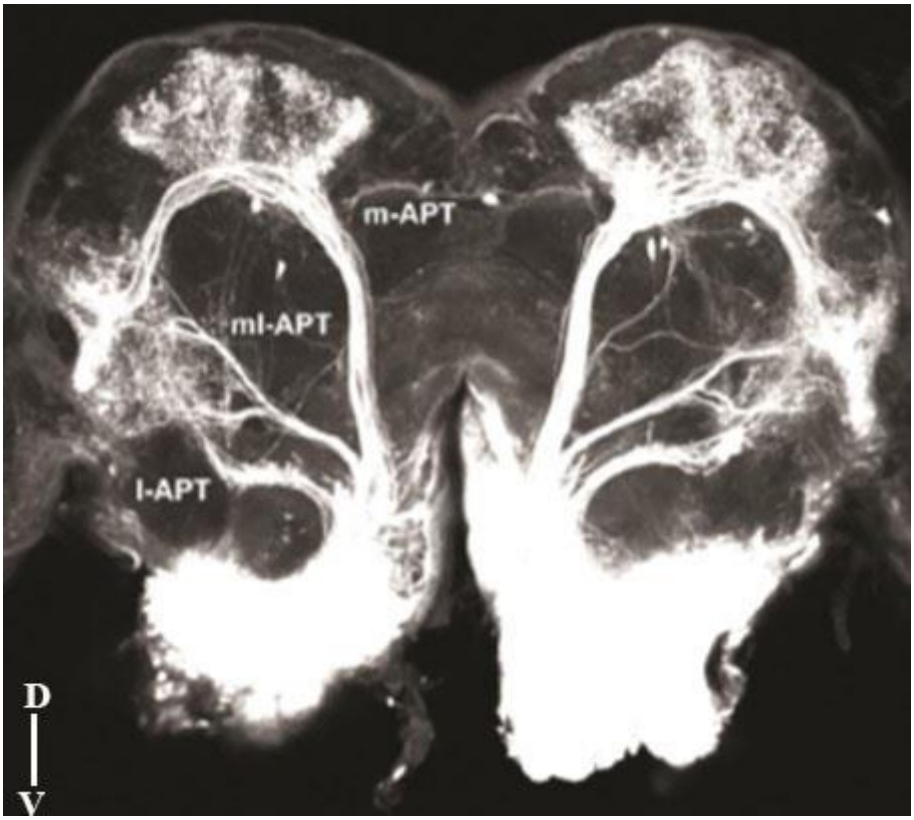


Figure 1. 2: Confocal image of the three main antennal lobe tract in a moth brain. l-ALT = lateral antennal lobe tract; ml-ALT = mediolateral antennal lobe tract; m-ALT = medial antennal love tract. Xin-Cheng Zhao (unpublished data)

The ml-ALT is comparatively smaller to the m-ALT and follows it for a short distance after leaving the AL. It bends at the edge of the central body and continues laterally in close proximity of the ventral edge of the pedunculus. The mediolateral ALT divides into several smaller branches terminating into different parts of the lateral protocerebrum (Homberg et al., 1988; Ro et al., 2007).

The l-ALT leaves the antennal lobe ventral to the m-ALT and ml-ALT. As soon as it leaves the antennal lobe, it bends laterally and divides into two branches. One branch with few fibers makes a dorsal turn ventral to the pedunculus and terminates in the area lateral to the alpha lobe, a mushroom body structure. The remaining fibers continue as a common tract in the

lateral protocerebrum and innervates the lateral horn. From the lateral horn, some of the fibers of the l-ALT turn dorsomedially innervating the calyces (Homberg et al., 1988; Ro et al., 2007).

The dm-ALT is the most dorsal tract. It continues dorsomedially after leaving the AL and turns posterolaterally towards the mushroom body calyces on the dorsal surface of the brain. This tract contains three large diameter fibers of protocerebral neurons apart from the neurites of SEG-neurons. These neurons have somata in the anterior dorsal protocerebrum, anterior to the median bundle. They project via dm-ALT into the contralateral protocerebrum (Homberg et al., 1988).

The AST is comprised of many small fibers; specifically it contains the axons of receptor cells from the labial palp organ projecting into the AL. These fibers have their soma in the SEG. They give off sub-branches in several glomeruli and then continue with dm-ALT towards the calyces (Homberg et al., 1988).

Apart from the above-described pathway, another tract called the second mediolateral tract has also been described for *H. vir.* in general olfactory system (Figure:A1) (Siri Lillevoll 2013, unpublished data).

1.4 CO2 - a special odor

Few odorants are very crucial. CO_2 is one of them, pheromones are the other ones. CO_2 is an important molecule and odorant that has been shown to influence behaviors, e.g. feeding, mating, ovipositioning and signaling of danger (Bracker et al., 2013). The behavior of insects is affected by the concentration of CO_2 in the atmosphere. Either attracted or repulsed behavior depending on the concentration of CO_2 was observed (Faucher, Forstreuter, Hilker, & de Bruyne, 2006). *H. virescens* larvae orientate towards photosynthetically active plants where the CO_2 concentration was greater compared to inactive plants (Rasch & Rembold,

1994). Similar orientation behaviour, towards higher CO_2 gradient was also observed in another moth species *Cactoblastis cactorum (*Strange, Monro, Stowe, & Osmond, 1995*)* . A study in D. melanogaster has shown behavior fluctuation depending on the minor change in CO_2 concentration. Drosophila in contrast to moth and mosquitoes are repelled by elevated CO2 level, which is mediated by specialized pathway (Faucher et al., 2006; Lin, Chu, Fu, Dickson, & Chiang, 2013). As the use of CO_2 information by insects is now established, better knowledge of the CO_2 pathway seems necessary for understanding the relationship of CO_2 and insect behavior.

Anatomically, in insects, the CO_2 receptors are located either on the antennae as in *Drosophila melanogaster*, or in the mouth parts, i.e. club shaped sensilla in distal segment of maxillary palps in mosquitos (de Bruyne, Foster, & Carlson, 2001; Distler & Boeckh, 1997), and labial palps in moths (Lepidoptera) (Kent, Harrow, Quartararo, & Hildebrand, 1986; Zhao et al., 2013). In our model species, the noctuid moth *H. vir.*, the CO_2 receptors are located in sensilla in the labial palp pit organ. In *H. vir.*, the labial palp pit organ is a bottle-shaped structure lying in the third segment of the labial palp, containing about 1200 sensilla (Fig 1.2) (Zhao et al., 2013). In another moth, *Rhodogastria*, the distal part of labial palp possesses a pit housing sensilla and are characterized by wall-pores and lamellated dendrite of the receptor cells (Bogner, Boppre, Ernst, & Boeckh, 1986). As shown in *M. sexta*, another noctuid moth, these sensory receptor cells in the labial palp pit organ are sensitive to CO_2 (Kent et al., 1986). Physiologically, these labial palp pit receptors are most excitable by CO_2 stimulation and show a characteristic steep dose-response, and are moderately excited by various other odorant. These receptors show spontaneous response to CO_2 as they are already in excited condition in an ambient atmosphere of ca. 0.03% CO_2. However, this activity decreases under N_2, O_2 and CO_2 free air (Bogner et al., 1986).

The CO_2 receptor seems to belong to the family of gustatory receptors. The ectopically expressed chemosensory receptors Gr21a and Gr63a, which together are responsible for sensation of CO_2 in *D. melanogaster,* are gustatory receptors (Kwon, Dahanukar, Weiss, & Carlson, 2007). In mice, taste cells respond to CO_2. In an experiment with mice, involving targeted genetic ablation and silencing of synapses in defined population of taste receptor cells, sour sensing taste cells were demonstrated to mediate response to CO_2. It was shown

that the sour-sensing cells act as the taste sensors for carbonation, and that carbonic anhydrase 4, a glycosylphosphatidylinositol-anchored enzyme, functions as the principal CO_2 taste sensor (Chandrashekar et al., 2009).

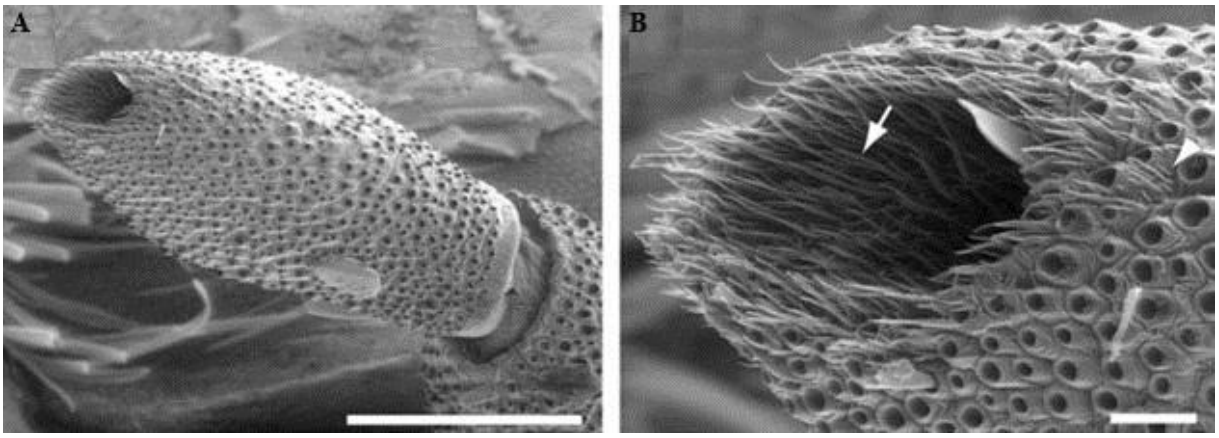


Figure 1. 3: Scanning electron micrographs of the third labial palp segment showing sensilla situated near the opening of the LPO, i.e., sensilla of the hairshaped type. (a) The third segment of the labial palp with the opening of the LPO. (b)High-magnification image of the opening showing hairshaped sensilla localized in the upper part (arrow). Arrowhead represents the presence of a small hair-shaped sensillum . Bars 100 µm (a), 20 µm (b) Scanning electronmicroscopy. Image from (Zhao et al., 2013)

1.4.1 The primary LPO pathway

The sensory axons originating from sensilla in the labial palp form a bundle that enters the subesophageal ganglion (SEG) ipsilaterally via the labial nerve. These axons project to three distinct areas: i) the labial palp pit organ glomerulus (LPOG) in the antennal lobe, ii) the suboesophageal ganglion and iii) the ventral nerve cord (Kent et al., 1986; Zhao et al., 2013). In the sphinx moth *Manduca sexta*, LPOG is the target region for the CO_2-sensitive neurons from the labial palp pit organ. The LPOG only receives afferents from the labial pit organ but no other sensory neurons (Kent et al., 1986). The same is true for *H. vir.*, our model species, (Kvello, Masterthesis; Moe Dahl, Masterthesis). This arrangement has also been described in *M. sexta* in which fibers from the labial nerve enter SEG and extend dorsally to LPOG (Kent et al., 1986). Sparse arborisation of the neurons in SEG has also been described. Kent et al. also described some neurons that leave the SEG through the ventral connective (VC) towards

ventral nerve cord (Kent et al., 1986). No studies have been found describing the terminating area of the neurons projecting to the ventral nerve cord.

Axons from these receptors in LPO project into the LPOG in both ipsi- and contralateral antennal lobe (Kent et al., 1986) unlike the general olfactory neurons which project only in the ipsilateral antennal lobe.The bilateral projection to a single glomerulus (LPOG) was observed in all the three species of *Manduca (M. quinquemaculata, M. dilucida,* and *M. lanuginosa)* and in the silkmoths *Antheraea polyphemus* and *Bombyx mori (*Kent et al., 1986*).* Similarly, CO_2 receptors in the antennae of *D. melanogaster* also project to a specialized neuropil, the V glomerulus, here too in both ipsi- and contralateral hemisphere (Bracker et al., 2013). Also maxillary palp receptor neurons in the mosquitoe *Anopheles gambiae* project to a dorso-medial area in both the ipsi- and contralateral antennal lobe (Anton et al., 2003).The bilateral projection of neurons from the CO_2 receptor to one of the glomeruli in both hemispheres indicates that CO_2 information is processed bilaterally already at the first processing stage. Bilaterality of antennal projections has not been described yet, and thus it is not the case for plant odors in the moth olfactory pathway.

The projection of CO_2 neurons from the labial palp to one of the glomerulus in the Antennal Lobe is quite surprising as the information from the labial palp could be expected to be processed in the labial neuromere in the SEG (Kent et al., 1986). The LPOG seems to be the only dedicated target for the sensory neurons from the labial palp pit organ as it receives no afferent input from the antennae (Kent, Oland, & Hildebrand, 1999). In mosquitoes, CO_2 information from the maxillary palps acts synergistically with the host-emitted volatiles detected by the antennae to find the host (Dekker, Geier, & Carde, 2005) which shows integration of antennal information with inputs from maxillary palp CO_2 neurons in mosquitoes.

1.4.2 Secondary LPOG pathway

As described above, the antennal lobe is also the target for the CO_2 neurons projecting from LPO. All neurons from LPO project into one glomerulus, called LPOG, in the antennal lobe (Kent et al., 1986). From the LPOG the projection neurons, putatively CO_2 sensitive, have been described to project to higher centers via two main tracts: i) the lateral antennal lobe tract (l-ALT) and ii) the mediolateral antennal lobe tract (ml-ALT) (Ingrid Moe Dahl, 2013). The main target areas of the CO_2 neurons are the superior protocerebrum and the ipsi- as well as the contralateral lateral protocerebrum. No projections in the MB calyces were observed indicating the processing of CO_2 to be experience independent (Ingrid Moe Dahl 2013).

However, in *Drosophila melanogaster*, the CO_2 neurons from the V glomerulus project to higher brain centers through all of the three antennal lobe tracts: inner, medial and outer antenna cerebral tracts (Lin et al., 2013). Also, the projection of CO_2 neurons in *D. melanogaster* are observed in MB calyces, two out of four projection neurons follow the medial antennal cerebral tract, that bypass the mushroom body (MB) to connect the Lateral Horn (LH), a structure similar to the moth's lateral protocerebrum. The two remaining projection follow the outer antenno-cerebral tract. These are the typical bilateral PNs that bifurcate and extend into LH an MB calyx one on each side of the brain (Bracker et al., 2013). Relay of CO_2 signals to the MB suggests the possibilities of integration of other stimuli with the CO_2 information (Bracker et al., 2013) in the MB. Ingrid Moe Dahl investigated the LPO secondary pathway experimentally, whereas this thesis focuses on reconstructing of the LPO secondary pathway to gain additional information. This was achieved by intensifying, immunostaining, and rescanning of well-stained preparations from I. Moe Dahl. These rescanned samples were then processed in AMIRA.

Table 1: Comparison of the CO2 processing in our model species and the fruit fly

Heliothis virescens	Drosophila melanogaster
Receptors are present in third segment of labial palp, named the labial palp pit organ (LPO)(Zhao et al., 2013)	Small, CO_2 sensitive subpopulation of olfactory sensory neurons designated ab1C(de Bruyne et al., 2001)in antenna
The target regions of CO_2 neurons are all within the LPO-Glomerulus	The target regions of CO_2 neurons are all within the V glomerulus
Moths show attraction behaviour to CO_2	CO_2 avoidance
PN follow lateral-ALT and medio-lateral ALT *(Ingrid Moe Dahl,2013)* but see result section	PN follow all the three pathways (Lin et al., 2013)
CO_2 neurons in Calyces (G. Pfuhl)	Projection neurons from mACT bypass mushroom bodies to LH

2. Materials and methods

2.1 The Insects

The pupae of the experimental animal *H. virescens* (Heliothine; Lepidoptera; Noctuidae) were received from Syngenta Laboratory culture (Syngenta AS, Basel, Switzerland). In our lab, the pupae were sorted according to sex and then incubated at 22-23° Celsius, 70% air humidity, and a phase-shifted photoperiod (14 hr light: 10 hr dark). After hatching, the moths were kept in a Plexiglas container with continuous access to 0.15M sucrose solution.

2.2 Preparations

The confocal images used for the reconstruction of the secondary CO2 pathway are based on Ingrid Moe Dahl's experimental data (Moe Dahl, Master thesis 2013).

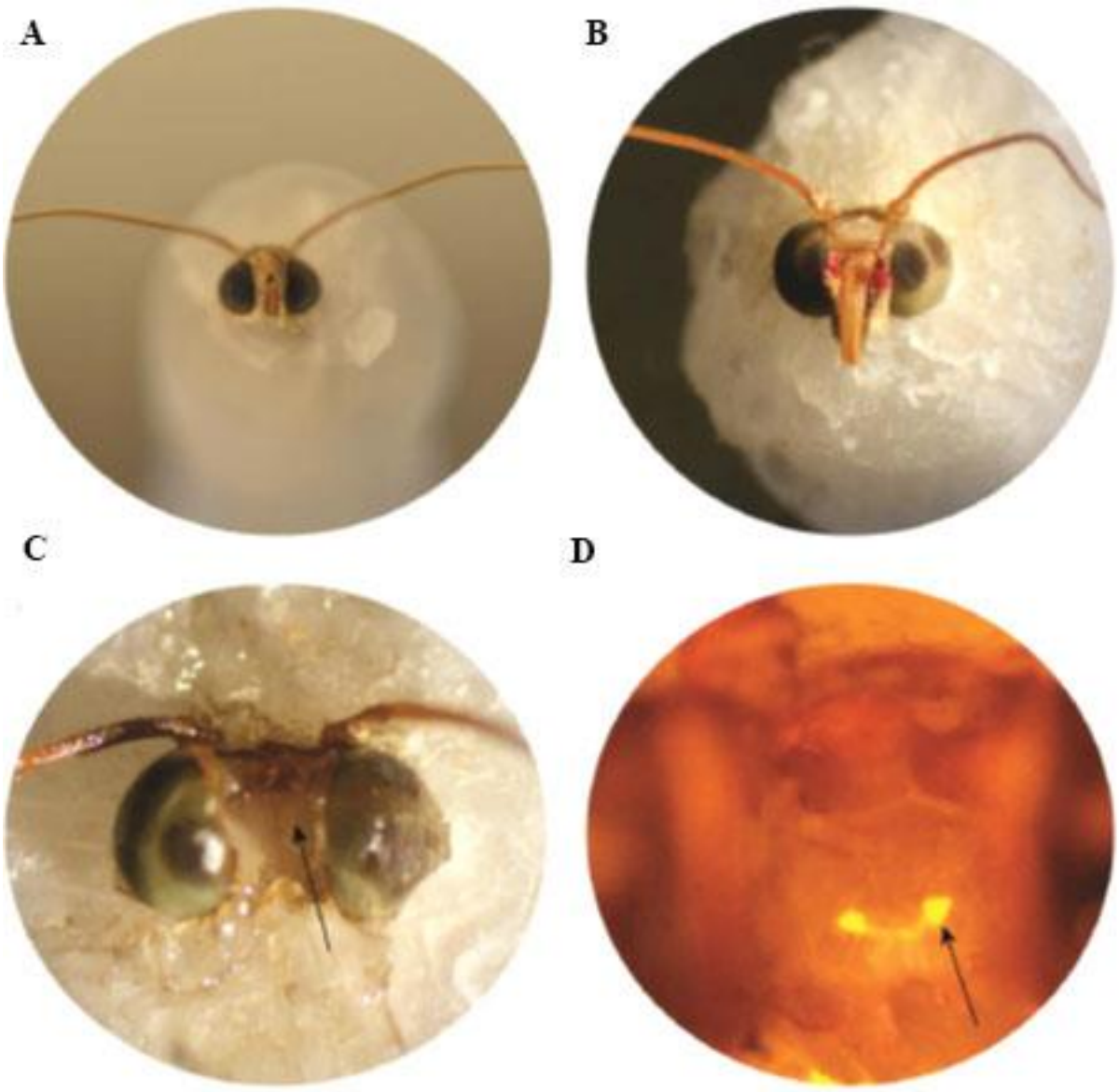


Figure 2. 1: A: The moth is fastened and immobilized with a dental wax with only the head protruding. B: The Labial pit organ (LPO) is stained with micro-Ruby until the palp turned pink. The animal was kept cool overnight before dissection. The proboscis, mouth parts, all cuticula between the antennae and eye lobes including the intracranial muscle tissue and trachea are removed so that the brain remains exposed. Black arrow indicating the position of left antennal lobe. D. Arrow indicates the position of Pre-staining of labial pit organ glomerulus (LPOG) from the LPO was visualized using a stereo microscope with green fluorescence filter. Visualization of LPOG allows navigation of the glass electrode for retrograde staining of the LPOG. Ingrid Moe Dahl (unpublished data, 2013)

Ingrid Moe Dahl stained the LPOG sensory neurons and projection neurons in two steps. Firstly, in order to display the LPOG in a fluorescent light microscope, the glomeruli were pre-marked by applying fluorescent dye (micro-ruby: dextran, tretramethylrhodamine and

biotin, 3000 MW, Lysine Fixable, D-7162, Invitrogen, Germany) onto the labial palps. This visualized the primary pathway. In the second step, the CO_2 projection neurons innervating the LPOG in one AL were stained by injecting dye (micro-ruby) into the LPOG, i.e. by retrograde staining of the LPOG.

Two methods have been used for the retrograde staining of the LPOG. One method includes the staining of LPOG with micro ruby crystals added on the outer tip of a glass electrode. The second method includes the iontophoretic staining of LPOG. In this process, the tip of the Glass electrode was filled with a solution of potassium acetate (0.24) containing 4% of microruby.

2.3 Confocal laser-scanning microscopy

The stained preparations were scanned using a laser-scanning confocal microscope (Zeiss, LSM 510 META, Carl Zeiss Microscopy, GmbH, Jena, Germany) at the Biophysics Department, NTNU. A helium neon laser with wavelength 543 nm was used to excite the projection neurons stained with Micro-ruby (Ex/em: 555/580). A transmission rate of 49.5%, 1mV mas) was used, and filtering through a band-pass filter (rhodamine BP565-615). The brain preparations were scanned with a plan-Neofluar 10x/0.5 dry objective, a plan-Neofluar 20×0.7 dry objective and a few areas of special interest were scanned using a C-Achroplan 40×0.8 water immersion objective (refractive correction:1.15). The resolution frame size was set to 1024×1024 pixels with a scan speed of 6 and average of 4 scans. The stacks of images obtained from scanning were saved as Zeiss image files (.lsm).

This scanning protocol was, however, not optimal for the AMIRA reconstructions. Therefore, some of the good preparations were rescanned. To acquire optimal raw data for AMIRA the brains were scanned with a higher transmission rate, smaller z-stack distance, scan speed of 6 but only one scan, i.e. no averaging occurred. Were necessary brains were also scanned frontally or dorsally, respectively. In total five preparations were re-scanned: 055, 075, 078, 104, and 107. Two of them were also intensified with immunocytochemistry and re-scanned a third time (see below).

2.4 Intensification and rescanning

To better identify neuropil, the samples were intensified and rescanned (20x) for fine details. Already prepared samples were rehydrated in ethanol series at room temperature (100%: 100%: 96%: 90%: 70%: 50%) for 10 minutes each. The samples were washed in PBS (pH 7.2, 10mins at room temperature) and incubated in streptavidin-cy3 (1:200 in 0.1M PBS, pH 7.2 for 2 hr in room temperature or overnight at 4 ˚C). Then the samples were washed in PBS (pH 7.2, 10mins at room temperature) and dehydrated in series of ethanol before being embedded in methyl salicylate plates. The samples were rescanned in different sections.

2.5 Immunocytochemistry

The preparations were rehydrated in a decreasing ethanol series (100%, 100%, 96%, 90%, 70%, 50%). The brains were rinsed in PBS for 10 minutes and then dehydrated in a series of ethanol (50%, 70%, 90%, 96%, 100%, 100%). The preparations were then degreased in Xylol for 5 min, rehydrated in decreasing ethanol series (100%, 100%, 96%, 90%, 70%, 50%) and rinsed with PBS for 10 minutes. The preparations were pre-incubated in normal goat serum (NGS; Sigma, ST. Louis, MO, USA; 10 %) diluted in triton x PBS (PBStx; 1%) for 30 min to minimize the non-specific staining. The preparation were then incubated in SYNORF 1 for 48 hours at 4 ˚C (SYNORF 1, diluted in PBStx (0,1 %) and NGS (10 %)). SYNORF 1 is a monoclonal mouse antibody against the presynALTic terminal protein synapsin and thus aids the identification of synALTic neuropiles. NGS blocks binding of unspecific proteins. The preparation was then rinsed in PBS for 6 x 20 minutes (2 hours) and incubated with CY5-conjugated goat anti-mouse secondary antibody (Jackson Immunoresearch), diluted in PBStx (1:500), for 48 hours at 4˚C. CY5 is a hydrophilic fluorescent dye that binds to the primary antibody. Then the samples were rinsed in PBS for 6 x 1 hours (6 hours), dehydrated (50%, 70%, 90%, 96%, 100%, 100%), cleared with methylsalicylate and mounted on aluminum slides.

2.6 Reconstruction of the brain and brain structures

The dataset, which was originally in (.lsm), was converted to (.am) format using Amira 4.1. and 5.3 (Visage imaging, Germany). Deletion of superfluous slides as well as cropping was

performed to reduce file size in LSM software and in Amira 4.1, respectively. The confocal image stacks in .am format were opened. Depending on necessity, the sections were merged before reconstruction of the surface and the neurons. The desired structure was labeled in every second slide and interpolated. The resulting label field was edited by removing islands (voxel size 3) in all slices, smoothing labels, (size 15 pixels) and saved as (*-labels.am). The file *.-labels.am was down-sampled before a surface was generated with the compute surface generate function inbuilt in Amira. Thus generated surface was saved as (*-surf.am). The generated surface was then scaled with a factor 1.6 in the z-axis.

The LPOG was reconstructed as a glomerulus into a single labeled structure. In the deutocereberal structures, the antennal lobe glomeruli have been collectively assigned as one labeled region. The calyces of mushroom bodies, which was clearly distinguished from the protocerebrum, was labeled as a distinct structure. The central body was distinguishable and assigned a distinct label. In the optic lobe, the medulla, the lobula and the lobula plate was labeled as one single structure.Different labels were given distinct colour to avoid confusion. The label for the whole brain is given a blue color, the antennal lobe label is given a light blue color, the LPOG is given a pink color, the mushroom body calyces a green color and the optic lobe a red color.

2.7 Neuronal Reconstruction

The confocal image stacks were examined section by section and the neurons were constructed semi-automatically in AMIRA 4.1 and AMIRA 5.3.3 using the skeleton tool option. Each pathway was reconstructed individually. For clarification not all neurons within a pathway were reconstructed. The pathways were scaled with a factor 1.6 in z-axis. The neurons were given a distinct color based on the pathways they belong to for easier identification i.e. red for l-ALT, green for ml-ALT, blue for second ml-ALT, black for dm-ACT, yellow for individual tract, black for interneurons, orange for neurons going in the antennal nerve.

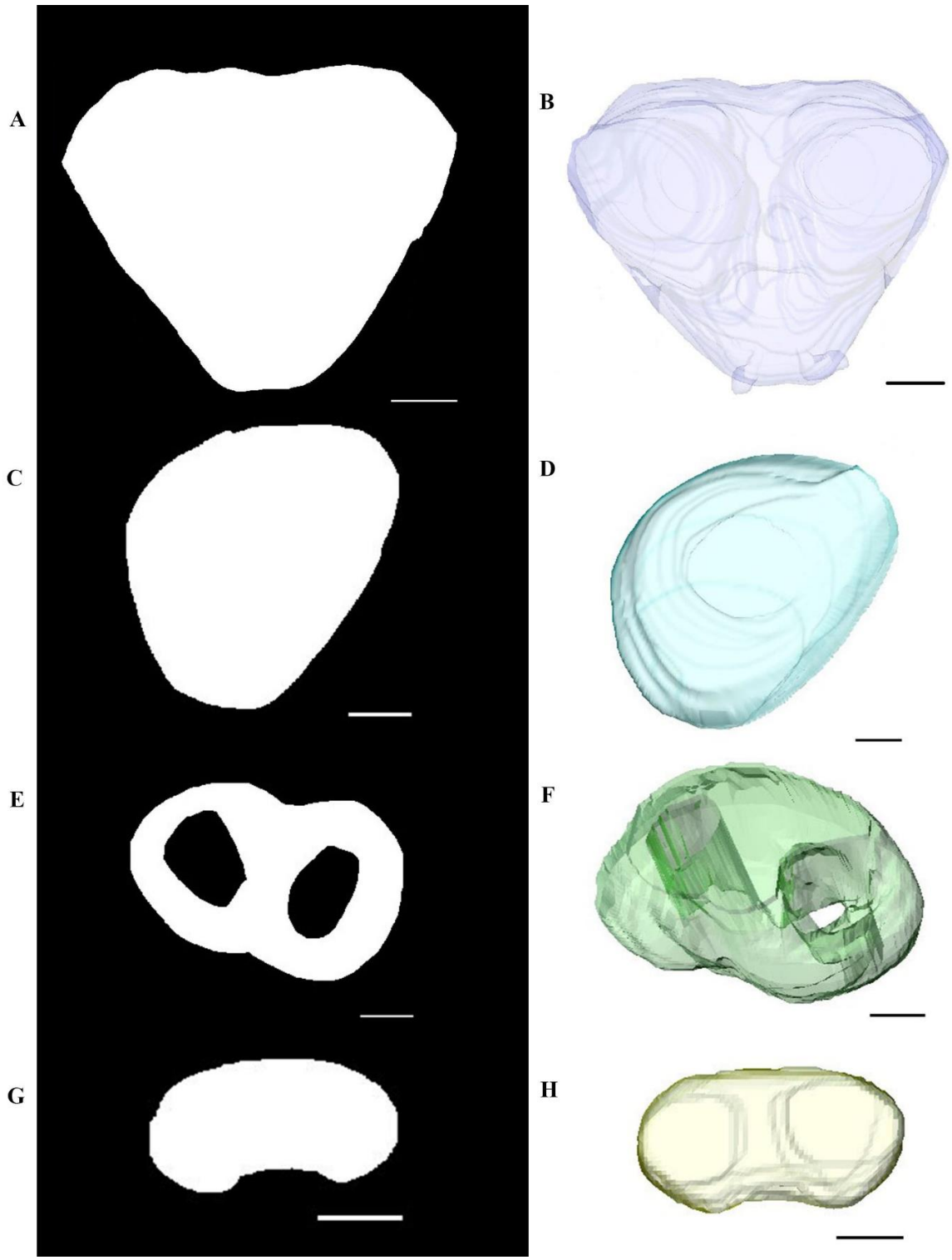


Figure 2. 2 The structures included in the *Heliothis virescens* brain visualized by confocal image. The labelled outline of brain regions (left) and surface reconstructions (right). The images are from a single brain preparation (78). A,B: Midbrain region. C,D: Right antennal lobe. E,F: Mushroom body calyces. G,H: Central body. Scale bars: A,B:100µm, C,D,E,F,G;H:50µm

3 Results

Intensification, immunostaining and rescanning of the preparation from Ingrid Moe Dahl's masters' thesis experiment have yielded novel results. In this study, LPOG neurons were found to project to l-ALT, ml-ALT and second ml-ALT. In addition to that, we have also observed projection neurons in the mushroom body calyces and interneurons projecting from LPOG towards other glomeruli. The results of this study are as follows:

3.1 Primary pathway:

The LPOG primary pathway originates from the LPO receptor neurons in the labial pit organ. It passes to the subesophageal ganglion dorsally through the labial nerve. It gives off some branches in the SEG. The neuron bifurcates ventral to the esophageal foramen and projects dorsally to LPOG in both the ipsi- and contralateral antennal lobe.

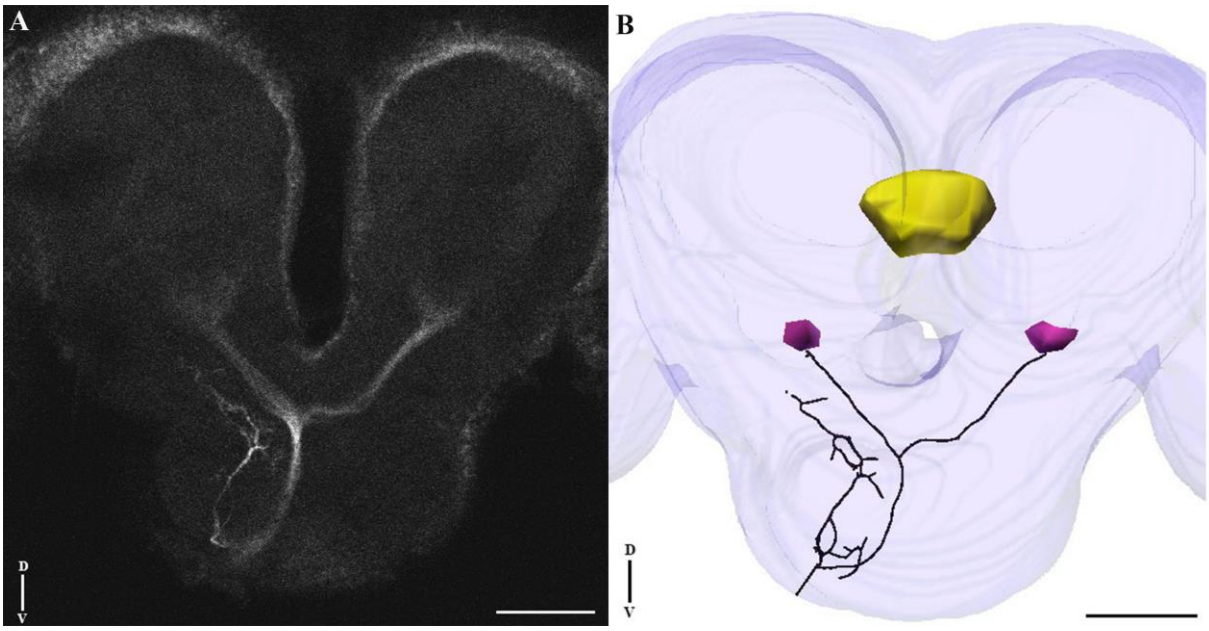


Figure 3. 1 confocal image and AMIRA construction showing the LPOG primary pathway. Scale bars: 100µm

3.2 Secondary pathway and LPOG projection neurons:

The neurons from the LPOG project to higher brain centers through different tracts:

- LPOG-Projection neurons following the lateral antennal lobe tract
- LPOG-Projection neurons following the Medio lateral antennal lobe tract
- LPOG-Projection neurons following the Second Medio lateral antennal lobe tract
- LPOG-projection neurons following the dorsomedial antennal lobe tract
- LPOG Individual projection neuron
- LPOG-processes projecting towards antennal nerve
- LPOG interneurons

3.2.1 Projection neurons following the lateral antennal lobe tract (l-ALT)

The l-ALT leaves the LPOG medio-ventrally, see red tract in the Figure 3.2. Shortly after leaving the LPOG, the CO_2 projection neurons running in the l-ALT bifurcates into two sub-bundles; one terminating into the lateral protocerebrum and the other projecting dorsally and terminating in the superior protocerebrum. As shown in Figure 3.2, the l-ALT continues laterally projecting towards the optic lobe as a loosely defined bundle of thin fibers. Shortly after bifurcating, a significant number of these fibers form a sub-bundle that terminate into the lateral protocerebrum. Another sub-bundle consisting of loosely defined side-branches bending off from the lateral path and projects dorsally terminating relatively medial and anterior in the superior protocerebrum. This bundle stretches in dorsal-ventral direction. Within the superior protocerebrum it forms a characteristic columnar structure. The fibers in this sub-bundle are often weakly stained compared to the sub-bundle projecting to the lateral protocerebrum and only appear in a few well stained preparations (Figure 3.2).

Similar projection neurons with a columnar structure were observed in mass staining study of projection neurons in general olfactory system, ongoing masters' project in our lab (Pramod KC; Siri Lillevoll)

3.2.2 Projection neurons following the mediolateral antennal lobe tract (ml-ALT)

The ml-ALT leaves the LPOG postero-medially compared to the l-ALT, represented as a green tract in the Figure 3.3. After leaving the LPOG it turns laterally at the edge of the central body, ventrally bypassing the pedunculus. This tract comprises a nicely stained bundle of neurons which projects dorso-medially and then continues ventro-laterally towards the optic lobe finally terminating in the lateral protocerebrum.

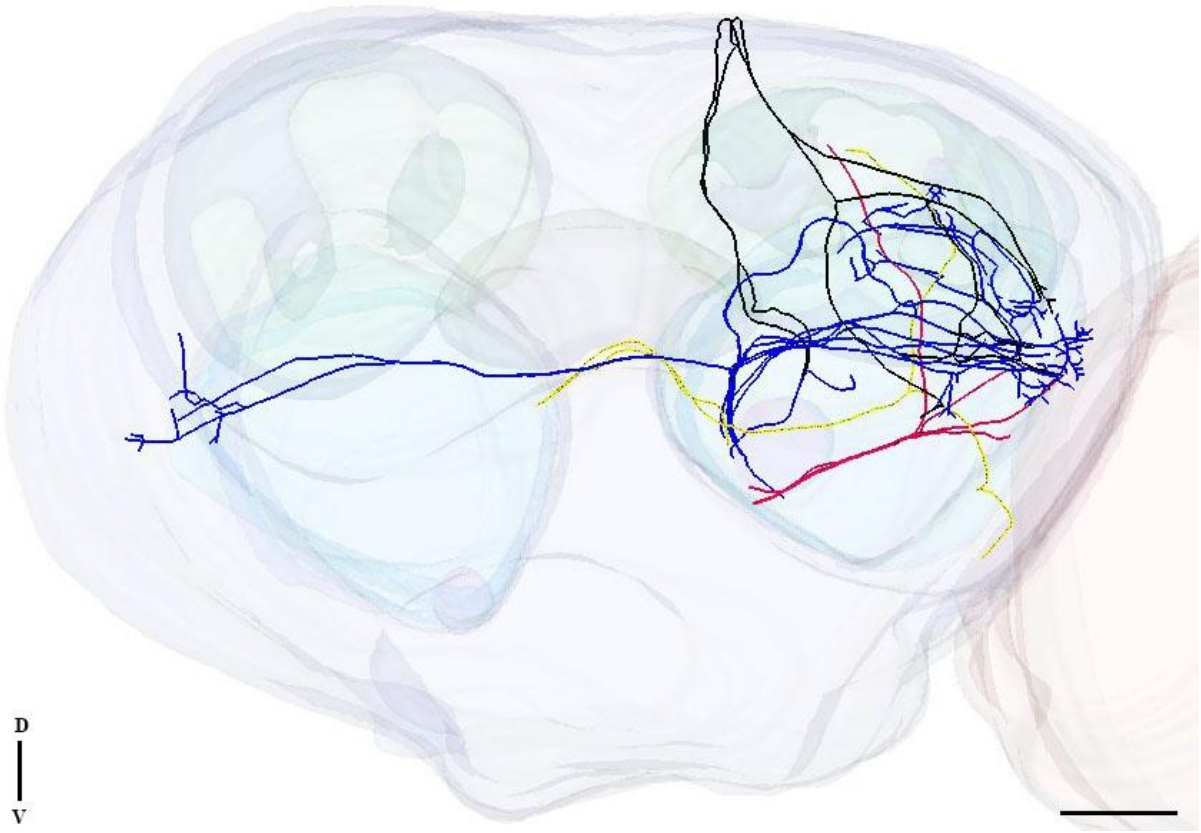


Figure 3.2: AMIRA reconstruction of preparation 78 (female) showing the antennocerebral pathways; lateral antennoprotocerebral pathways (red), second mediolateral antennocerebral pathway(blue), dorsomedial pathway (black), individual neuronal loop (yellow). Scale bar: 100µm

3.2.3 Projection neurons following second mediolateral antennal lobe tract (2ndml-ALT)

The second mediolateral ALT projects more medially compared to the mediolateral pathway, represented by blue tract in Figure 3.2 and 3.3. After leaving LPOG, the tract projects dorsally and divides into several branches at the ventral edge of the central body. These different

branches continue to various parts of the brain, terminating in separate regions, some in the ipsi- others in the contralateral brain hemisphere. The fibers in this pathway project into the superior lateral protocerebrum in ipsi- as well as contralateral hemisphere, and in the mushroom body calyces (Figure 3.2 and 3.3).

Compared to the l-ALT and ml-ALT, the number of neurons running in this tract is larger. From LPOG they continue dorsally up to the edge of the central body and divide into two branches; i) projecting to the ipsilateral hemisphere, and ii) Projecting in the contralateral hemisphere.

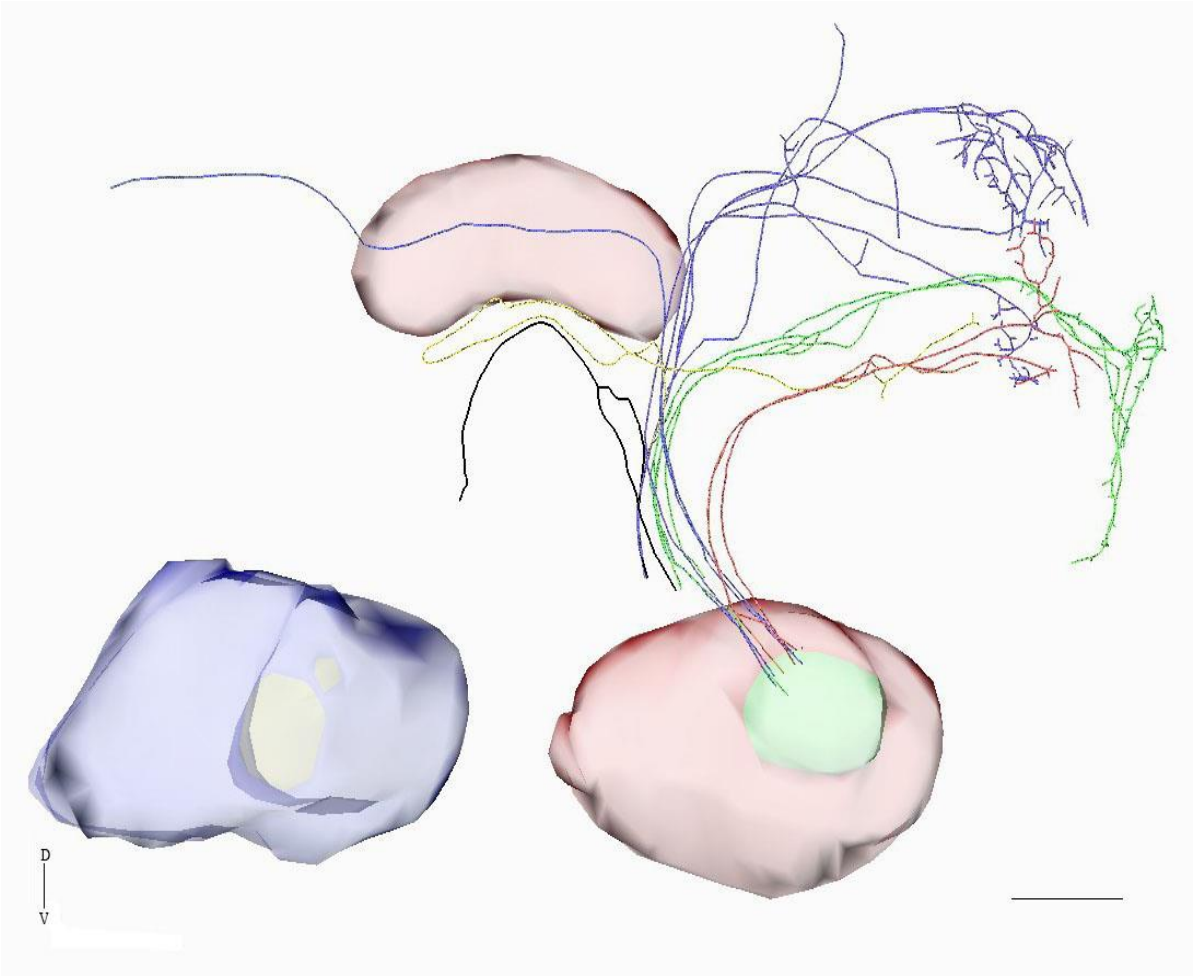


Figure 3. 3: AMIRA reconstruction of LPOG projection pathways from preparation 78 (female); lateral antennocerebral pathway (red), medial antennocerebral pathway(green), second mediolateral antennocerebral pathway (blue), individual neuronal loop (yellow), neurons from the somata dorsal to the SEG (black). Scale bar: 100µm

The number of neurons in this pathway projecting toward the ipsilateral hemisphere is at least 7. One or two of these tracts project to the lateral protocerebrum. The remaining tracts initially project in lateral direction and continue dorsally to form the characteristic loop like structure anterior to the mushroom body calyces (Figure 3.5, 3.6). The tract also sends few fibers posteriorly to the mushroom body calyces (Figure 3.7). One of these fibers has a characteristic wave like structure. This fiber projects dorso-laterally twice giving it a wave-like structure before it finally bends laterally. It continues laterally and divides into two branches; one of them continues to innervate the lateral protocerebrum and other bends dorsally terminating in the superior protocerebrum.

The tract that follows the contralateral pathway projects laterally towards the contralateral hemisphere from the ventral edge of the central body. It takes a straight path anterior to the CB and continues to the lateral protocerebrum in the contralateral hemisphere (Figure 3.10). In the contralateral hemisphere, this tract terminates in the symmetrical region as in the ipsilateral hemisphere giving off some branches.

3.2.4 Projection neurons following dorsomedial antennal lobe tract (dm-ALT)

After originating in the LPOG, the neurons in this tract projects medio-dorsally. The tract is represented by black color (Figure 3.1). It continues until the anterior edge and turns ventro-laterally. It continues in this direction and terminates in the superior protocerebrum. One of the branches bifurcates, contributing to the circular loop-like structure anterior to the mushroom body calyces.

3.2.5. LPOG Individual projection neuron

One fiber originating from the LPOG continues to the lower edge of the CB, and bends medially. The tract is represented by yellow color (Figure 3.3). It continues to the contralateral hemisphere, curving further along the edge of CB on the contralateral hemisphere. The fiber then bends backward and continues its path back on the same path. At about the edge of the CB, where it started, it bends ventrally and then turns laterally. It continues laterally and

divides into two branches: i) one branch continues ventro-laterally and terminates in the ventral part of the lateral hemisphere, ii) the other branch initially bends dorso-laterally and terminates in superior protocerebrum.

3.2.6 LPOG processes towards the antennal nerve

In addition to the other described tracts, some neurons were observed to project to the antennal nerve from LPOG (Figure 3.8). Few neurons around 5 or 6 in number, in a bundle project laterally from the LPOG and are part of one of the antennal nerve.

3.2.7 LPOG Interneurons in the antennal lobe

Interneurons have been observed between the LPOG and other glomeruli (Figure 3.9). Two interneurons seem to project dorsally among few glomeruli and terminate in one of the glomeruli.

The summary of the results in comparison to the general olfactory system is recapitulated in figure 3.11.

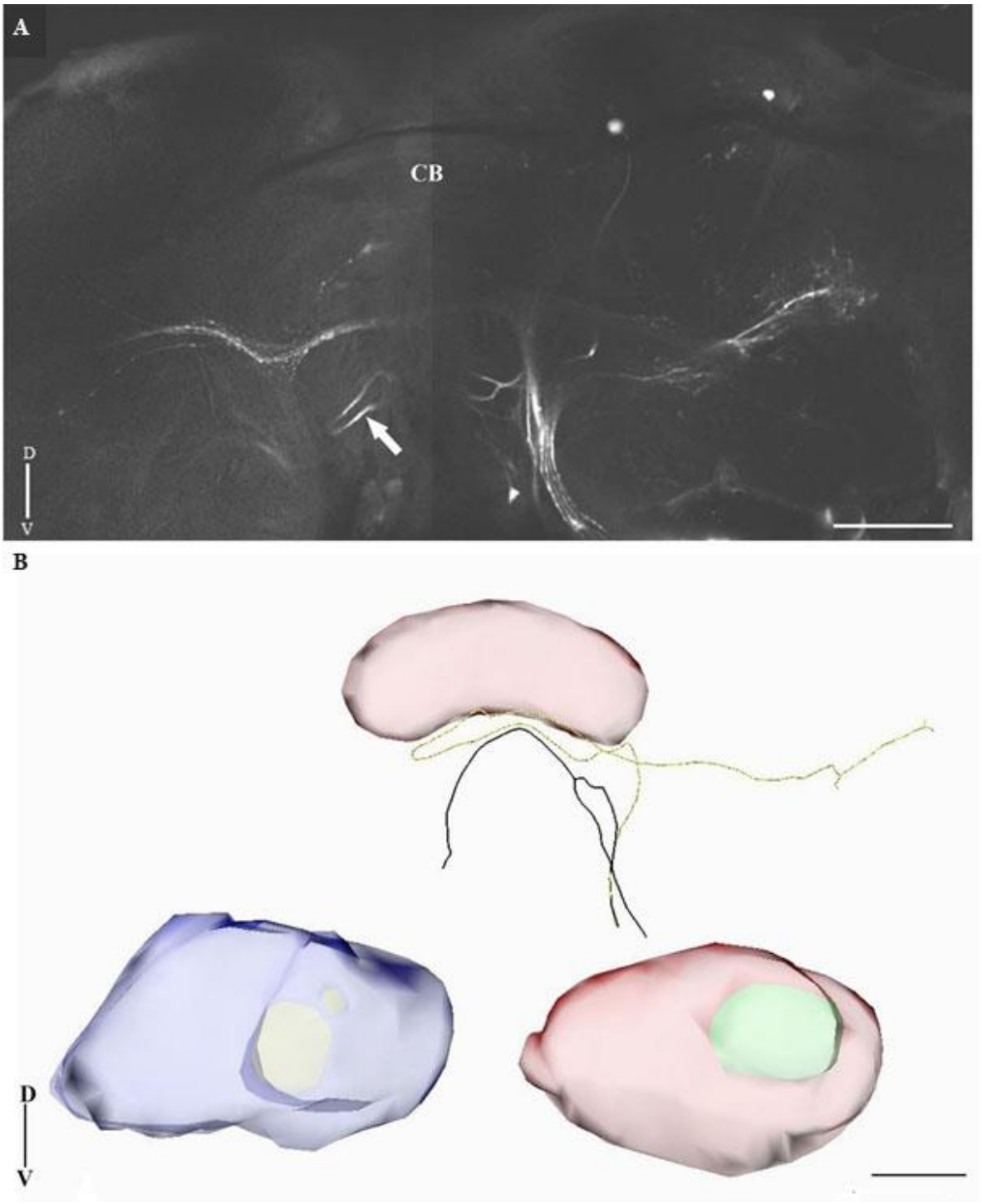


Figure 3.4 Confocal image and AMIRA reconstruction showing the individual loop (yellow tract). A: confocal image of preparation 78 (female); CB = central body. B: AMIRA reconstruction of the individual loop. Scale bars: 100µm

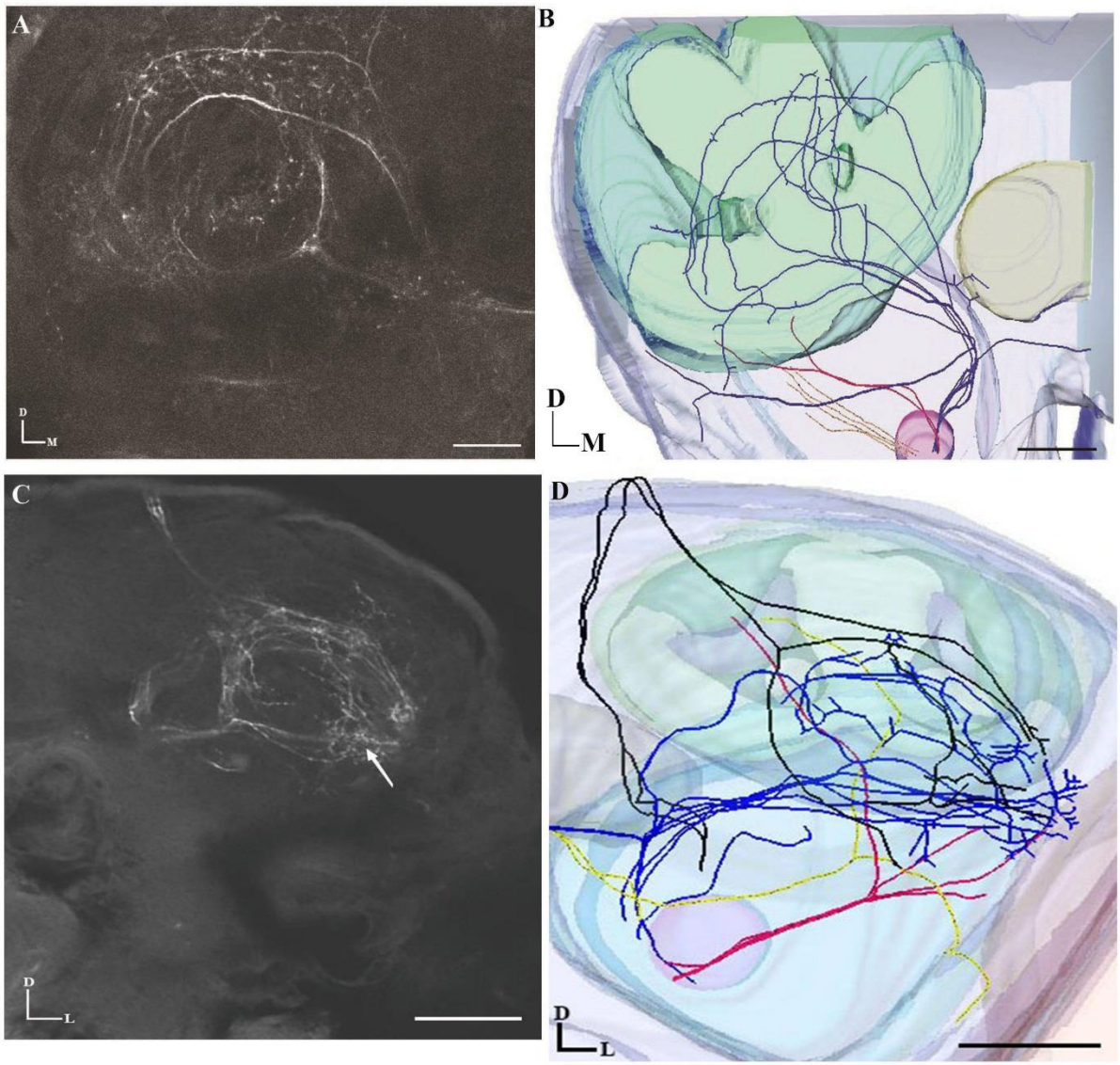


Figure 3. 5: Confocal image and AMIRA reconstruction showing the projection neurons projecting from LPOG second mediolateral tract. This tract bends dorsal lateral direction and projects to the superiror protocerebrum and terminates just anterior to the mushroom body. A-B: Confocal scan AMIRA reconstruction brain preparation 5 4(male) . C-D confocal scan and AMIRA reconstruction of brain preparation 78 (female). Scale bars: A-B: 50µm and C-D: 100µm

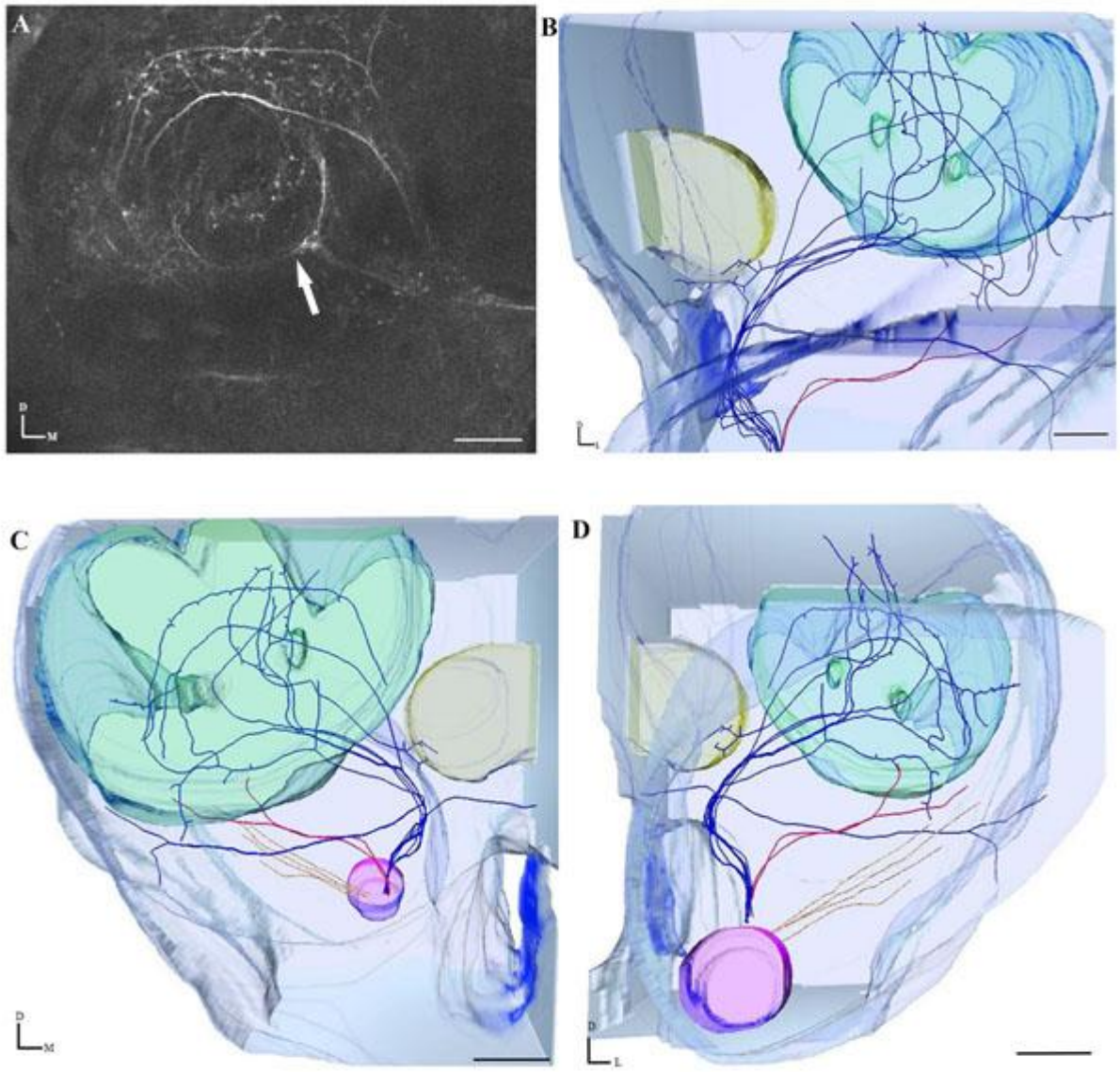


Figure 3. 6: Confocal image and Amira construction showing the neuron in second mediolateral tract forming a circular loop in superior protocerebrum anterior to the mushroom body calyces. A: confocal image of preparation 54 (male), white arrow showing the circular loop B: Frontal view C: posterior ciew D: frontal view. Scale bars: A: 50µm, B,C,D: 100µm

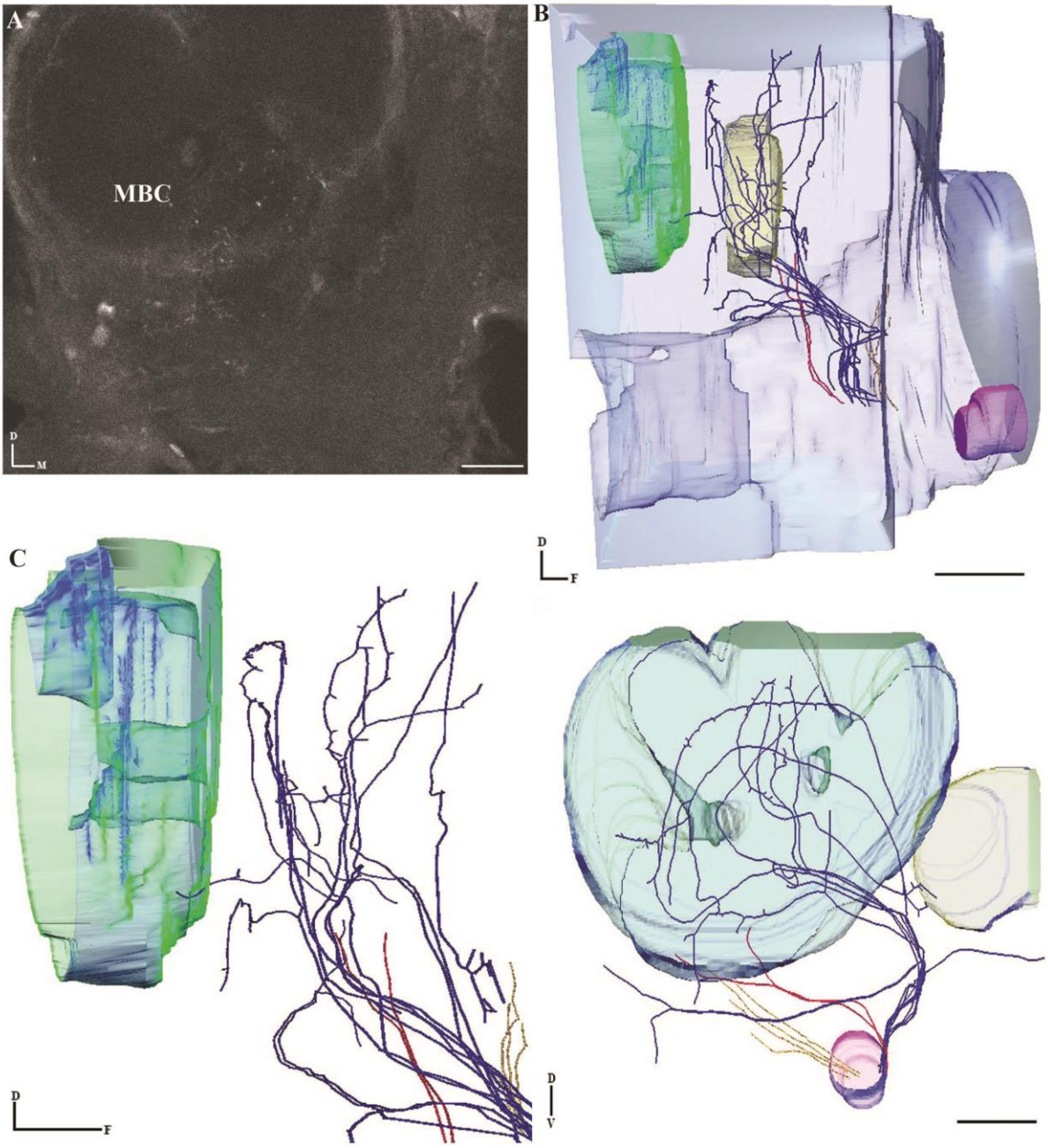


Figure 3.7 Confocal image and AMIRA reconstruction of neurons in second mediolateral tract projecting into mushroom body.A: confocal image of preparation 54 (male). , B: sagittal view showing neurons going into mushroom body calyces. C: Close up view of neuron projecting into mushroom body calyces. D: posterior view). MBC = mushroom body calycesScale bars: A: 50µm; B,C,D: 100µm

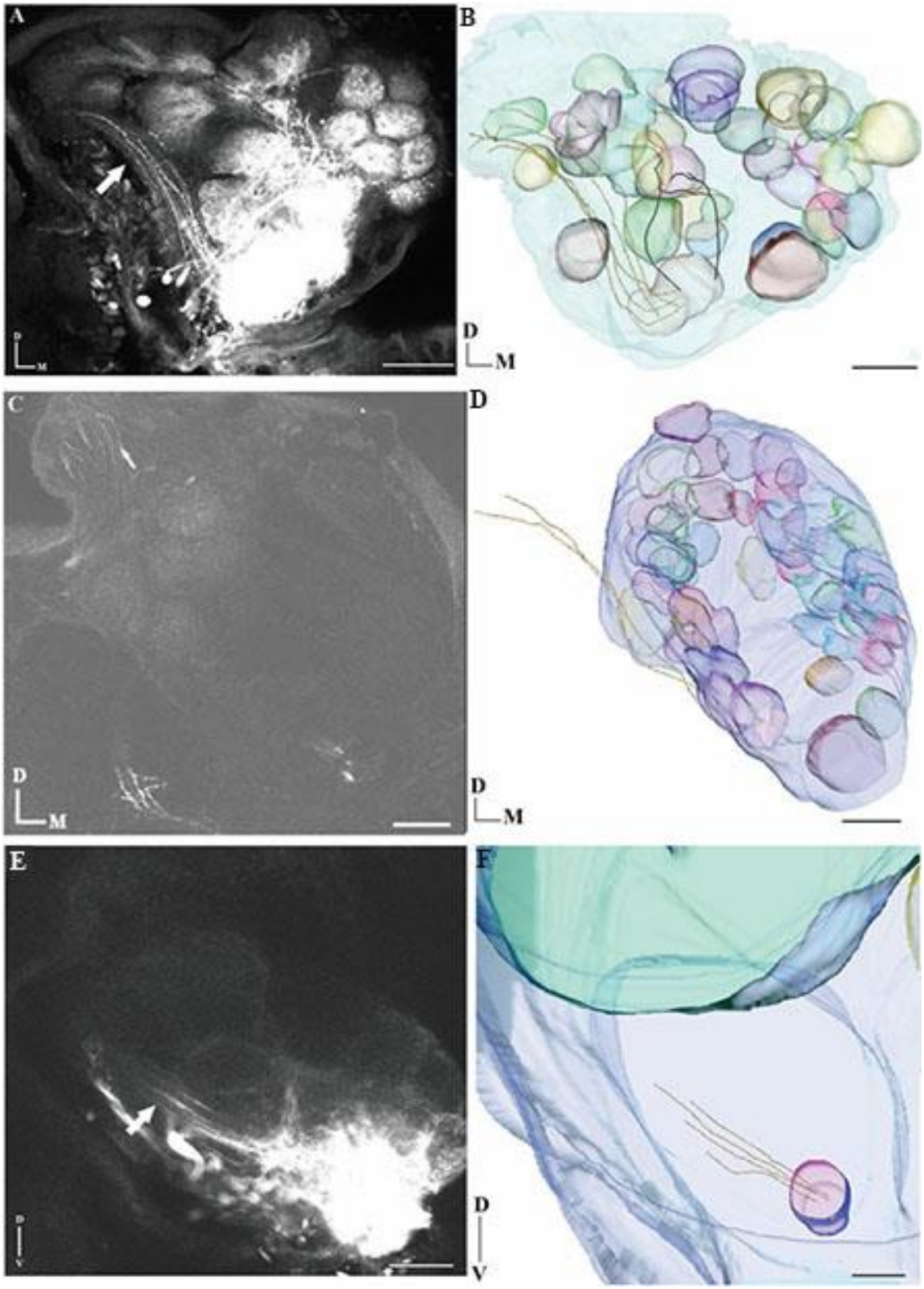
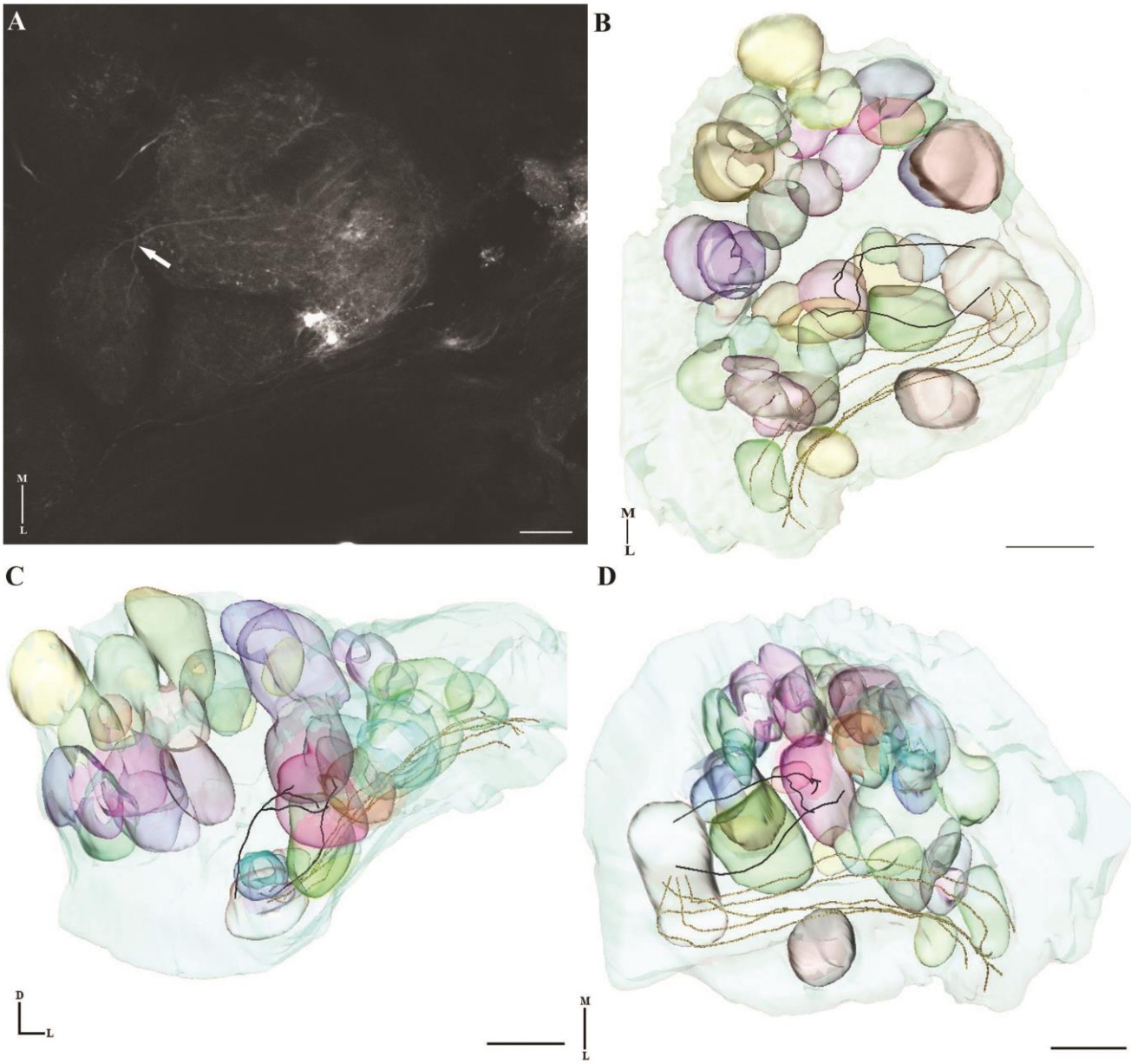


Figure 3.8 Confocal images and AMIRA reconstructions showing neurons projecting towards antennal nerve (white arrows in confocal images and orange tracts in AMIRA reconstruction. A,B: preparation 78 (female); C,D: preparation 104 (female); E,F: preparation 54 (male). Scale bars: 50µm



B: Posterior view, LPOG interneuron (Black). C: Frontal view of antennal lobe with LPOG interneuron D: Posterior view. Scale bars: A: 20µm; B,C,D:50µm

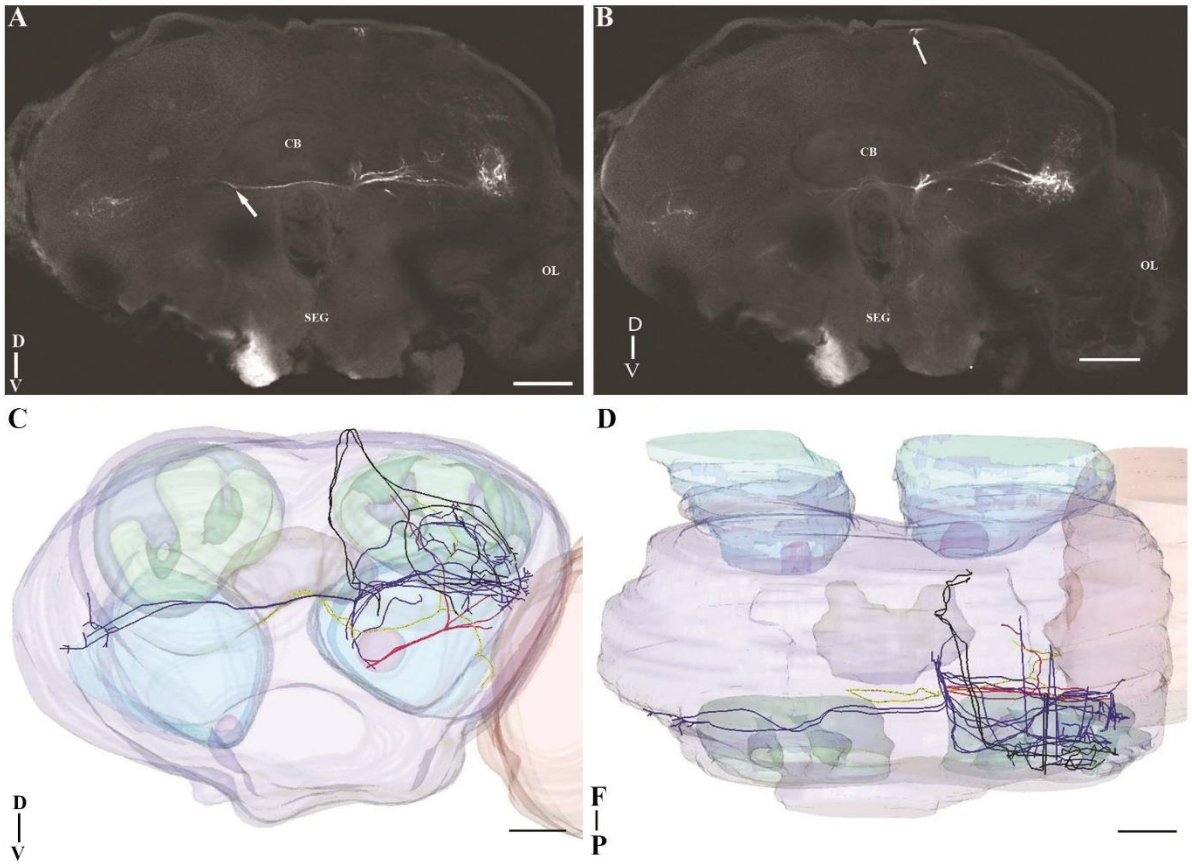


Figure 3. 10: Confocal scans and AMIRA reconstruction showing the main tracts projecting from LPOG preparation 78 (female). A: white arrow showing bilateral pathway. B: white arrow showing dorsomedial pathway. CB = central body, OL = olfactory lobe, SEG = Suboesophageal ganglion. C: AMIRA reconstruction posterior view. D: Anterior view. Note the gap in between the neurons and the LPOG is due to overintensification of dye in the LPOG. Scale bars: 100µm

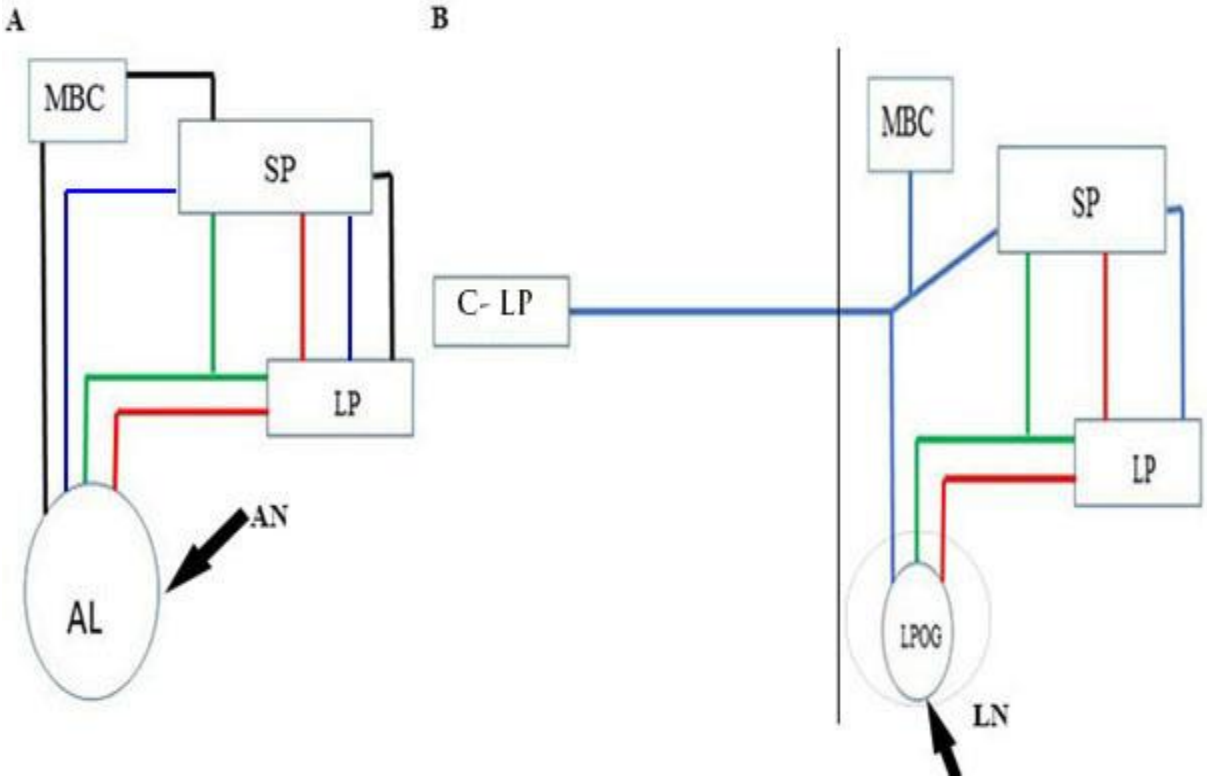


Figure 3.11 Schematic representation of information processing in general olfactory system(A) and LPOG system(B). AL=antennal lobe, AN=antennal nerve, MBC= mushroom body calyces, SP=superior protocerebrum, LP=lateral protocerebrum, LPOG=labial pit organ glomerulus, LN=labial nerve, C-LP= contralateral lateral protocerebrum. The antennal lobe tracts are represented by colors. Medial antennal lobe tract (black), second mediolateral antennal lobe tract (blue), medilateral antennal lobe tract (green), lateral antennal lobe tract (red)

4 DISCUSSION

The present Master's project is based on further analysis and reconstruction of the result obtained from work by Ingrid Moe Dahl (2013), on mapping of LPOG neurons of the moth *H. virescens*. How LPOG neurons represent information in the primary olfactory center and this topographic organization is further reflected in higher centers, were central questions in this study.

It is important to emphasize that our study does not include electrophysiological stimulation of LPO receptors or LPOG projection neurons. Thus, the physiological properties of the projection neurons are not known. The projection neurons might or might not respond to CO_2 or it might respond to other compounds besides CO_2. Studies have suggested that the receptors on the LPO are sensitive to CO_2. LPO receptors are able to detect extremely small fluctuations in CO2 concentration in the environment (Bogner et al., 1986; Guerenstein, Christensen, & Hildebrand, 2004; Homberg et al., 1988). Thus, in this thesis, the LPOG system is referred to CO_2 system at some instances.

The findings, on LPOG primary pathway, in this study were not significantly different from the previous findings. However, significant differences were observed in the secondary pathway compared to previous results in LPOG pathway as well as general olfactory pathway (Figure 3.11). Thus, the discussion of the secondary LPOG pathway will be focused in this thesis.

4.1 Secondary Pathway

The second order pathway of the CO_2 system shows both similarities and disparities with the general olfactory system. Apart from few contributing research (Guerenstein et al., 2004; Homberg et al., 1988; Ro et al., 2007), the knowledge on higher order processing of CO_2 in Lepidoptera is very limited. The result in this study shows difference in the arrangement of the LPOG system compared to the findings in the general olfactory pathway

The Projection neurons originating in the LPOG project via four main pathways: the l-ALT, the ml-ALT, the second ml-ALT and the dm-AlT. This compliments the previous study on mapping of CO_2 projection pathway (I. Moe Dahl,2013). The fibers from these tracts terminate in distinct brain regions, including the lateral protocerebrum (both ipsi- and contralateral hemisphere), superior protocerebrum, mushroom body calyces and also to the antennae. The finding is also contradictory to the finding in *D. melanogaster*, where the V glomerular neurons are observed in all the three main tracts: the inner antenno-cerebral tract (iACT), the middle antenno-cerebral tract (mACT) and the outer antenno-cerebral tract (oACT) (Lin et al., 2013).

Another interesting finding is an individual projection neuron that projected to the contralateral hemisphere and then follows its path back to ipsilateral hemisphere terminating in the superior protocerebrum and lateral protocerebrum. Also, neuronal arborization from the LPOG was observed in other glomeruli in antennal lobe. A schematic representation of CO_2 pathways is presented in (Figure 3.2, 3.3, 3.10). The projection pattern of the second order neurons origination from LPOG seems to be similar in males and females.

4.1.1 LPOG projection neurons in l-ALT

The l-ALT neurons project to the lateral and superior protocerebrum of the ipsilateral hemisphere. The projection pathway consists of two distinct sub-bundle; one branch targets the lateral protocerebrum nearly up to the border of the optic lobe, another sub bundle projects dorsally forming a distinct columnar structure. The projection pattern of subbundle projecting to lateral protocerebrum is comparable with projection pattern of POb neuron described by Homberg and Rø (Homberg et al., 1988; Ro et al., 2007). The so called POb neuron projects to very lateral areas of lateral protocerebrum via the l-ALT. Another branch has the characteristic columnar structure and projects to the superior protocerebrum. This bundle is comparable to the POa fibre in a previous study, which shows similar branching pattern with a columnar structure targeting the superior protocerebrum (Homberg et al., 1988; Ro et al., 2007). In our lab, a similar columnar structure in l-ALT has been observed in the study

involving mass staining of the PNs in various tracts of *H. virescens* (Lillevoll, S., 2013, unpublished data), and by Pramod KC(Masterproject, 2014).

Homberg (1988) has described both uni and multiglomerular PN in l-ALT and each of them are connected either to the ordinary glomeruli detecting plant odors or to the glomeruli found in the MGC-area detecting pheromones. However, in this study all PNs running in this tract are uniglomerular and are connected to LPOG. Thus, it can be concluded that l-ALT consists of three functionally different neurons; linked to pheromones, plant odors and CO_2. None of the neuron in this tract originating from LPOG show innervation to MBC as that in the corresponding general olfactory system (Homberg et al., 1988). The description of arrangement of tracts as a loosely connected bundle is similar to that of the study of *M. sexta* in general olfactory system (Homberg et al., 1988).

4.1.2 LPOG projection neurons in the ml- ALT

At least three PNs from this tract project to the lateral and the superior protocerebrum. The morphology of this pathway is in agreement with that in corresponding general olfactory pathway (Homberg et al., 1988; Ro et al., 2007). A previous study has pointed out that ml-ALT is more directly linked to the assumed premotoric areas in the brain; i.e. the lateral protocerebrum (Ro et al., 2007).

Studies in *M. sexta* and *H. virescens* have described the presence of only multiglomerular PN in this tract (Homberg et al., 1988; Ro et al., 2007). However, this study shows the projection of uniglomerular neurons in this tract.

4.1.3 LPOG projection neurons in Second ml-ALT

At least seven PN in this tract project seemingly randomly to different areas in the brain: the lateral protocerebrum, the superior protocerebrum, the contralateral hemisphere and the mushroom body calyces. The second ml-ALT in this study however does not correspond to the one described in general olfactory system. But this tract has been recently described in a

study involving the mass staining of the projection neurons in various tracts of *H. virescens* (Lillevoll, S. 2013, unpublished data).

One of the interesting findings in this pathway is projection of one of the neurons to the contralateral hemisphere. The neuron targets the symmetrical area as that targeted by it in the ipsilateral hemisphere. No comparative data to this system is available at the present moment as this arrangement is different from the corresponding general olfactory pathway of most insect species. The information from LPOG however seems to be processed bilaterally already in primary pathway i.e. from LPO to LPOGs in both AL.

Another fiber in second ml-ALT bends dorsally giving rise to a characteristic circular pattern anterior to the mushroom body calyces (Figure 3.5, 3.6). Similar findings have been observed in *Apis mellifera* describing a neuropil in the form of a ring surrounding the area of the alpha-lobe of the mushroom bodies (Abel, Rybak, & Menzel, 2001).

4.1.4 LPOG projection neurons in dm-ALT:

At least three neurons project in this tract. This tract projects medio-dorsally, straight to the anterior edge of the brain. It then bend ventro-laterally terminating in the superior protocerebrum. . A similar arrangement has been described in *M. sexta* general olfactory system (Homberg et al., 1988).

4.1.5 LPOG projection neurons in the Mushroom body calyces

In contrast to the previous findings by Ingrid Moe Dahl (2013), we observed projection neurons in the mushroom body calyces. However, the projection neurons do not follow the route as described in case of general olfactory system, i.e. through the m-ALT into calyces and terminating in lateral protocerebrum (Homberg et al., 1988; Ro et al., 2007). The neurons rather follow the second mediolateral tract and project to the mushroom body calyces. The neuronal pathway to the mushroom body calyces is an experience dependent pathway and

plays a vital role in learning and memory (McGuire, Le, & Davis, 2001). The presence of neurons in the calyces indicates that the CO_2 processing system is experience dependent in moths. This is similar to data from the *D. melanogaster*, here CO_2 information is processed in the mushroom body calyces in generating a behaviour (Bracker et al., 2013).

4.1.6 LPOG individual projection neuron

One neuron projects from LPOG ventral to the edge of the central body and bends towards the contralateral hemisphere. It then follows its path back to the ipsilateral hemisphere. In the ipsilateral hemisphere, it projects ventro- laterally giving two branches; one terminating in lateral protocerebrum and another in the superior protocerebrum. No comparable arrangementas such is described for the general olfactory system in moth, yet.

4.1.7 LPOG processes to the antennal nerve

At least five neuronal processes were seen in the antennal nerve. It is not clear whether they are afferents or efferents, i.e. process from or to the LPOG. CO2 receptors are present in the antennae in drosophila however no sensory neurons are sensitive to CO_2 in antennae in moth. Furthermore, mass staining of the antennal nerve shows no staining of the LPOG. However, there are two more possibilities. CO_2 information from the LPOG may modulate the antennal muscles, or on the other hand, CO_2 information may be detected at the base of the antenna. These axons join the antennal nerve, i.e. the antennal nerve is a bundle of nerves (Kloppenburg, Camazine, Sun, Randolph, & Hildebrand, 1997).

4.1.8 Interneurons

Interneurons between the LPOG and other glomeruli were observed. Findings in the honeybee suggest that a dense network of local interneurons (REF) interconnects a large number of glomeruli that receive sensory input. A study involving suppression of activity in a single glomerulus with γ-aminobutyric acid (GABA) and presenting an odor reveals the existence of inhibitory interactions between the glomeruli. Whereas stimulating a glomerulus with

acetylcholine (ACh) activates inhibitory interglomerular connections that can reduce odor-evoked responses. This study suggests individual glomeruli inhibit other glomeruli with graded strength, but in a spatially dis-continuous manner (Girardin, Kreissl, & Galizia, 2013; Meyer, Galizia, & Nawrot, 2013). Similarly, serotonin-immunoreactive neurons in the antennal lobe of male oriental tobacco budworm, *Helicoverpa assulta* were also inhibitory in nature (Zhao & Berg, 2009). The interneurons projecting from LPOG to the other glomeruli might also possibly be inhibitory in nature as well. This could be answered with GABA immunostaining.

4.2 reconstruction

The reconstruction was performed in AMIRA 5.3. The selection of structures for reconstruction was based on staining quality and relevance. The SEG, tritocerebum and protocerebral lobes, lateral accessory lobes, the protocerebral bridge and the lateral protocerebrum were collectively included into one midbrain label. Similarly, SEG, tritocerebrum and protocerebral lobes are also constructed as one label in *H. virescens*, *Apis mellifera* and *D. melanogaster* standard atlases (Brandt et al., 2005; Kvello et al., 2009; K. Rein et al., 2002) due to the lack of distinct borderlines. However, the lateral horn is given a unique label in case of the *Schistocerca gregaria* and *D. melanogaster* (Kurylas et al., 2008; K. Rein et al., 2002).

The antennal lobe glomeruli appeared as distinct structures, however all of the glomeruli were collectively assigned as one label. Also, few of the glomeruli have been constructed individually to show the interneurons between the glomeruli. Three dimensional atlases of the antennal lobe glomeruli of *H. virescens* has been constructed in our lab (Berg et al., 2002). Detailed analysis of the relative position of the glomeruli was useful for the identification of the glomeruli innervated by a neuron. The mushroom body is allocated as a single label with it calyces and peduncle/lobe system. The standard brain atlas of *H. virescens* consists of mushroom body calyces as a separate label and peduncle and lobe system as a separate label (Kvello et al., 2009). However, the number and quality of samples limited our study for the detailed construction of structures as such.

4.3 Methodological consideration

The selection of the samples was based on staining quality and degree of damage. The staining quality of very few preparations was found to be relevant for reconstruction of the neurons. Also, repeated scanning, intensification and immunostaining of the samples caused more damage to the brain and weakening of the dye. Also, the absence of interneurons in the I. M. Dahls thesis can be attributed to procedural shortcomings. Immunostaining of the preparation, done here, proved to be a helpful procedure for the visualization of the interneurons.

It is important to consider that our study does not include electrophysiological stimulation of LPO receptors or LPOG projection neurons. Thus, the physiological properties neurons are not certain. The projection neurons might or might not respond to CO_2 or it might respond to other compounds besides CO_2. As suggested by studies, LPO receptors are able to detect extremely small fluctuations in CO_2 concentration in the environment (Bogner et al., 1986; Guerenstein et al., 2004; Homberg et al., 1988).

The insects are either attracted or repulsed by certain concentration of CO_2. A study in *D. melanogaster* has shown avoidance behavior for a slight rise in ambient CO_2 concentration (Faucher et al., 2006). In *D. melanogaster*, two pathways PN_v-1 (one of the PN in oACT) and PN_v-2 (one of the PN in iACT) are important for the avoidance response to low and high CO_2 concentrations respectively. Low concentration of CO_2 activates PN_v-1 and high concentrations activate both PN_v-1, PN_v-2as well as GABAergic PN_v-3 (one of the PN in mACT). The PN_v-3 may inhibit the PN_v-1 pathway meditated avoidance response to CO_2 (Lin et al., 2013). Comparision of CO_2 system in our model species *H. vir*. and *D. melanogaster* is represented in table 1. Behaviourally, CO_2 acts as distant attractor in both males and females in *M. sexta* . In this case, CO_2 affects male appetitive responses, but the response of females is context-dependent. This study suggests females may use floral CO_2 as a distance indicator of host plant quality for feeding as well as oviposition on Datura and Nicotiana plants

(Goyret, Markwell, & Raguso, 2008). It is therefore important to emphasize that the alteration of CO_2 in the environment disrupts the feeding and ovipositining behavior of insects. It is suggested that ultra-prolonged activators of CO_2 mediating neurons can completely disrupt CO_2-mediated activation and source-finding behaviour in *Aedes* mosquitoes, even after the odour is no longer present (Turner et al., 2011), indicating the effect of alterations in CO_2 in environment.

In sum, a further understanding of the CO_2 pathway in the "pest" H. virescens may open new, cleaner options for protection of the crops the larva feed on.

5 Conclusion

This master project presents the 3D reconstruction of LPOG projection neurons from the preparations of Ingrid Moe Dahl's experiment. We have further performed intensification, immunostaining and rescanning of well stained preparation in order to gain additional information. In contrast to the previous research, LPOG projection neurons were found to project to higher centres via three main projection pathways, l-ALT, ml-ALT, second ml-ALT tract. In addition to this, the projection neurons also follows dm-ACT. No projection neuron were observed in the m-ALT. The main termination area of these neurons are the superior protocerebrum and the lateral protocerebrum in both ipsi- and contralateral hemisphere indicating that LPOG information is processed bilaterally. Similarly, LPOG neurons were also observed to be innervating the mushroom body calyces. The projection to the mushroom body calyces through the second mediolateral pathway indicates that the LPOG processing pathway is experience dependent in moths too. In addition to this, LPOG interneurons were observed to be projecting to other glomeruli within the antennal lobe. The neurons projecting to the antennal nerve give an impression that there could be LPOG sensory neurons on the base of antennae, however further data is required to verify this. No sexual dimorphism was observed in LPOG pathway, i.e. no difference in the pattern of the pathway was seen between male and females.

Notably, some significant differences were observed between the general olfactory projections and the LPOG projections. This fact makes it very interesting to explore further to gain a

better understanding of this pathway. Though this project has explored some information on the LPOG projection pathway, there are still many question that need to be answered. The electrophysiological study on the response characteristic of the LPOG projection pathway is most needed to understand the physiological characteristic of this system. To understand whether it is only a CO_2 mediating system or whether it is also influence by other odorants. Immunostaining could possibly be used to interpret the nature of the interneurons from LPOG projecting to the other glomeruli.

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Appendix:

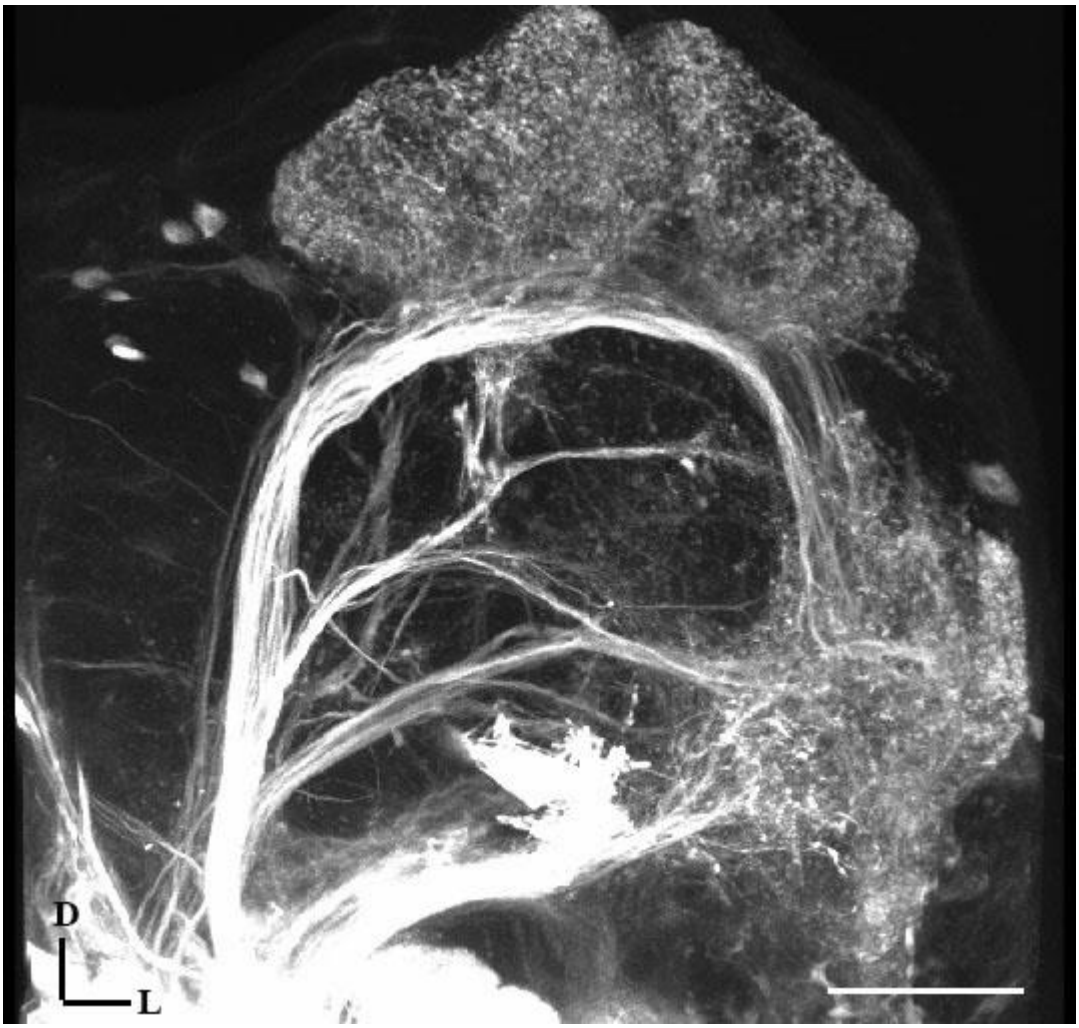

Figure A1 Confocal image showing four antennal lobe tracts; Lateral antennal lobe tract, mediolateral antennal lobe tract, second mediolateral antennal lobe tract, medial antennal lobe tract.