Pramod KC

Mapping of second order olfactory neurons and ventral-cord neurons; double-fluorescence labeling performed from the noctuid moth, *Heliothis virescens*

Master's thesis in Neuroscience

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ABSTRACT

Detection of chemical stimuli from the external environment is performed by all living organisms. Of all senses, the chemical sense is the evolutionary oldest. Also, the neural system devoted to process chemosensory information is strikingly well conserved across the different species, in particular the olfactory pathways. Due to their highly specialized ability of detecting air-born molecules over long distances, plus an accessible nervous system, noctuid moths have served as favorable model organisms for exploring basic neural principles underlying chemosensory information processing. Among the most extensively studied moths, are the heliothines, comprising numerous species distributed in all five continents. Whereas the first and second order levels of the moth olfactory pathway have been relatively thoroughly explored, less is known about the subsequent levels. In particular, the connection between the brain circuit and the motoric system, being positioned in the ventral-cord ganglia, is not described in the current insect group. In this study, fluorescence staining of the axon terminals of the second-order neurons, i.e. antennal-lobe projection neurons, was combined with staining of ventral-cord neurons for the purpose of exploring putative connections between the two neural arrangements in the Heliothine moth, *Heliothis virescens*. Dye application to the primary olfactory center of the brain, the antennal lobe, showed three major tracts projecting to higher processing regions, mainly to the calyces and the lateral horn, a particular part of the lateral protocerebrum that is innervated by medial antennal-lobe projection neurons. Labeling of the ventral cord, on the other hand, resulted in visualization of several neuropil regions in the brain, one of which being located in the ventrolateral area of the lateral protocerebrum was of specific interest. Double-labeling experiments performed in the same individual demonstrated, however, that there is no overlap between terminal regions of second-order olfactory neurons and neural processes projecting in the ventral cord, meaning that the main portion of odor information is carried to the ventral cord, not via third order neurons projecting from the lateral horn, but via fourth or higher order neurons originating in another synaptic region of the brain.

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1. INTRODUCTION

1.1. Introduction

Organisms use their sensory systems for finding food, shelter, and a mate. Also, sensory information is generally important for avoiding predators and danger. Thus, the external environmental cues (chemical signals, electromagnetic waves etc.) are converted to electrical signal in the sensory neurons and thus represented internally in neural networks in the brain displaying sophisticated behavioral responses that are adaptive to the particular situation (Hansson & Stensmyr, 2011; Martin et al., 2011). Among the sensory systems, that dedicated to detection of odor information is the evolutionary oldest and also the one being possessed by all organisms, including bacteria and humans. Actually, information about odor blends in the environment is detected and selectively discriminated by neural pathways that are remarkably similarly organized across various species. Due their highly advanced sense of smell and a nervous system being relatively easily accessible for experimental research, insects have been used as biological models for achieving general knowledge about the olfactory system (Martin et al., 2011).

Insects are the most diverse of all animal groups on our planet, comprising about one million different species. These various creatures occurring in distinct ecological niches have evolved diverse behaviors being perfectly adapted to the environment they inhabit. Male moths, for example, are equipped with an olfactory system enabling them to recognize a conspecific female and orient towards the source over remarkable long distances. Several moth species have therefore been attractive organisms for scientists dedicated to studying olfaction. Among the moths species most thoroughly studied are particular members of the sub-family Heliothinae.

1.2. Anatomical organization of the insect olfactory system

1.2.1. Peripheral pathways

In insects, the large repertoire of odors in the environment is detected by olfactory sensory neurons (OSN) housed in about 100,000 hairlike structures, so-called sensilla, covering the antenna. The olfactory sensory neuron is bipolar extending one dendritic branch inside the sensillum and a second branch (an unmyelinated axon) projecting into the primary olfactory center of the brain, similarly to OSNs of vertebrates. Male moths have a large

number of long hairs, *sensilla trichodea*, containing sensory neurons tuned to female-produced pheromones specifically. In addition, they have shorter sensilla housing plant odor detecting neurons. The latter category seems to be similar to those possessed by the female.

The olfactory receptors in insects differ somewhat from those identified in vertebrates by consisting of a heterodimeric complex, including one typical odorant-binding unit and one ubiquitous co-receptor (Orco protein) (Sato et al., 2008; Wicher et al., 2008). Also, the insect odorant receptor has an inverted topology compared with that of mammals by having the amino terminus located intracellularly. Recently, it has been suggested that this heterodimeric complex serves as a ligand gated ion channel and cyclic nucleotide activated protein, thus, forming a unique strategy for responding to the olfactory environment (Sato et al., 2008). The rapid detection of odor information is enabled by the ionotrophic pathway differing from the slow and sustained odor detection via the conventional G-protein pathway (Wicher et al., 2008).

The odorant receptor proteins are positioned on the dendrites of OSNs. The odor molecules pass through the pore in the cuticular wall and reach the aqueous sensillum lymph. The lipophilic odorant molecules are carried to the receptor protein by special proteins, so-called odorant-binding proteins. The binding of odorant molecules to the receptor initiates the transduction process finally depolarizing the neuron and generating action potentials by opening the voltage gated ion channels. The action potentials are transferred to the primary olfactory center for further processing.

1.2.2. The primary olfactory center

The olfactory afferents from the antenna project to the primary olfactory center of the insect brain, called the antennal lobe. Here, the axon terminals target characteristic spherical neuropils structure termed glomeruli. The OSNs synapse with axons of second order neurons (Anton &Hansson, 2000). The number of glomeruli differs across the species and also in gender. For example, in male noctuid moths, the large number of male-specific receptor neurons being selectively tuned to female-produced pheromones project to a few enlarged glomeruli located dorsally in the antennal lobe, close to the entrance of the antennal nerve (Anton & Homberg, 1999). This arrangement of male-specific glomeruli has been termed the macro-glomerular complex (MGC).

In the heliothine moth, *Heliothis virescens*, the MGC consists of four units, two large glomeruli located dorsally receiving input about the two principal pheromone compounds

while two smaller compartments located ventrally are the target region of so-called interspecific information, meaning signals emitted from heterospecific females (Baker et al., 2004; Berg et al., 1998). In addition to the MGC-units, the male moth has a group of more numerous ordinary glomeruli receiving input from the plant odor neurons. In *H. virescens*, there are approximately 60 ordinary glomeruli (Berg et al., 2002; Lofaldli et al., 2010). This number corresponds to that reported in several other moth species. Among the ordinary glomeruli, there is one large unit, located ventrally in the antennal lobe, the so-called labial pit organ glomerulus being responsible for processing of CO_2 information (Kent et al., 1986; (Zhao et al., 2013); Zhao et al., submitted article).

In addition to the sensory axon terminals, the glomeruli are innervated by two main types of antennal-lobe neurons, i.e. projection neurons and local interneurons (Homberg et al., 1988). The projection neurons carry the olfactory information to higher brain centers, mainly to the mushroom body calyces and the lateral horn. The neurites of local interneurons, on the other hand, are restricted to the antennal lobe. Many of the local interneurons are GABAergic, being responsible for interglomerular exchange of information, providing mainly lateral inhibition in the antennal lobe (Das et al., 2011). The local neurons receive input signals both directly from the sensory axon terminals and via other local neurons.

In addition to the two main types of antennal-lobe neurons, a relatively small group of centrifugal neurons having dendrites outside the antennal lobe constitutes a third category. Particularly, in *H. virescens*, two categories of centrifugal neurons have been identified physiologically and morphologically: one is the serotonin immune reactive neuron (Zhao et al., 2009) and the other a multisensensory neuron responding to odor and sound (Zhao et al., 2013).

1.2.3. Antennal-lobe pathways

The olfactory information is carried from the antennal lobe to higher brain centers via projection neurons, as shown in figure 1.1. The two main target regions of antennal-lobe projections are the mushroom body calyces (MBC), an area responsible for associative learning of odors (Müller, 2002), and the lateral horn, a region of the lateral protocerebrum (Homberg et al., 1988; RØ et al. 2007). Previous studies have reported about three parallel antennal-lobe tracts in moths, namely the medial antennal lobe tract (mALT), the mediolateral antennal lobe tract (mlALT), and the lateral antennal lobe (lALT) (Homberg et al., 1988; Ro et al., 2007, for terminology, see Ito et al., 2014).

The most prominent tract, the mALT, houses projection neurons arborizing in one single glomerulus, i.e. so-called uni-glomerular neurons. The mALT leaves the antennal lobe dorso-medially and runs posteriorly passing the central body before making a lateral turn in order to reach its first target, the mushroom body calyces. The axon bundle projects further laterally and terminates in a region of the lateral protocerebrum named the lateral horn.

The mlALT, being considerably thinner than the mALT, consists of mainly multiglomerular projection neurons. The current tract exits the antennal lobe together with the mALT, but bends laterally at the level of central body. Different from the mALT, it projects directly to the lateral horn without forming any contact with the calyces (Homberg et al., 1988; Ro et al., 2007). A considerable portion of the projection neurons passing in the current tract is reported to be GABAergic (Berg et al., 2009)

The third tract, the lALT, contains both uni- and multiglomerular projection neurons. This path exits the antennal lobe ventrally and passes laterally toward the lateral horn where it has most of its terminal projections. Also, some fibers are reported to run dorsomedially from the lateral horn to the mushroom body calyces (Homberg et al., 1988; Ro et al., 2007).

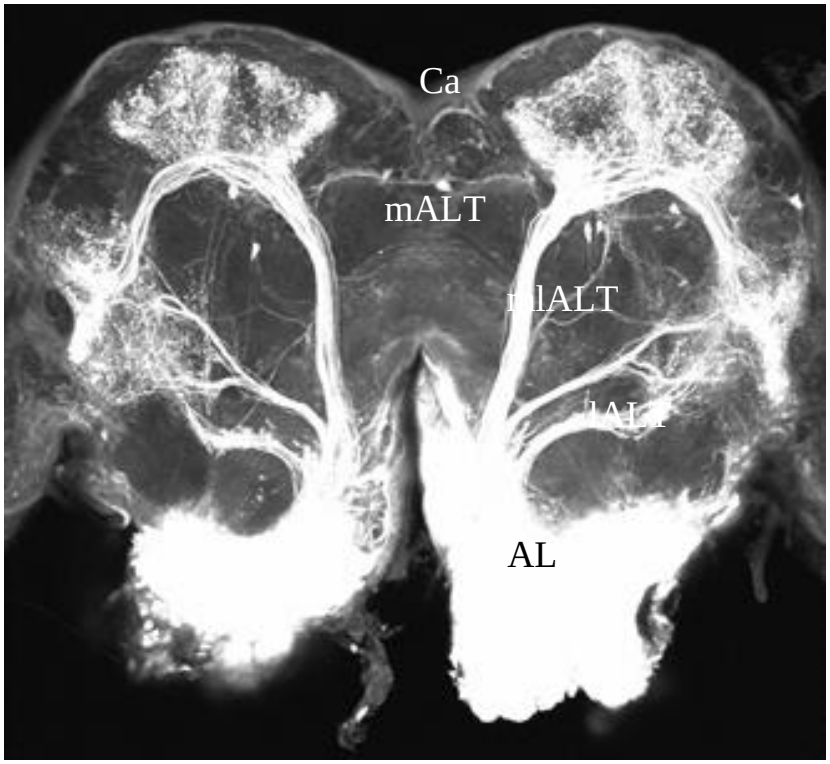

Figure 1.1: Confocal image showing the three antennal lobe tracts, the medial antennal lobe tracts (mALT), the mediolateral antennal lobe tract (mlALT), the lateral antennal lobe tract (lALT). AL antennal lobe; Ca calyces; LH lateral horn. Source: Xin-Cheng Zhao (unpublished data).

1.3. Higher Olfactory centers

As mention above, the three main antennal-lobe tracts project to two main regions in the protocerebrum, the mushroom body calyces and the lateral horn. The calyces receive odor information essentially via the mALT. In addition, some fibers from the lALT also target the calyces, however, after innervating the lateral horn. In the calyces, boutons of the antennal-lobe projections are surrounded by dendrites of Kenyon cells (Yasuyama et al., 2002). The mushroom bodies, which also include several other structures, are involved in learning and memory and multisensory integration, in particularly in experience-dependent odor learning (Menzel & Muller, 1996).

The lateral horn is defined as the particular area of the lateral protocerebrum being innervated by antennal-lobe projection neurons (see Ito et al., 2014). The current region lacks visible synaptic structures and relatively little is known about its neural connections. Pheromonal and plant odor information are reported be processed in different regions in the lateral horn (Homberg et al., 1988; Kanzaki et al., 2003; Zhao et al., submitted article). Furthermore, the lateral horn is assumed to be closely connected to the motoric system than the calyces, and it has been suggested that it is linked to innate behavioral responses. Actually, the lateral protocerebrum is often being termed as a pre-motoric region. The term premotoric refers to the projection of the descending neurons in the brain being connected to the motor centers in the ganglia of the ventral cord (analogous to the spinal cord in mammals). This descending pathway is responsible for different behavioral responses (Wada & Kanzaki, 2005).

However, no evidence for a direct connection between the lateral horn and the motoric system, being positioned in the ventral cord ganglia, has been demonstrated so far. Also, it should be mentioned that the lateral protocerebrum, including the lateral horn, is considered to be a multisensory integration center of the insect brain.

Whereas the first and second order level of the olfactory pathway of the moth is relatively thoroughly explored, the subsequent pathway is poorly described. However, the lateral accessory lobe, situated adjacently to the central body and posteriorly of the antennal lobe in each hemisphere, is reported to be innervated by descending neurons responding to odors. Thus, in the male silk moth, *Bombyx mori*, so-called “flip-flop”-interneurons having dendritic branches in the lateral accessory lobe (LAL) and an axon projecting in the ventral cord have been found (Sakurai et al., 2014). Furthermore, in the male fruit fly, *Drosophila*

melanogaster, descending ventral-cord neurons with dendrites in the lateral triangle of the LAL have also been reported (Ruta et al., 2010) In heliothine moths, one particular odor-responding neuron projecting from the ventrolateral protocerebrum into the ventral cord has been found (Lofaldli et al., 2012).

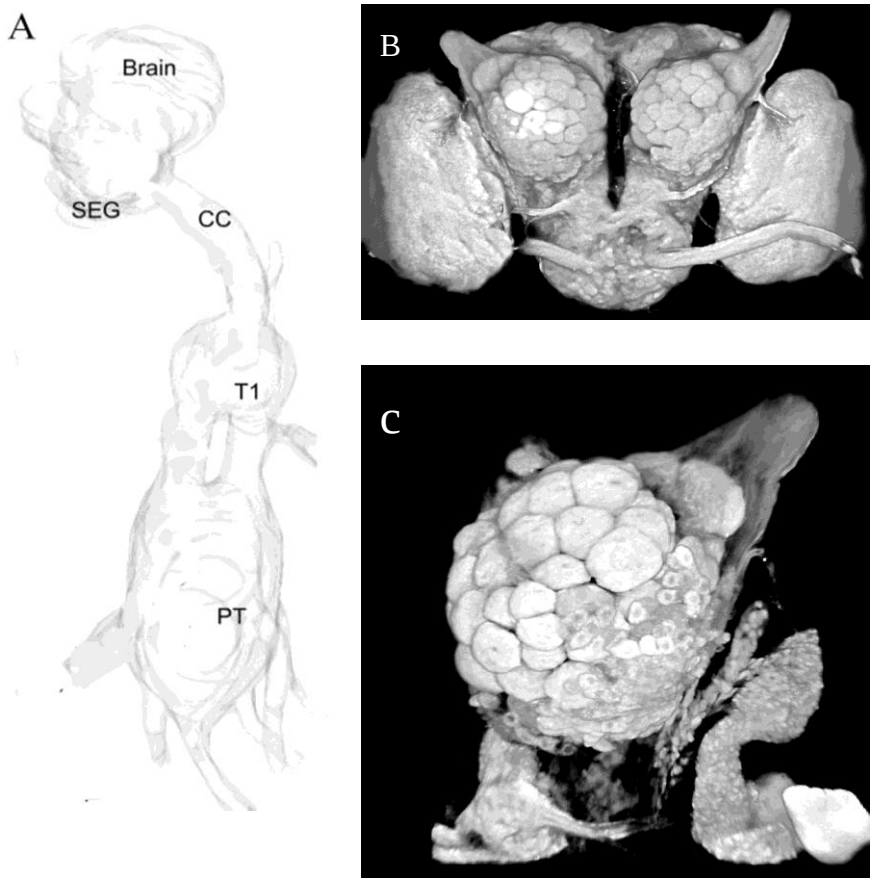

Figure 1.2: The central nervous system (CNS) of the heliothine moth. **A:** Reconstruction of the ventral cord including the ganglia. **B:** Confocal image of the moth brain in frontal view. **C**: Confocal image of the primary olfactory center of the moth brain, the antennal lobe. CC cervical connectives; SEG suboesophageal ganglion; T1 thoracic ganglion; OL olfactory lobe; Cam mushroom body calyx; caL Mushroom body lobe. Source Adapted from (Zhemchuzhnikov et al., 2014); (Berg et al., 2002).

1.4. Ventral cord neurons

The ventral cord consists of the cervical connectives and several ganglia. The cervical connectives contain axons of both ascending and descending neurons which connect to the central brain (Figure 1.2). The descending neurons are also named command neurons, by performing input to the motor centers. These neurons have their somata in the brain. In the cockroach, for example, cell bodies and dendritic arborisations of descending neurons are located in the lateral and the medial protocerebrum (Okada et al., 2003). The somata of most motoric neurons, on the other hand, are gathered in distinct ganglia in the ventral cord. Generally, the ascending ventral-cord neurons carry information to the brain about the state of activation in the ganglia (Cardona et al., 2009). These neurons usually have their somata in the ganglia as well.

1.5. Comparison of brain structures in insects and humans

A number of striking similarities have been found in the nervous system of insects and mammals, humans included. Figure 1.3 presents an overview of brain structures in the higher primate brain that are suggested to correspond with structures in the insect brain. In particular, the olfactory pathways seem to be well conserved during evolution. In addition to the general structure of the olfactory receptor neuron, being a small bipolar neuron extending a dendrite towards the external world and an unmyelinated axon directly into the brain, several similarities are found at various synaptic levels. In the primary olfactory center of the brain, the antennal lobe in insects and the olfactory bulb in mammals, the sensory neurons make synapses with second order neurons in characteristic structures, called glomeruli (Hildebrand, 1996). Furthermore, the folded structure of the mushroom body calyces has been compared with the mammalian cortex, including the piriform cortex, amygdala, and hippocampus.

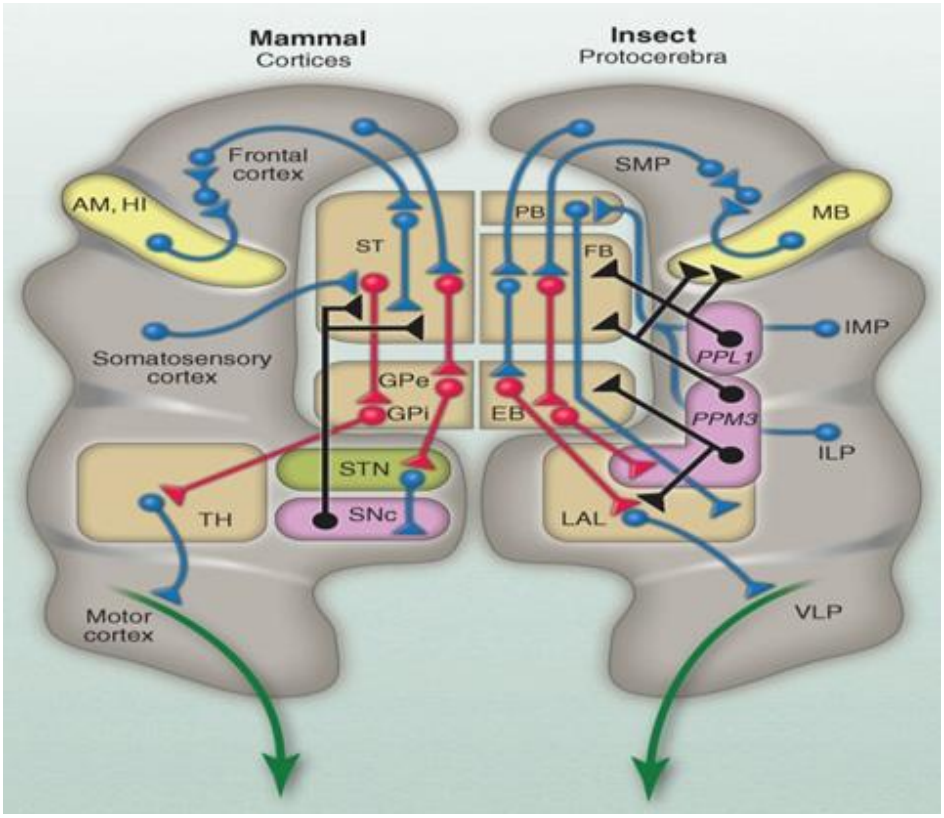

Figure 1.3: Distinct brain structures of higher primates that are suggested to correspond with brain structures of insects. VLP ventrolateral protocerebrum; LAL lateral accessory lobe; FB fan shaped body; PT protocerebral bridge; GPe external globus pallidus; GPi internal globus pallidus; EB ellipsoid body; STN subthalamic nucleus; SNc dopaminergic substantia nigra pars compacta; HI hippocampus; AM amygdala; SMP superior medial protocerebrum; TH thalamus; ILP inferior ventrolateral protocerebra Figure adopted from (Strausfeld et al., 2013).

1.6. Main aim of the thesis

There is a considerable amount of knowledge about the first and second order level of the moth olfactory pathway. However, little is known about the third order level and the olfactory neurons descending to the ventral nerve cord. In particular, it is still unclear whether there are third order neurons projecting directly to motor regions in the ventral cord. The main aim of this thesis is therefore to map second order olfactory neurons, i.e. antennal-lobe projection neurons, and ventral-cord neurons in order to investigate whether the two categories display any overlap in the lateral horn areas.

Specific goals of the study

1. To map terminal regions of antennal-lobe projection neurons in the lateral horn.
2. To map brain regions being innervated by ventral-cord neurons.
3. To establish a staining technique enabling simultaneous labeling of antennal-lobe projection neurons and ventral-cord neurons by using two different fluorescence dyes.
4. To investigate whether there are overlapping regions of antennal-lobe projection neurons and ventral-cord neurons in the lateral horn by performing double-labeling of the two neuron categories in the same preparation.

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2. MATERIALS AND METHODS

2.1. Insect cultures and selection of insects

The insects (*Heliothis virescens*; Lepidoptera; Noctuidae) used in the experiments originated from our lab culture (eggs kindly provided by Bayer CropScience, Monheim, Germany), taken care of by Dr. Quingbo Tang. The insect pupae were sorted by gender, transferred to hatching cages (18 x 12 x 17 cm) and kept separated in two heating cabinets (Refitherem 6E incubator, Struers) at 22-24 °C. The cabinets had a light-dark cycle of 14 – 10h and a humidity of 70 %. After hatching, the insects were transferred to cylinders of plexiglass (18 x 10 cm) and kept in this environment until they were used in experiments, usually 2 – 5 days old.

2.2. Ethical considerations

According to the Norwegian Law concerning animals used in research (Dyrevernloven), all vertebrates such as mammals, birds, reptiles, amphibians, and fish, and some invertebrates such as decapods, squid, and honey bees (www.lovdata.no) are included. Lepidoptera is not included, hence there are no restraints regarding the use of this particular order in research. However, the insects in our lab were treated with care. They were inspected daily, and they were regularly fed a mixture of honey solution or sucrose and water. Further, they had paper sheets to climb on and each plexiglass cylinder contained a maximum of 8 moths in order to avoid unnecessary space-related stress for the insects.

2.3. Selection of the fluorescent dyes

In total four fluorescent dyes from Life Technologies (www.lifetechologies.com) were used in the experiments. 1) Dextran tetramethylrodamine/biotin (3000MW; microruby; ext/emis: 490/508 nm), 2) Dextran fluorescein/biotin (3000 MW; microemerald; ext/emis: 550/570 nm), 3) Alexa fluor 488 (10000MW; ext/emis: 495/519 nm), and 4) Dextrantetramethylrodamine (3000MW, anionic, ext/emis: 550/570 nm). Of these, dextran tetramethylrodamine and biotin (microruby), and dextran fluorescein and biotin(microemerald) worked best. All dyes were stored at -20^{o}C in the crystalline form. Before the experiment began, the dye crystals were kept at room temperature for a shorter period, but in the dark in order to prevent the degradation of fluorescent entities.

2.4. Preparation of the insects

Both male and female moths were used in the current project. Before the experiments, the insects were anesthetized in the cold by keeping them in the fridge for about ½ hour. Incision of the three leg pairs was done to ease positioning of the insect inside the plastic tubes, i.e. a plastic pipette (100-1000 µl) that was cut at the tapering end and covered with utility wax (Kerr Corporation Romulus, MI, USA). After placing the insect inside the plastic tube, the protruded head was fixed by means of the wax as shown in figure 2.1. The cephalic scales and hairs were removed by fine scissors under suction (to prevent allergic reactions). The head cuticle between the eyes was removed with a sharp razor blade, using a microscope (e.g. Leica, MZ12.5). Next, the muscles, tracheas, and thin neuronal sheet covering the brain were removed using the fine forceps. Thereafter, the brain was supplied with Ringer's solution (Nacl: 150mM, cacl2:3mM, KCL: 3mM, TES buffer: 10mmol, Sucrose ($C_{12}H_{22}O_{11}$): 25mmol, p^H 6.8) to keep the neural tissue alive and prevent dehydration.

2.5. Staining of the antennal lobe

Two approaches were applied for inserting dye into the antennal lobe. In the first method, a micro needle was used to pick up dye crystals (microemarald) that were subsequently injected into the AL of the moth brain.

The second approach included usage of a glass electrode made from a flaming-brown horizontal puller (P97; Sutter instrument, Novarto, CA, USA). The tip of the glass electrode was used to pick up the dye being applied into the antennal lobe.

During both procedures, Ringer's solution was applied immediately after dye injection. Then the solution was soaked up by medical wipes to uncover whether the region of interest was marked with the color of the relevant dye (for dextran microemarald, green). Finally, wet medical wipes, soaked in Ringer's solution, were placed on the brain to apply nutrition and keep it humid.

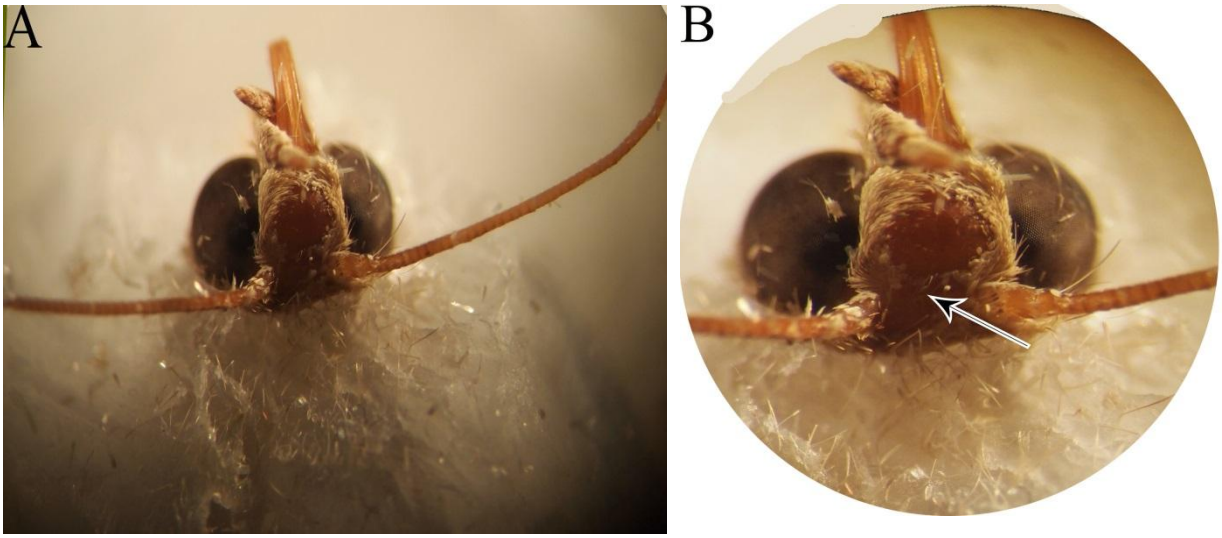

Figure 2.1: Images showing the head of the moth *Heliothine virescens,* immobilize with wax during the preparation procedure.

2.6. Staining of the cervical connectives

The moth was mounted on wax with staple pins, with its ventral side facing upwards. Then the ventral sclerite of the prothorax was removed by cutting it horizontally. That exposed the prothoracic ganglion. Next, the cerebral connectives joining the suboesophageal ganglion (SOG) and the prothoracic ganglion was cut with the scissor. A micro needle was used to apply the dextran microruby dye at the cut end of the cervical connectives (Figure 2.2). As for the brain, the cerebral connectives were rinsed with Ringer's solution immediately after the dye was applied.

2.7. Double labelling experiments

For the double labeling experiments, the preparation with the pre-stained antennal–lobe neurons was taken out of the plastic tube and mounted in the wax, as described above. Then the micro ruby dye was applied to the cut end of the cerebral connectives before the insect was kept in a refrigerator at 4^{o}C for 16-24 hours.

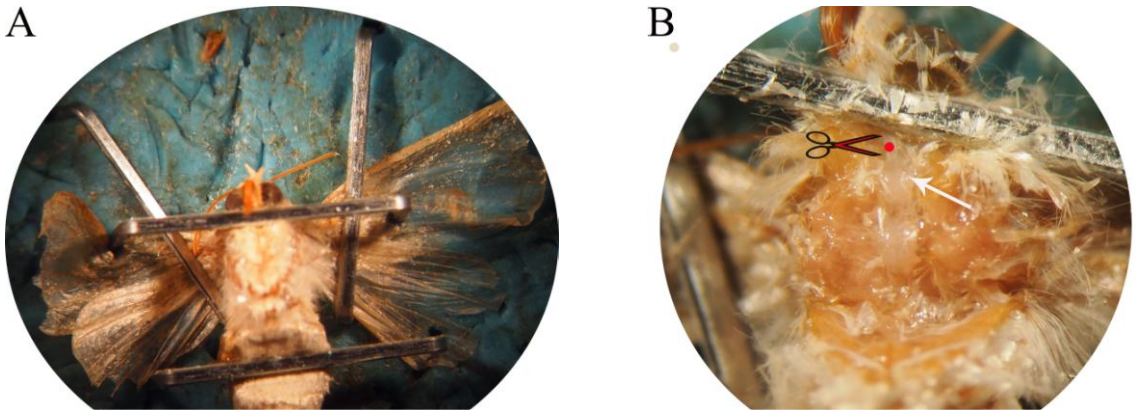

Figure 2.2: Images demonstrating the position of the preparation when applying microruby in the ventral nerve cord. **A:** Image of the moth mounted with its ventral side facing upwards. **B:** Image indicating the application site for the dye (red dot). The white arrow points at the prothoracic ganglion.

2.8. Dissection and fixation

The insect was decapitated by cutting at the neck region with fine scissors. Forceps were used to remove the maxilla, proboscis, cuticles, muscles, tracheas, and a pigmented layer of the compound eyes. . The maximum duration for the dissection never exceeded 30 minutes. After the dissection, the brain was fixed in 4 % paraformaldehyde (Roti, Histiofix pH 7) for 1-2 hours or overnight, i.e. 16-24 hours, in the refrigerator. The fixed brain was dehydrated in a series of alcohol (50%, 70%, 90%, 96% 10min each; 100%, 100% 10 min each). Then the brain was immersed in methyl salicylate for making it transparent, and put them on a metal plate. After about 10 minutes a coverslip was applied.

2.9. Intensification of brains

Only preparations containing one dextran dye were intensified. This because both microruby and microemarald contains biotin, which is the element being visualized during intensification. Thus, the preparations stained by microruby only (usually ventral-cord neurons), were intensified by using CY3, which corresponds with tertramethylrhodamine, and the preparations stained by microemarald only (usually antennal-lobe neurons) with CY2.

The brain cleared in methylsalicylate was rehydrated in a series of alcohol (100%, 100%, 96%, 90%, 70%, and 50%: 10 min each). Then it is washed in PBS (0.1M, Ph7.2) for 10 minutes at room temperature before being incubated in streptavidin CY3 or CY2, at a concentration of 1:200 in PBS, for 2 hours or overnight at 4^{o}c. Then the brain was washed in PBS (0.1M, ph 7.2) for 2x10 min at room temperature. Thereafter, it was dehydrated in a

series of alcohol (50%, 70%, 90%, 96% 100%, 100%, 10 min each). Finally, the brain was cleared in the methyl salicylate.

2.10. Visualization of stained processes using confocal laser scanning microscope

Successfully stained preparations, selected from observations under a stereo fluorescence microscope (Carl stereo Discovery V12, motorized 12× zoom; pentafluor) were taken to a confocal microscope (LSM Zeiss 510 Meta Mira 900F, GmbH, Jena, Germany) for further analyzing. The preparations were scanned using three different objectives, 10×0.45W(to retrieve the overview of the moth brain), 20×0.5 dry objective plan-neofluor(to retrieve the detailed image), and 40×0.8W C-Achroplan(to retrieve the finer details of the staining process). The scanning of the preparation was made in the z- axis. The resolution of the image stacks was 1024×1024 pixels with a slice thickness of 1-5 µm. The scan speed is set up to 6. The optimal pin hole diameter selected. The helium neon laser (wavelength, 543um) and argon laser (wavelength, 488um) were used to excite the microruby/CY3 and microemerald/CY2, respectively. Detector gain and amplification was adjusted for the brain preparation to give good scan. The stack of images was saved as Zeiss image files (.lsm).

2.11. Image processing

The files containing the confocal scans were studied amd visualized by means of the LSM 510 image browser (Carl Zeiss Microscopy, Jena, Germany, version 3.5). The contrast was adjusted in Adobe Photoshop. Finally, the images were edited in Adobe Illustrators CS6 (Adobe systems, San Jose, CA).

2.12. Nomenclature

The naming of each olfactory tract and other neural structures are in accordance with nomenclature guideline proposed by (Ito et al., 2014). The previous medial, mediolateral and lateral antenno- protocerebral tracts are here named the medial, mediolateral, and lateral antennal-lobe tract (mALT, mlALT, and lALT, respectively). The nomenclature is based on the brain of *Drosophila melanogaster* as the reference standard.

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3. RESULTS

In order to visualize whether the processes of antennal-lobe projection neurons and ventral-cord neurons overlap within regions of the lateral horn, the two-neuron categories were stained using different labeling procedures. This includes separate staining of each neuron category as well as double labeling of the two neuron populations in the same preparation using different fluorescent dyes, one in the antennal lobe and the other in the ventral cord.

Staining of antennal-lobe projection neurons and ventral-cord neurons, either separately or simultaneously, was attempted on 80 *H. virescens* moths, including 37 males and 43 females. In addition, two heliothine male moths of the species, *Helicoverpa armigera,* and three of the species, Silver Y moth (*Autographa gamma),* were used during the experimental period. Details of the different species used in the experiments are listed in Appendix 1. Among the 80 preparations, 20 were successfully labeled (all of the species, *H. virescens*). Six of these were stained from the antennal lobe only (three males and three females), three from the ventral cord only (two males and one female), and 11 were double labelled (five males and six females).

The results are presented according to the following topics:

3.1. Projection pattern of the antennal-lobe tracts

3.2. Projection patterns formed by ventral-cord neurons

3.3. Projection patterns of the two neuron categories obtained by double labelling

3.1. Projection pattern of antennal-lobe projection neurons

Of the six successfully labelled preparations stained from the antennal lobe exclusively, four are presented in the current sub-section. Generally, all successfully stained brains showed the three main tracts projecting from the antennal lobe to the protocerebrum in the ipsilateral hemisphere. As indicated in figures 3.11, 3.12, and 3.13, these tracts were identified as the medial antennal lobe tract (mALT), the mediolateral antennal lobe tract (mlALT), and the lateral antennal lobe tract (lALT). The most distinctive tract, the mALT (Ø: 18.21±5.3 µm), ran posteriorly, bypassing the central body ventrally before turning laterally. The measurement of the diameter is shown in the Appendix I. It sent off collaterals to the calyces before terminating in the lateral horn as shown in figure 3.11.

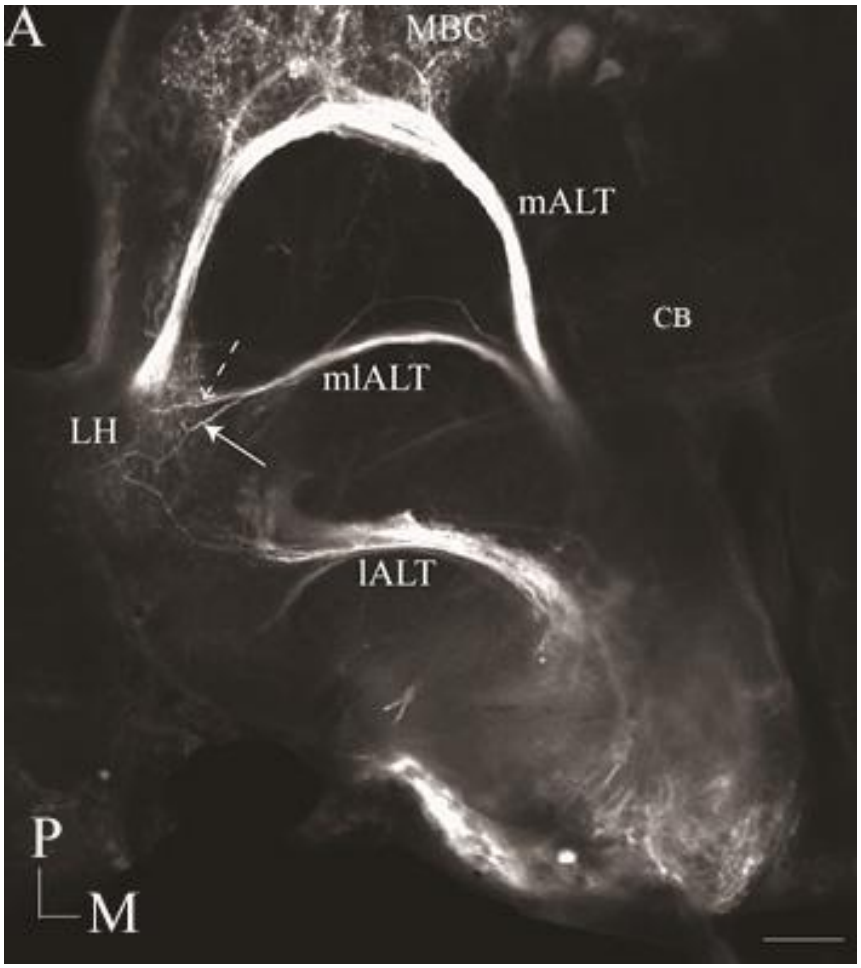
The somewhat thinner mlALT (Ø: 4.51±3.08 µm) projected together with the mALT for a short distance, but then turned laterally at the edge of the central body and passed directly to the lateral horn. This tract seemed to split into two axonal branches before terminating in the lateral horn as shown in figure 3.11A.

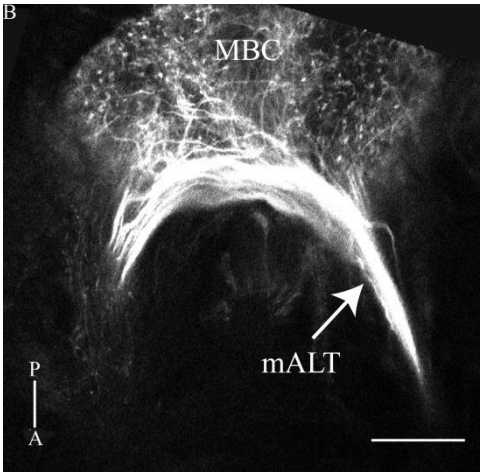
The lALT (Ø :11.11±2.15 µm) left the antennal lobe medioventrally, ran in a lateral direction and targeted regions of the lateral horn, particularly in an area located ventro-medially, as shown in figure 3.11A and C. One particular section of the lALT was strongly labelled. When rotating the dorsally oriented brain presented in figure 3.12A into a more sagittal position, the current region appeared as a pillar-like structure terminating in the superior protocerebrum (Figure. 3.12B).

In addition to the three main tracts, one additional, termed the 2nd medio- lateral antennal-lobe tract (Lillevoll, 2013), terminating in areas adjacent to the calyces, was also observed in some of the preparations (Figure. 3.12C). This tract also bent off from the prominent mALT, and it ran in parallel with the mlALT, but in a more posterior position, targeting the lateral horn and a medially located region anterior of the calyces.

The labelled projection neurons from the three main tracts targeted various regions of the lateral horn, which to some extent seemed to overlap. Particular areas were innervated by the lALT only, including a ventrally located region of the lateral protocerebrum (Figure. 3.11A).

In some preparations, a difference was observed between males and females as regards the projections from the calyces to the lateral horn. As shown in the male brain in figure 3.13A and B, the projection neurons confined to the mALT terminates in two areas of the lateral horn, one located more anteriorly, and medially (indicated by a white dotted circle in figure 3.13A) than the other (indicated by a black dotted circle in figure 3.13B). In females, on the other hand, only one target region was observed for the corresponding projection neuron type (Figure. 3.13 C, black dotted circle).





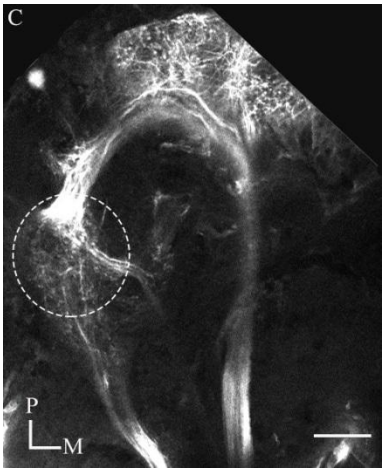


Figure 3.11: Confocal images of one brain hemisphere showing the three main antennal-lobe tracts (ALTs), the medial ALT (mALT), the mediolateral ALT (mlALT), and the lateral ALT (lALT). **A:**.Three tracts project to the lateral horn (LH), the mlALT divide before terminating in the lateral horn indicated by a solid and a dotted arrow, **B-C**: The mALT projects to the mushroom body calyces (MBC) giving off collaterals in the current structure before terminating in the LH (dotted circle in C). The tracts were visualized by staining the antennal-lobe projection neurons anterogradely, i.e. applying dye into the antennal lobe (AL). CB central body; P posterior; M medial; A anterior. Scale bar: 50 µm.

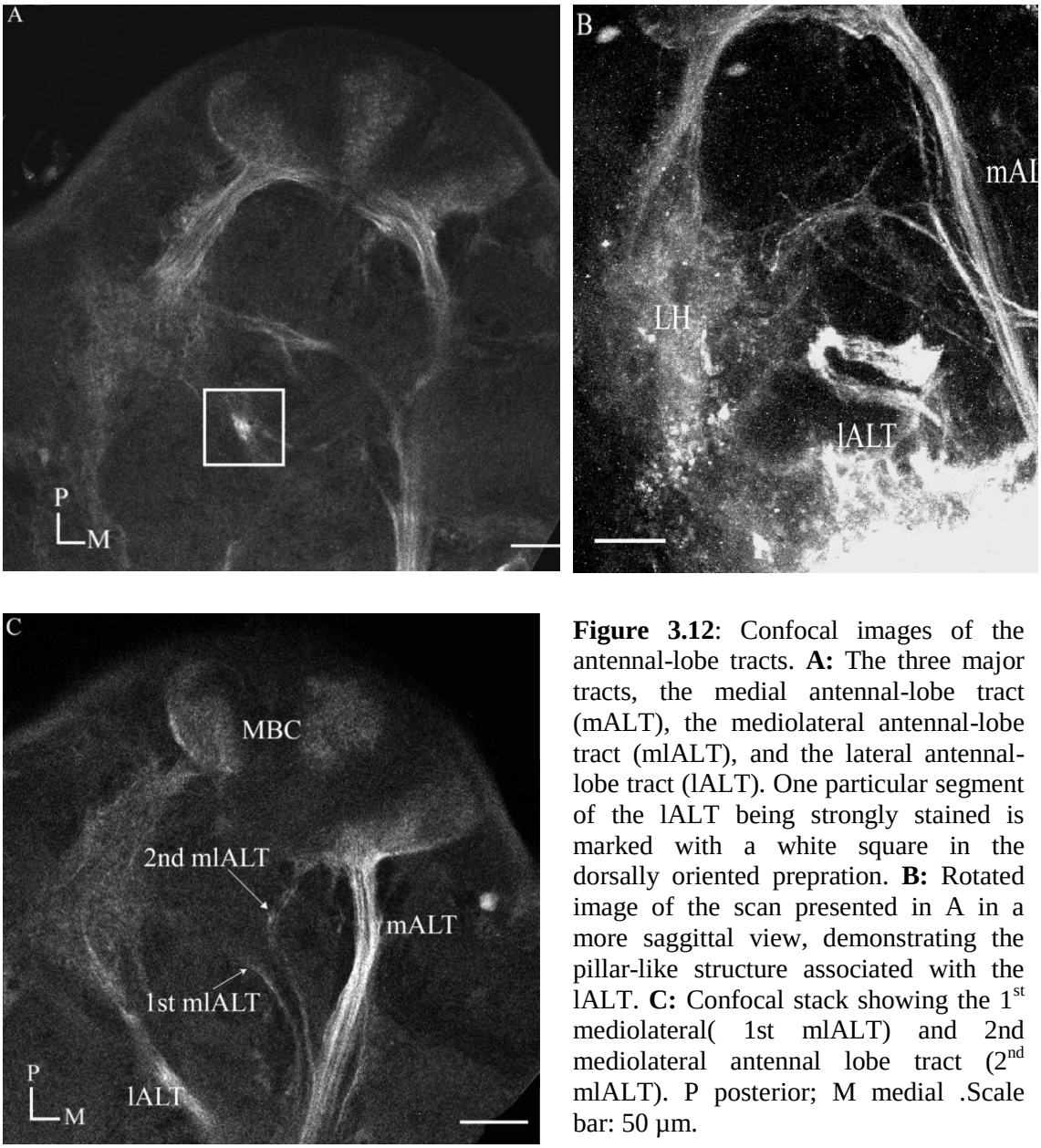


Figure 3.12: Confocal images of the antennal-lobe tracts. **A:** The three major tracts, the medial antennal-lobe tract (mALT), the mediolateral antennal-lobe tract (mlALT), and the lateral antennal-lobe tract (lALT). One particular segment of the lALT being strongly stained is marked with a white square in the dorsally oriented prepration. **B:** Rotated image of the scan presented in A in a more saggittal view, demonstrating the pillar-like structure associated with the lALT. **C:** Confocal stack showing the 1st mediolateral(1st mlALT) and 2nd mediolateral antennal lobe tract (2nd mlALT). P posterior; M medial .Scale bar: 50 µm.

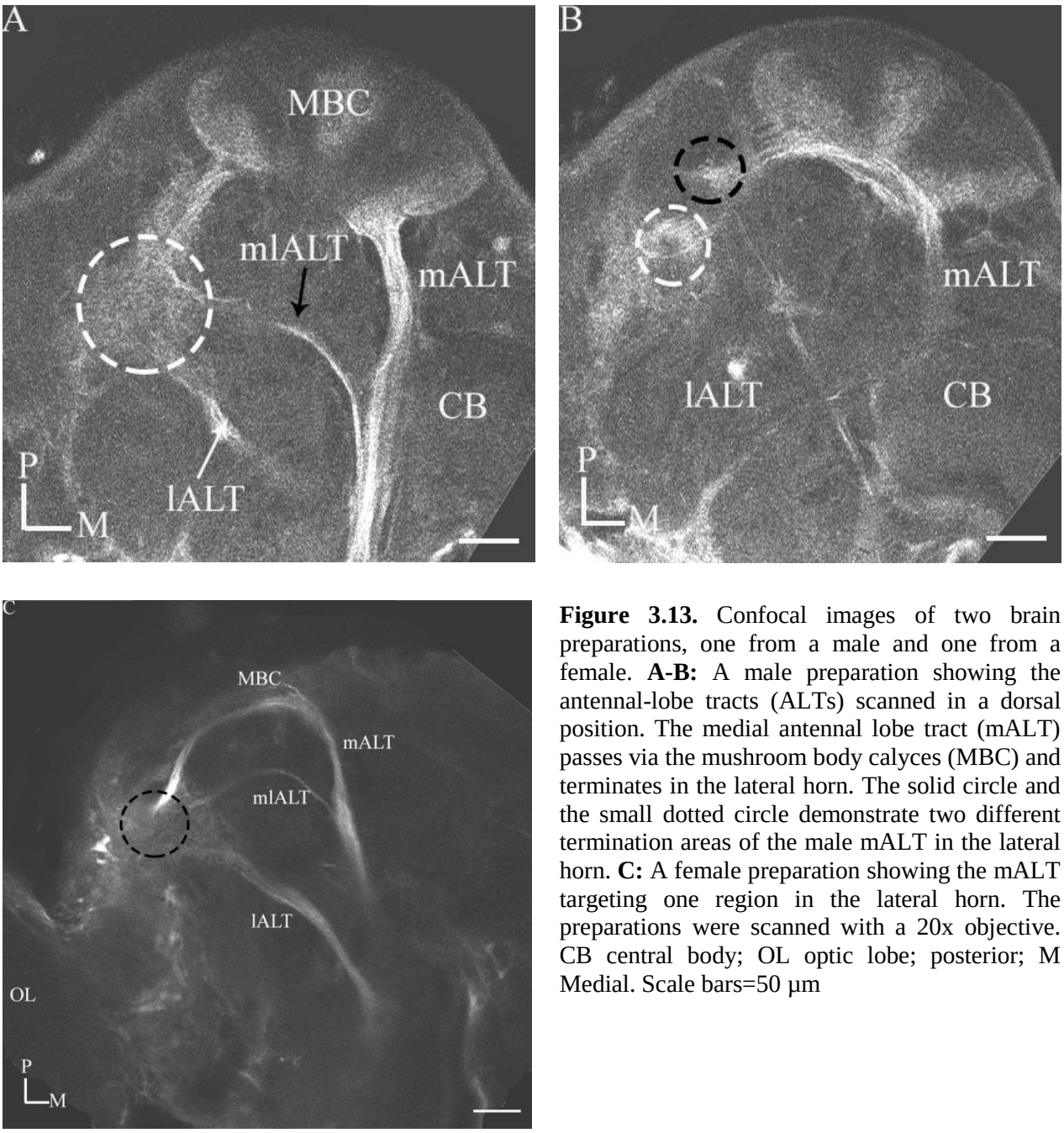


Figure 3.13. Confocal images of two brain preparations, one from a male and one from a female. **A-B:** A male preparation showing the antennal-lobe tracts (ALTs) scanned in a dorsal position. The medial antennal lobe tract (mALT) passes via the mushroom body calyces (MBC) and terminates in the lateral horn. The solid circle and the small dotted circle demonstrate two different termination areas of the male mALT in the lateral horn. **C:** A female preparation showing the mALT targeting one region in the lateral horn. The preparations were scanned with a 20x objective. CB central body; OL optic lobe; posterior; M Medial. Scale bars=50 µm

3.2. Projection patterns formed by ventral-cord neurons

The current paragraph presents data from two of the three successfully labelled preparations stained from the ventral cord exclusively. In addition, two preparations from the double-labelling experiments are also included.

3.2.1. Brain neuropils formed by stained ventral-cord neurons

Dye application into the ventral cord resulted in distinct staining pattern in protocerebral areas of the brain. Particularly interesting for the current investigation was the labelling of one spherical neurophil structure being located in the ventro-lateral protocerebrum of each hemisphere, as shown in figure 3.21A. This neuropils structure is connected to a prominent tract projecting in a medial-lateral direction. In addition, one densely stained region located laterally to the central body identified as the lateral accessory lobe was found (Figure. 3.21B).

3.2.2. Distribution of stained somata in the brain

As shown in figure 3.22, several stained somata located in different brain regions were stained. The current staining technique did not enable identification of descending versus ascending ventral-cord neurons. The staining from the ventral cord showing the axonal fibers with the somata is presented in Appendix III. However, as descending neurons usually have somata in different areas of the brain whereas ascending neurons have their somata located in the ganglia of the ventral cord, the stained somata seen here assumingly belong to descending ventral-cord neurons. Many of the stained somata were found in the dorso-medial region of the brain, around the mushroom body calyces. In addition, the subesophageal ganglion (SOG) contained numerous labeled somata. One particular group of cell bodies positioned dorsally in each hemisphere was observed. This cell cluster was connected to a small fiber bundle projecting in a dorsal- ventral direction in the protocerebrum.

3.2.3. Axonal fibers connecting the ventral cord and the antenna

A few stained fibers connected the ventral cord and the antenna, as shown in figure 3.23. In each hemisphere, the stained fibers by-passed the antennal lobe on its lateral side and projected through the ventral part of the SOG. Here, the current axons passed through one heavily labeled region identified as the antennal mechanosensory and motor center (AMMC)

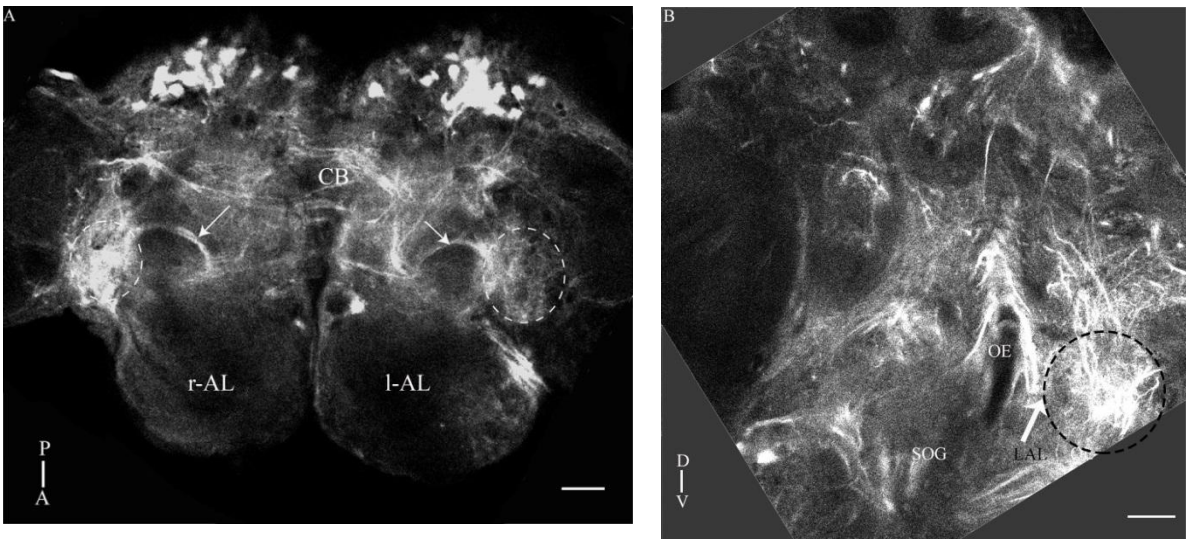


Figure 3.21: Confocal (projection) images of the brain after having performed staining from the cervical connectives. **A:** Image of a moth brain in a dorsal position. The dotted circle demonstrates the ventro-lateral part of the protocerebrum, being distinctly stained. The white arrows point to one labelled fiber bundle that is stained in each hemisphere. **B:** Image of the same preparation scanned with a 20X objective showing the stained ventral-cord neurons, plus stained somata in a higher resolution. The large dotted circle in black indicates the lateral accessory lobe. The white arrow points to one descending axon having its cell body located in the posterior medial part of protocerebrum. P posterior; A anterior. Scale bars=50 µm.

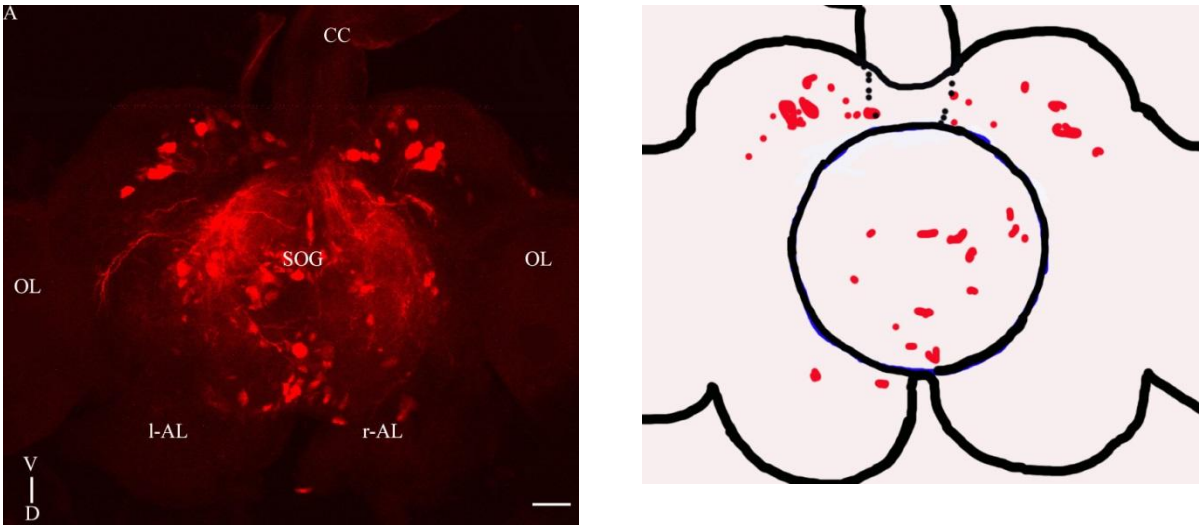


Figure 3.22: Images indicating the distribution of stained somata in different parts of the moth brain after having applied dye into the ventral cord. A: Confocal image showing the arrangement of somata in the protocerebrum and the suboesophageal ganglion (SOG). Many somata are distributed around the mushroom body calyces. B: Schematic diagram showing the arrangement of somata linked to the ventral cord. D dorsal; V ventral; OL optic lobe; CC cervical connectives; r-AL right antennal lobe; l.AL left antennal lobe. Scale bar=50 µm.

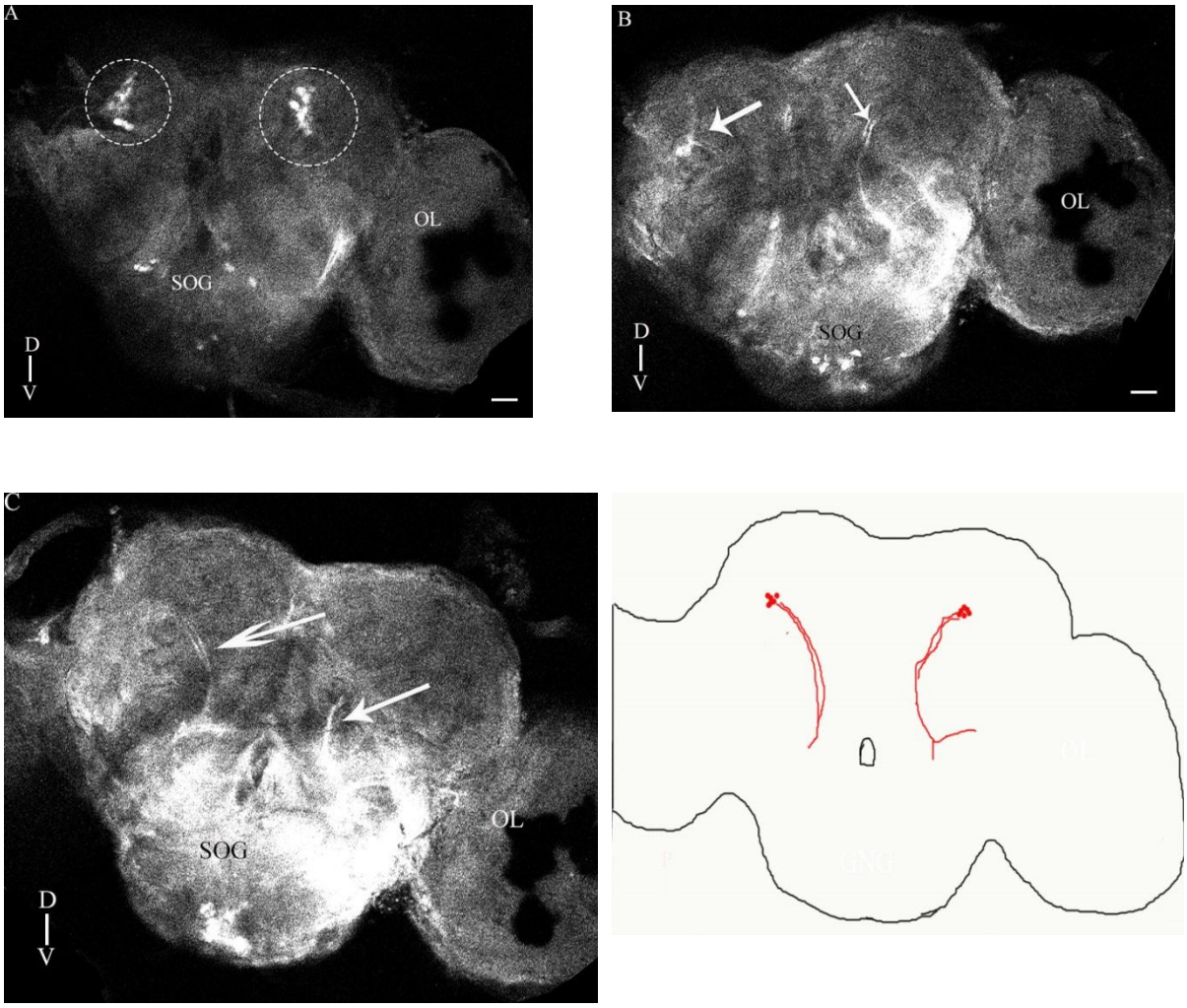


Figure 3.23: Images showing a small cluster of stained somata being connected to a particular fiber bundle in each hemisphere**. A-C:** Confocal scans showing the cluster of stained somata and their associated axons. The confocal images are scanned with a 10x objective. The schematic diagram indicates the stained cell clusters with their fiber bundles. D dorsal; V ventral; OL optic lobe; SOG suboesophageal ganglia. Scale bars= 50 µm.

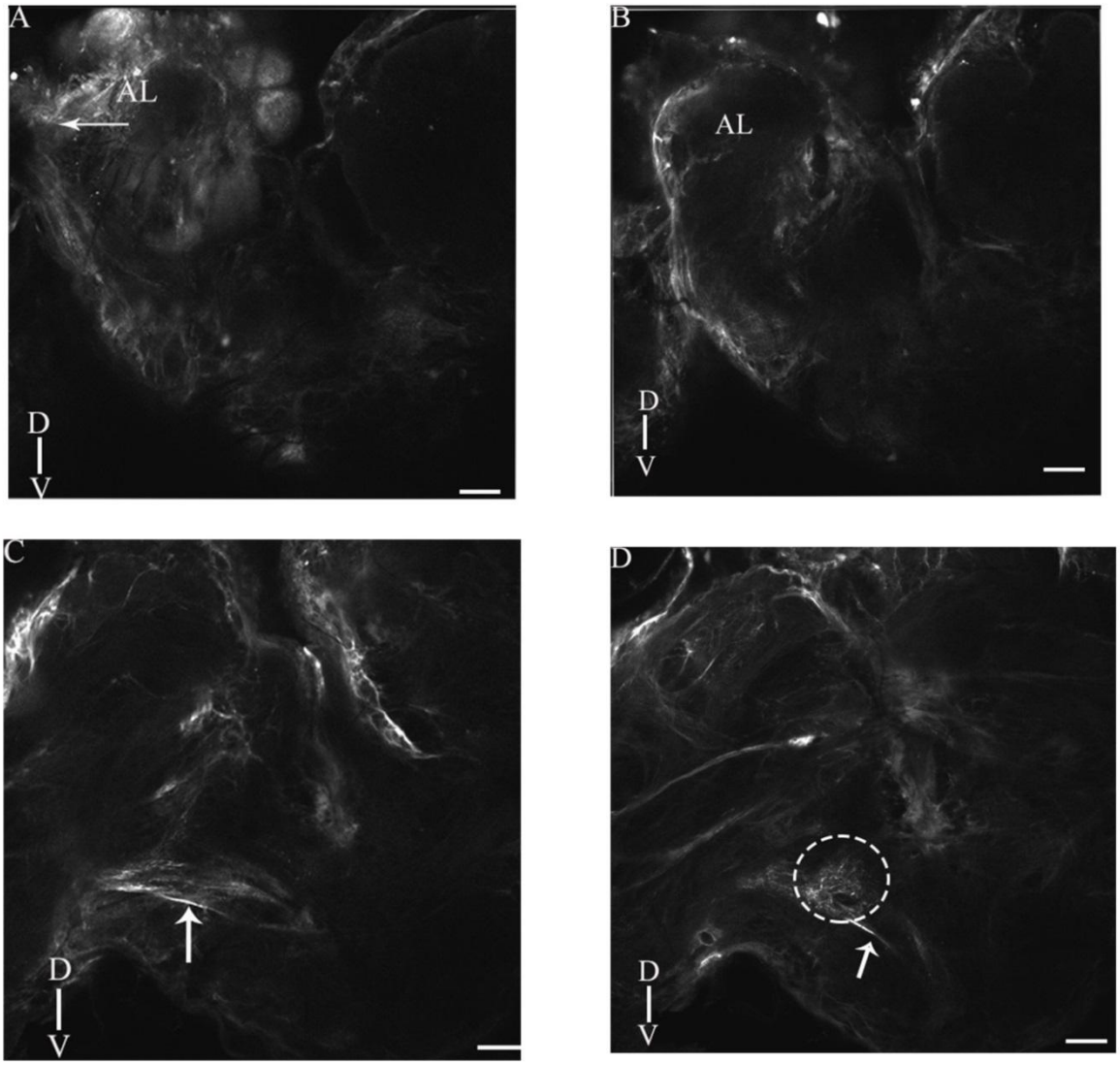


Figure 3.24: Confocal images from one stack showing stained ventral-cord fibers connecting with the antenna. **A:** The arrow points to the stained fibers at the base of the antennal nerve. **B:** The stained axons bypass the antennal lobe (AL) on its lateral side. **C-D**: The stained axons project via the antennal mechanosensory centre (AMMC, dotted circle in D). D dorsal; V ventral; Scale bars=50 µm

3.3. Projection patterns of the two neuron categories obtained by double labelling

Among the 11 successfully double-labelled preparations, image materials from four preparations are presented in the current sub-section.

3.3.1. General staining pattern in the lateral protocerebrum

The double-labelling experiments showed no overlap of antennal-lobe projection neurons and ventral-cord neurons in the lateral horn. As shown by the confocal scans in figure 3.31 - red indicating ventral cord neurons and green antennal-lobe projection neurons - there is no overlap of the two dyes.

3.3.2. Co-localisation of ventral-cord projections and antennal-lobe projections passing in the lALT

The results from the double-labeling experiments showed that the lALT and a fiber-bundle connected with the ventral cord followed the same path. The projections from the ventral cord were positioned ventrally to the lALT, as shown in figures 3.31C, 3.32A and B. The ventral-cord projections were connected with the strongly innervated region in the ventro-lateral protocerebrum.

3.3.3. Co-localisation of ventral-cord projections and the antennal-lobe projections passing in the mALT

In addition to the co-localisation of projections from the ventral cord and the lALT, respectively, a similar type of paired projections was found for one of the other antennal-lobe tracts. As shown in figure 3.32C, the mALT passed adjacently to projections from the ventral cord, though; the course of the two fiber bundles was not as tightly joined as for those mentioned above. The ventral-cord fibers could be traced and since they were connected with one particular cluster of somata located posteriorly in the protocerebrum, the current fiber bundle thus consists of descending neurons (see figure 3.23 and figure 3.32C).

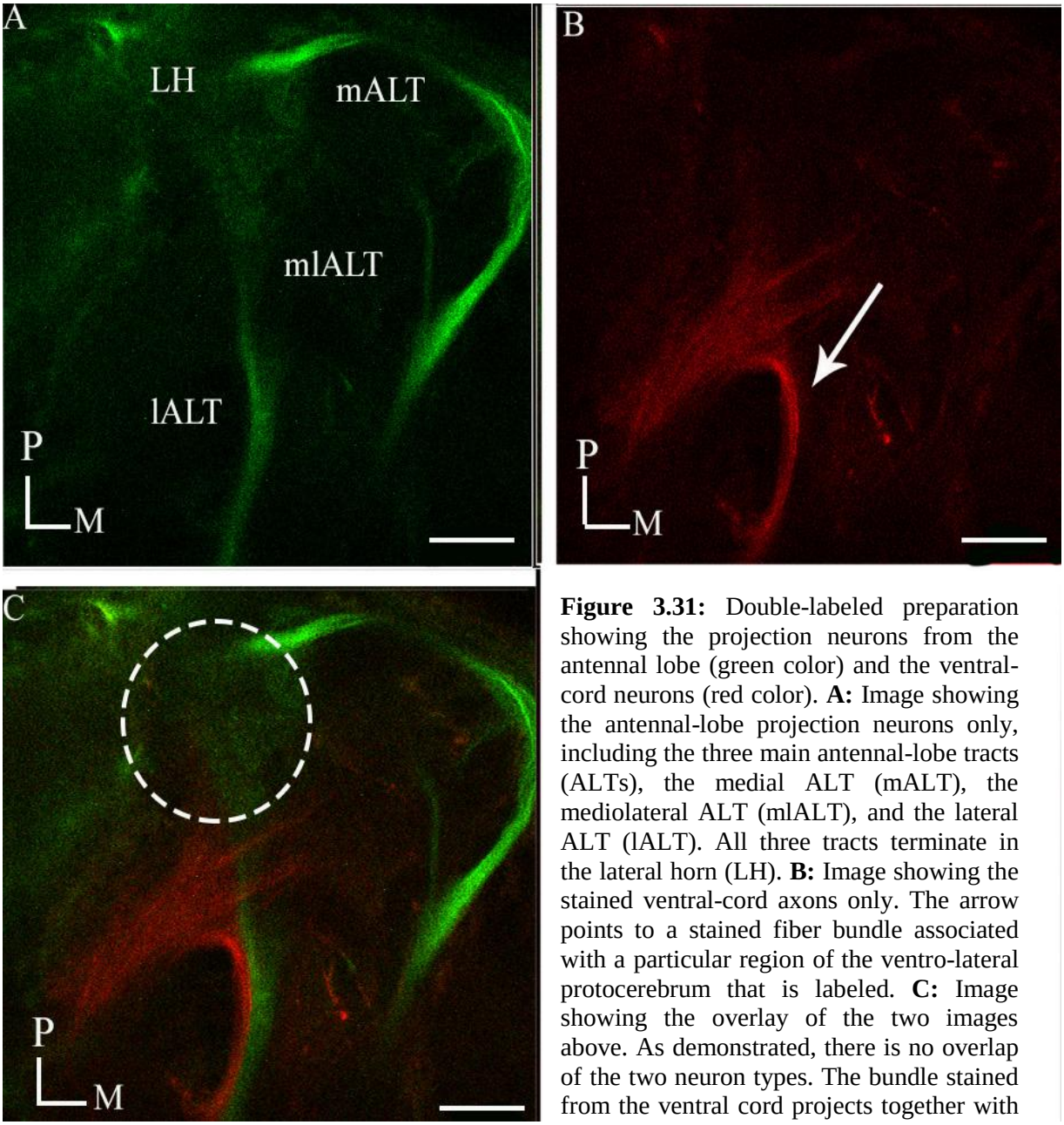


Figure 3.31: Double-labeled preparation showing the projection neurons from the antennal lobe (green color) and the ventral-cord neurons (red color). **A:** Image showing the antennal-lobe projection neurons only, including the three main antennal-lobe tracts (ALTs), the medial ALT (mALT), the mediolateral ALT (mlALT), and the lateral ALT (lALT). All three tracts terminate in the lateral horn (LH). **B:** Image showing the stained ventral-cord axons only. The arrow points to a stained fiber bundle associated with a particular region of the ventro-lateral protocerebrum that is labeled. **C:** Image showing the overlay of the two images above. As demonstrated, there is no overlap of the two neuron types. The bundle stained from the ventral cord projects together with the lALT, The dotted circle indicates the lateral horn. P posterior, M medial. Scale bars: 50 µm

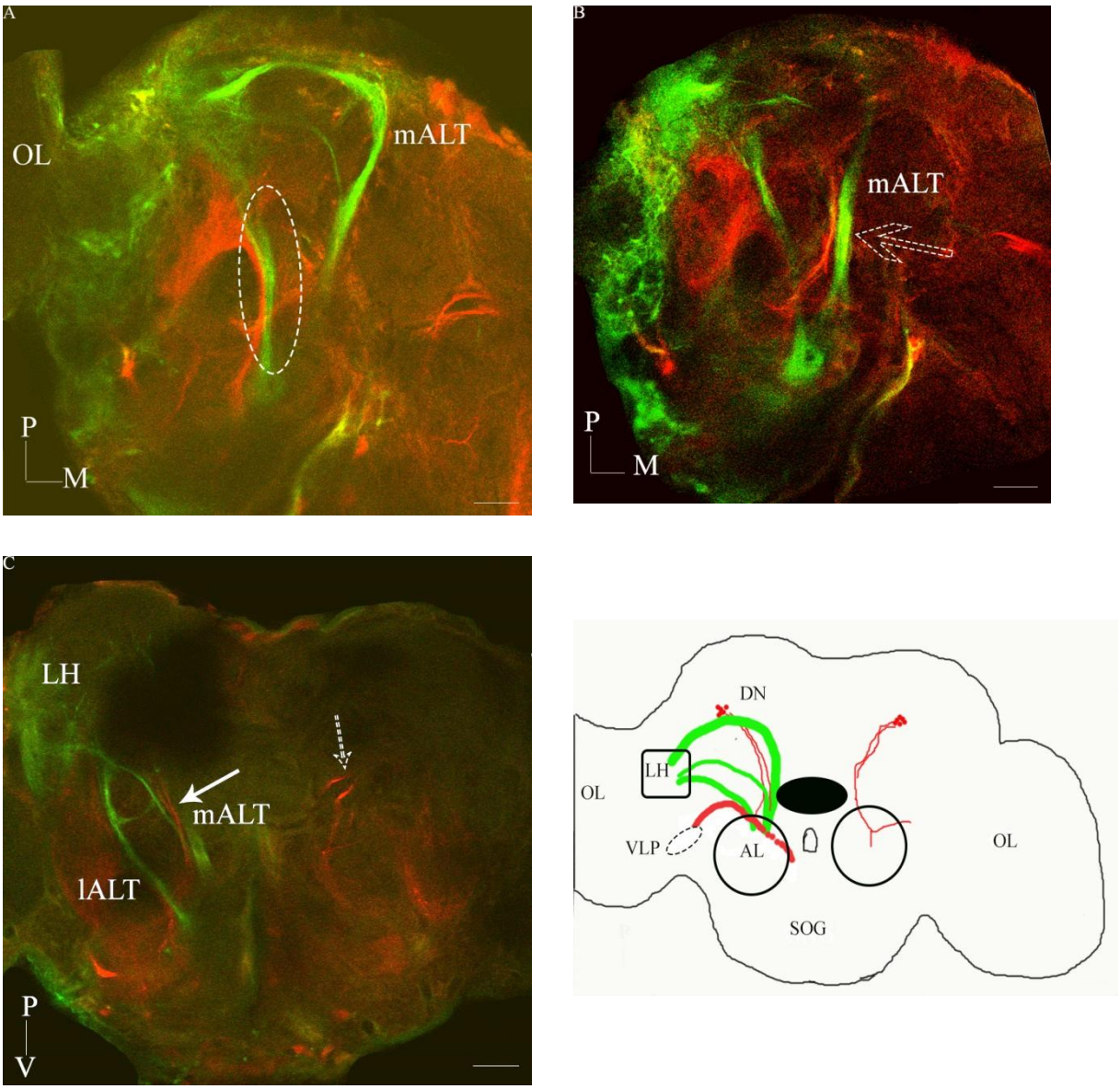


Figure 3.32: Confocal images of double-labeled preparations showing antennal–lobe projection neurons in green and ventral-cord neurons in red. **A:** Image showing co-localisation of the two neuron categories in the lateral antennal lobe tract (lALT) (dotted ellipse). **B:** Image from the same preparation showing co-localisation of the two neuron categories in a short part of the medial antennal lobe tract (mALT), indicated by a dotted arrow. **C:** Another preparation showing co-localisation of the mALT and the ventral-cord neurons. **D:** Schematic diagram of the co-localised neuron categories. The ventral-cord neurons could be traced to their somata, thus being descending neurons(DN).AL antennal lobe; P posterior; V ventral;OL optic lobe; SOG. Suboesphageal ganglia. Scale bars: 50 µm.

3.4 Additional observation

In an attempt to inject dye into the antennal lobe, some of the antennal nerve neurons, which descend to the ventral nerve cord, were stained(Figure 3.4)., This could be the mechanosensory neurons that project director from antenna to the ventral nerve cord(Dr. M Zhemchznikov, pers. Comm).

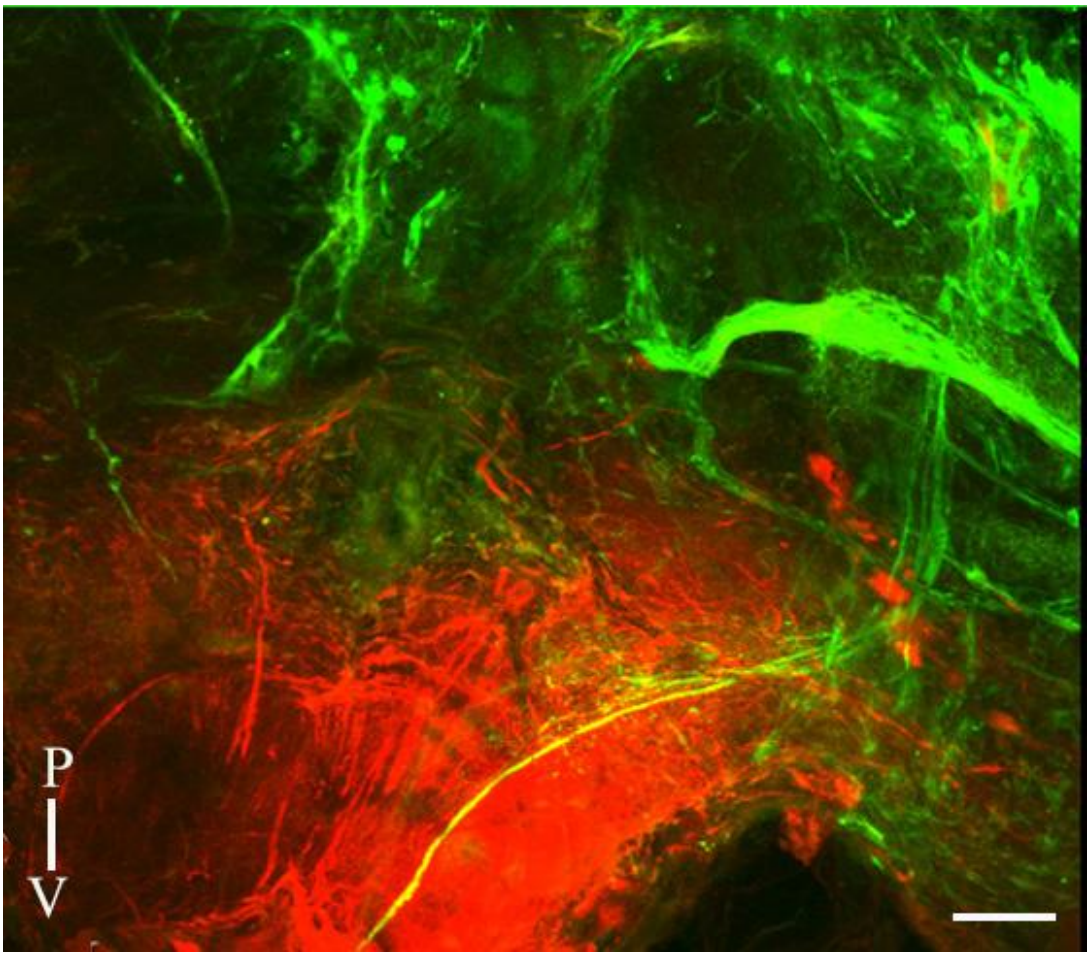


Figure 3.4: Sensory neurons from the antenna projecting to the ventral cord. P posterior; V. ventral. Scale 50µm.

4. DISCUSSION

The result of the present master thesis contributes to the knowledge about the organization of input and output regions in the lateral horn of the *heliothine moth, H. virescens.*The findings concerns possible overlap of antennal-lobe projection neuron axonal terminals and dendritic arborisations of ventral-cord neurons. The discovery of a descending neurons responding to a 10 component plant odor mixture (Lofaldli et al., 2012), formed the basis for performing double- labeling experiments of the antennal lobe neurons and the ventral nerve cord neurons.

4.1. Result summary

The successful staining of antennal-lobe projection neurons revealed the three main tracts, the medial antennal lobe tract (mALT), the mediolateral antennal lobe tract (mlALT) and the lateral antennal lobe tract (lALT). All these tracts targeted the particular region of the protocerebrum called the lateral horn. In addition, some preperations showed a fourth tract, the socalled 2nd mediolateral antennal lobe tract. As the main goal of the current study was to investigate whether a significant part of the 3rd order olfactory neurons projects to the ventral cord, labeling of the ventral-cord neurons was of particular interest. Among other stained regions in the brain, this revealed one characteristic neuropil structure in the ventro-lateral protocerebrum. However, successful double-labeling experiments showed no overlap of the stained antennal-lobe projection neurons and the ventral-cord neurons in the lateral protocerebrum.

4.2. Staining pattern of antennal- lobe neurons

The results from injection of fluorescent dye in the antennal lobe, clearly visualized the major antennal-lobe tracts, the mALT, the mlALT, and the lALT. The current result corresponds with previous findings in different species of moth, *H virescens, Helicoverpa assulta,* and *Munduca sexta* (Homberg et al., 1988; Ro et al., 2007; Zhao et al., submitted article). The present finding of a fourth tract in some preparations is also in agreement with the results from a previous study by (Lillevoll, 2013), As suggested by Lillevoll in her master thesis, the projection neurons confined to the current tract may innervate just a few glomeruli of the antennal lobe, explaining why it appears only in some of the stained preperations. The manual injection of dye in the antennal lobe depends on the visual and motoric skills, trying to

target with the tip of the glass electrode in the same position in the antenna lobe obviously hitting exactly the same place is difficult. The fourth tract called 2nd mlALT is reported to include PN axons carrying CO_2 information from the labial pit organ glomerulus (LPOG) in the antennal lobe as well (Dahl, 2013).

4.2.1. Projection pattern in the lateral horn

All the three major tracts projected to the lateral horn. The results showed that there are different projection patterns for the medial antennal lobe tract (mALT) in males and females. In males, two separate fibers bundles projected in two distinct areas of the LH, one antero-medially and the other postero-laterally. In females, however, only one fiber bundle projected to one continuous region in the LH. These findings are in agreement with previous results in other moths species including *H. virescens*. Thus, the mALT is shown to contain separate PNs mediating pheromone information and plant odor information (Homberg et al., 1988; Kanzaki et al., 2003, zhao et.al., submitted article). Whereas the finding of two distinct target regions for the male are in agreement with previous results in other moth species, the discovery of two separate fibers bundles leaving the calyces, as found here, has not been previously reported. Also in the LH of the fruit fly, *Drosophila melanogaster*, spatial separation of axon terminals of the pheromone and the plant odor projection neurons are demonstrated. Here, the pheromonal projection neurons terminate in the anterior ventral position of the LH and the plant odor projection neurons in the posterior ventral area (Jefferis et al., 2007). The segregation of the two types of projection neurons demonstrates that the information is separated in the LH, possibly meaning that odors are represented in the current brain region according to their behavioral significance. Actually, double labeling from the MGC and the ordinary glomeruli, respectively, was carried out in *H. virescens* by Lillevold in her master thesis (Lillevold, 2013) also demonstrating separate projection of the two kinds of PNs in the LH. Applying dye specifically in the MGC and the ordinary glomeuli is a challenging task and time did not allow for this kind of experiments in the current master thesis that had a different focus. However, to carry out more specific staining of the MGC and the ordinary glomeruli in order to visualize the distinct projection pattens associated with the two categories of antennal-lobe glomeruli and to compare these findings with corresponding data from females would be interesting in future experiments. The reason why the two lateral horn regions was visualized in the current study, including injection of only one fluorescent dye in the antennal lobe, was probably due to hitting both the MGC fibers and those

arborizing in the ordinary glomeruli during dye application, plus an optimal orientation of the brain preparation.

4.3. The main portion of odor information seems to be carried to the ventral cord from another region than the lateral horn

Labeling with two fluorescent dyes made it possible to distinguish the terminals/processes of the two neuron categories, antennal-lobe projection neurons and ventral-cord neurons, by using two-channel confocal scanning. The confocal images revealed no overlap of the projection–neuron terminals and the processes of ventral-cord neurons in the LH. This result indicates that the main portion of the odor information from the brain is not transmitted directly to the ventral cord, but is passed on via other brain interneurons taking part in processing the odor signals. Thus, the odor information is carried from the brain to motoric neurons in the ventral nerve cord via 4th order or higher order neurons.

In the fruit fly *D. melanogaster,* particular mapping experiments using photoactivable green fluorescent protein (PA-GFP) combined with electrophysiology and optical imaging, revealed that the pheromonal information is transferred from the lateral horn to the ventral nerve cord via 4th order neurons (Ruta et al., 2010). Here, the cell clusters of 3rd order olfactory neurons in the lateral horn were discovered. The neurons associated with the current cell clusters were shown to project to a particular region of the lateral accessory lobe called the lateral triangle. The lateral triangle in turn, possesses descending neurons (4th order) projecting to the ventral nerve cord (Ruta et al., 2010). Similarly, pheromone neurons projecting from the lateral accessory lobe to the ventral nerve cord have been physiologically and morphologically described in the silk moth, *B. mori* (Ryohei Kanzaki & Shibuya, 1986). However, the current neuron population has not been identified as 4th order neurons in the silk moth because the connections between the lateral accessory lobe and the lateral horn have not been established in this insect species.

It is still a matter of research whether there are additional brain regions harboring dendrites of odor neurons descending to the ventral nerve cord in the fruit fly and the silk moth. Noticeably, the descending neurons identified in the two species responded to pheromone information, i.e. 11-cis-vaccenyl acetate (cVA) in the fruit fly and bombykol in the silk moth. Interestingly, is in the *H. virescens* female, one plant-odor responding neuron projecting from the lateral protocerebum to the ventral cord has previously been physiologically and morphologically identified (Lofaldli et al., 2012). However, based on the

data from the current study, it can be concluded that the majority of descending neurons projecting from the brain to the ventral cord in the heliothine moth, including both pheromone neurons and plant odor neurons, is not constituted by 3rd order neurons passing from the LH.

4.4. One fiber bundle of ventral cord neurons project together with the lALT

Application of.two different fluorescent dyes, one in the antennal lobe and the other in the ventral cord, revealed tiling of two fiber bundles associated with the two regions, respectively – more precisely, a particular assembly of axons connected to the ventral nerve cord projected together with the lALT. The current staining method did not reflect whether the marked ventral-cord fibers belong to ascending or descending neurons. One of the macro distinguishing characteristics for such a categorization is the soma location. The labelled ventral-cord bundle was obviously connected with the characteristic spherical neuropil region in the ventro-lateral protocerebrum (see figure 3.21). No cell body cluster linked to the current processes could be observed in the brain preparations. This alone does not necessarily indicate that the current fiber tract belongs to ascending neurons. However, at least some of them may in fact be ascending since a population of sound-responding neurons in several heliothine species, *H. virescens* included, is reported to project from the ventral cord to the strongly stained region of the ventrolateral protocerebrum via this particular fiber bundle (Pfuhl et al., 2014). The projection pattern of individually stained sound neurons, being identified via intracellular recordings, coincides with the mass staining result from the ventral nerve cord. Furthermore, the joint projections of the two neuron categories have been formerly reported in the master thesis of Børø (Børø, 2012). some of the antennal-lobe projection neurons confined to the lALT are reported to extend short neural processes from their axons already immediately after leaving the antennal lobe (Homberg et al., 1988) Elena Ian, personal communication). However, whether there are any synaptic connections between the two-neuron bundles stained here is an open question.

The co-localisation of ventral cord neuron with the lAlT reveals how the nervous system is optimally organized within the small brain. There is also co-localisation of the mALT with fibers of ventral-cord neuron that have soma in the superior lateral protocerebrum, thus being descending neurons (Figure 3.23 and figure 3.32C

4.5. Fibers connecting the antenna to the ventral cord

In some preparations, a few stained fibers connecting the antennal nerve to the ventral cord was observed. A pair of similarly thick projections appearing at the antennal-nerve base

has been previously reported in *H. virescens* when applying dye to the ventral cord (Pfuhl et al., 2014). Furthermore, large diameter giant fibers of similar morphology have been identified in a number of insect species (Bacon et al., 1986). Whether the stained neurons are ascending or descending is not determined. However, they may be ascending, as these neurons were not observed when applying dye into the antennal nerve (Mihail Zhemzhuchnikov, pers. comm). The stained neurons may thus terminate at the base of the antennal nerve. Interestingly, in the honeybee, octopaminergic neurons having their cell bodies in the SOG are reported to project into the antenna (Schroter et al., 2007). The modulatory effect of the large stained neurons is not yet known.

4.6 Methodological consideration

The main aim of this investigation was to stain the projection neurons from antennal lobe and ventral-cord neurons for studying whether the two neurons categories had neural branches in overlapping regions of the lateral protocerebrum. Several different dyes were tested during the experimental work. Generally, microruby worked well both when applied into the antennal lobe and the ventral cord. As the most challenging part of the staining procedure was to stain from the ventral cord, being located relatively far from the protocerebral region of interest here, the microruby was applied in the ventral cord. The second dye used, which was applied into the antennal lobe, was micro-emerald. In spite of the lower photostability of this dye as compared to that of Alexa 488, it gave better results. Although the emission wavelength of micro-emerald overlaps with the autofluorescence of the moth brain, it gave good quality staining of the antennal-lobe tracts

The double-staining procedure always included initial labeling of the antennal lobe and then of the ventral cord. This because it would be difficult to fix the head of the moth after having exposed the ventral nerve cord. Altogether, a particular procedure for performing successful double-labeling from the antennal lobe and the ventral cord was established during the first period of the experimental period.

The insect that were kept overnight at the 4^{o}C showed good results. The quality of the staining was to a certain extent related to the duration allowing for dye transportation. The transportation of the dye also depends on its the molecular weight. Generally, dyes, which are lower in molecular weight, are transported faster than higher molecular weight dyes.

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5. CONCLUSION

1. Anterograde staining of the antennal-lobe projection neurons demonstrated three main tracts, all of which targeted the lateral horn.

2. Different projection patterns of the axons passing in the medial antennal-lobe tract were found in males and females; in males, the axon terminals targeted two distinct regions in the lateral horn whereas one continuous target area was found in females.

3. Staining of ventral-cord neurons resulted in visualization of distinct neuropil regions in the brain, one of which located in the ventro-lateral protocerebrum was of particularly interest in the current study.

4. Double-labeling experiments demonstrated no overlap of neural branches from antennal-lobe projection neurons and ventral-cord neurons in the lateral horn, meaning that the main portion of odour information is carried to the ventral cord via other neurons than the third order category arborizing in the lateral horn.

5. A particular fiber bundle connecting the ventral cord to the heavily stained region in the ventro-lateral protocerebrum projected tightly together with antennal-lobe projection neurons passing in the lateral antennal-lobe tract.

6. A fiber bundle of descending ventral-cord neurons projected relatively close to the medial antennal-lobe tract on its route towards the calyces.

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6. ABBREVIATION

1st mlALT- 1st Mediolateral antennal-lobe tract

2nd mlALT- 2nd Mediolateral antennal-lobe tract

AL-Antennal lobe tract

AMMC-Antennomechanical and motor center

AN- Ascending Neuron

CB- Central body

CC- Cervical connectives

DN- Descending Neuron

GABA- Gamma amino butyric acid

LAL- Lateral accessory lobe

lALT- Lateral antennal- lobe tract

LH- Lateral horn

mALT- Medial antennal-lobe tract

MBC- Mushroom body calyces

MGC- Macroglomerular complex

OG- Ordinary glomerulus

OL- Optic lobe

OsN- Olfactory sensory neurons

PN-Projection Neuron

VLP- Ventrolateral protocerebrum

VNC- Ventral nerve cord

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APPENDIX I

In order to obtain good optimization to have better method and also best use of the available resources, long trials are being performed. In this particular section the selection of insects, dyes, different method used are presented.

Insects

Three species of moth species were used in the experiment,

Heliothine virescens: 80 moths (37 males and 43 females), 2 moths of unknown sex at our lab

Helioverpa armigera 2 male moths at our lab and 14 moths at Nepal and

Silver Y moth (*Autographa gamma)*: 3 moths, sex not known, collected at field at Låde, Trondheim.

The experiment done at Nepal (at Kathmandu University) did not produce any significant results; the number of collection of moth species from the tomato field was hindered by rain.

Selection of dyes

Four dyes were used for good staining procedure.

1. Dextran tetramethylrodamine/biotin(3000MW; microruby; ext/emis: 490/508 nm)
2) Dextran fluorescein/biotin (3000 MW; microemerald; ext/emis:550/570nm)
3) Alexa fluor 488 (10000MW; ext/emis: 495/519nm)
4) Dextrantetramethylrodamine (3000MW, anionic, ext/emis: 550/570nm)

Six different combinations were used for dyes in two areas of brain (Antennal lobe and cerebral connectives).

Combination a. Alexa in AL and microruby in cerebral connectives(CC): 4 preparations
Combination b. Alexa in CC and microruby in AL: 8 preparations
Combination c. Tetramethylrodamine in AL and Alexa in CC: 2 preparations
Combination d. Tetramethylrhodamine in CC and Alexa in AL: 3 preparations
Combination e. Microruby in AL and Microemerald in CC: 5 preparations
Combination f: Microruby in CC and Microemerald in AL: 45 preparations

In the above combinations, microruby in cerebral connectives and microemerald in antennal lobe best work.

APPENDIX II

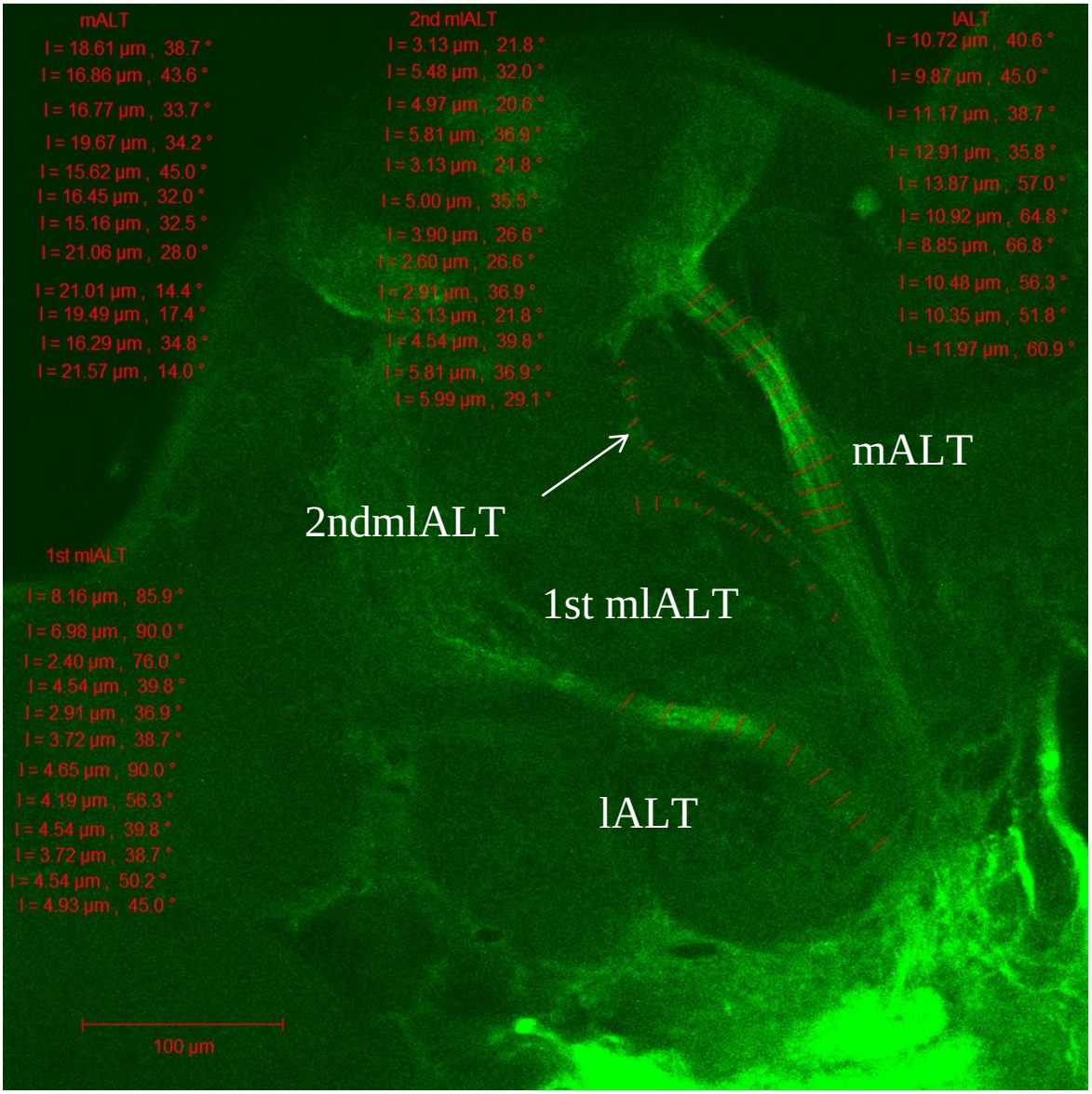

Figure A1: Confocal image of dorsally oriented brain of male moth. The diameter of the three anxonal tract, the medial antennal lobe tract (mALT), 1st mediolateral antennal lobe tract (1st mlALT), 2nd mediolateral antennal lobe tract (2nd ml ALT), and lateral antennal lobe tract (lALT). The diameters are measured from the lsm image brower brower directly and indicated here in the picture

APPENDIX III

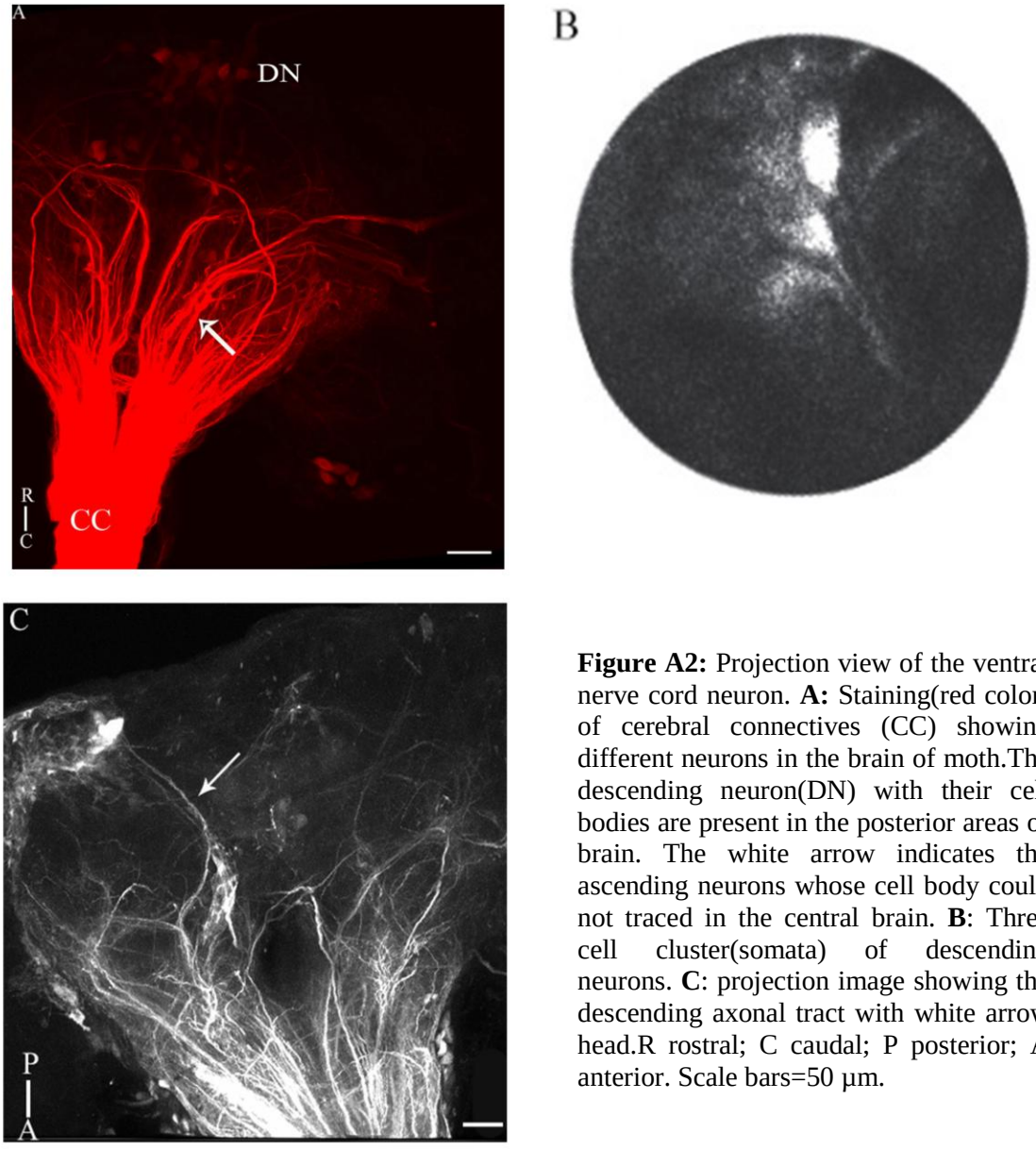

Figure A2: Projection view of the ventral nerve cord neuron. **A:** Staining(red color) of cerebral connectives (CC) showing different neurons in the brain of moth.The descending neuron(DN) with their cell bodies are present in the posterior areas of brain. The white arrow indicates the ascending neurons whose cell body could not traced in the central brain. **B**: Three cell cluster(somata) of descending neurons. **C**: projection image showing the descending axonal tract with white arrow head.R rostral; C caudal; P posterior; A anterior. Scale bars=50 µm.