Morphological characterization of antennal-lobe projection neurons in the olfactory pathway of heliothine moths

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Master's thesis in Neuroscience

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Submissioin date: June 2015

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Preface

This Master's thesis has been written at the Department of Neuroscience at the Norwegian Univercity of Science and Technology (NTNU), Trondheim. All parts of this experimental study have been carried out at the Chemosensory Laboratory at the Department of Psychology. The experimental work was conducted in close collaboration with another master's student, namely the lovely miss Ann Charlen Sveum. Our masters' projects have to a large degree been similar in method, but different in aim. This highlights the rationality of working closely together during the experimental parts of our studies, and explains why miss Sveums' method section in effect might be very similar to my own. Using the same experimental method, miss Sveum has been collecting local interneurons of the antennal-lobe, while I have been collecting antennal-lobe projection neurons. It has thus been convenient and fruitful to share results, and explains why Table 1 in the results section is presented as a cooperative effort.

Preliminary Acknowledgments

Firstly, I would like to thank my supervisor, Professor Bente Gunveig Berg, for her continuous support throughout this year, and for her apposite philosophy of "learning through failing". It has been a privilege and an inspiration to be able to learn and work under the guidance of such a genuine, knowledgeable, and positive spirit. I would also like to thank my co-supervisor, Elena Ian, for showing me how to perform all of the lab procedures, and for always being positive and welcoming; and my partner in moth crime, Ann Charlen, for working closely together with me this entire year, and for making it that much more fun!
To all the other people working in the lab this year, especially Professor Emeritus Hanna Mustaparta, thank you for fascinating conversations and for sharing your knowledge.

In addition I would like to thank my family for always supporting and believing in me, and particularly my sister Cathrine for going moth-hunting with me, even though she probably had better things to do. To my partner Eirik, thank you for your love, support, and for putting up with me.

Abstract

The olfactory sensory system is a phylogenetically old system highly important for both the survival and reproduction in most animals. Its organization and function shows striking similarities across phyla, making it possible to study its general underlying properties through the use of different species. The moth has proven to be a particularly suitable model organism for studying this system, as it has an exceptionally well-developed sense of smell, and a relatively moderate brain complexity. In this thesis, the part of the central olfactory pathway forming connections between the primary brain center and higher processing centers has been studied through the use of the heliothine moths *Heliothis virescens* and *Helicoverpa armigera*. Second-order olfactory projection neurons have been labeled through a dual electrophoretic staining technique, where one approach targeted the dendrites of projection neurons in the antennal-lobe, and one targeted the projection neuron somata confined within the lateral cell cluster of the antennal-lobe. In addition to complementing previous reports on morphological characteristics typifying antennal-lobe projection neurons confined within three primary antennal-lobe tracts, the present study contributes new findings previously not described in heliothine moths.

Table of contents

Introduction 1
What the sense of smell means to us 1
Pheromones 1
Similarities across phyla 2
The moth olfactory system 3
The antennae and olfactory sensory neurons 3
The antennal-lobe 3
Antennal-lobe circuitry 4
The antennal-lobe tracts 5
The mushroom body calyces and the lateral horn 6
The human olfactory system 7
The olfactory epithelium and olfactory sensory neurons 7
The olfactory bulb 8
The olfactory cortex 9
Neural coding and the odotopic map 9
Expanding knowledge by using model organisms 10
Main aim of the thesis 11
Materials and Method 12
Insects 12
Ethics 12
Preparation of model organsims 13
Preparation for intracellular recording and electrophoretic staining 14
Staining from the lateral cell cluster 14
Staining from the dendrites of antennal-lobe neurons 14
Dissection 15
Dehydration and mounting 15
Confocal microscopy 15
Intensification and immunohistochemistry 16
Image processing 17
Results 18
Cataloguing 18
Staining from the somata in the lateral cell cluster versus dendrites in the glomerular region 19
Arborization and innervation patterns of successfully stained projection neurons in the three ALTs 19
Projection neurons in the lALT 19
Projection neurons in the mALT 20
Projection neurons in the mlALT 20
Immunohistochemistry of preparations 21
Figures 22
Discussion 32
Morphology of mALT projection neurons 32
Morphology of lALT projection neurons 34
Morphology of single mlALT projection neuron 36
None-overlapping innervations in the LH 36
Parallel tracts organization 37
Methodological considerations 39
Conclusion 41
Abbrevations 42
Literature cited 44

Introduction

The sense of smell (olfaction) provides humans and other living organisms alike with the ability to detect and respond to the rich information held within the realm of airborne molecules in the environment. The stimuli detectable through the sense of smell are small, airborne, volatile molecules called odorants, capable of influencing the behavior of both vertebrates and invertebrates. Odorants can carry vast amounts of information, for instance about the location and safety of food, the presence of predators or competitors, and the proximity or location of a potential mating partner. The ability to detect chemical compounds thus plays a key role in both survival and reproduction for most animals.

What the sense of smell means to us

For human beings, the most prominent and informatory aspect of smell relates to food (Finger et al., 2000). We have all experienced being drawn towards the pleasant smell of freshly baked cookies in a bakery, or a newly brewed cup of coffee early in the morning, luring us in an almost seductive way, producing reflexes like salvation, and preparing our bodies for food consumption. On the other hand, most of us have also experienced the highly unpleasant smell of spoiled or rotting food (methane), which effectively elicits nausea, and if potent enough, even the gag-reflex. These reflexes are highly adaptive and advantageous, effectively repelling most hazardous foods, while drawing one towards compounds of high nutrition. However beneficial, the importance of this ability rarely becomes apparent to us unless we are deprived of it. General anosmia (complete lack of olfactory sensation) or hyposmia (diminished sense of smell) have been experienced by most of us as a transient symptom of the common cold (Brodal, 2009; Kandel et al., 2013), where it effectively renders any kind of food or beverage tasteless, unattractive, and dull. This is because olfaction, together with the sense of taste, is what gives rise to the perception of flavor. Loosing this highly enriching aspect of daily life, however briefly, is what sometimes makes us rightfully appreciate the significance of this sense.

Pheromones

For many organisms, like mammals, fish, and insects, a second aspect of the olfactory stimuli is highly valuable, namely that which carries with it specie-specific information. These olfactory compounds, called pheromones, are used as means of interspecies-specific

communication, influencing both the behavior and physiology of individuals of the same species (Kandel et al., 2013; Reece et al., 2011; Wyatt, 2014). Pheromones are released through excretory glands or blended with urine, and can among other things inform animals about territorial boundaries, injuries of conspecifics, the whereabouts of competitors, or the proximity of a potential mating partner. In addition, pheromone signals can more directly affect an individual's physiology through modulating its levels of reproductive hormones, stimulating to sexual behavior and/or to aggression. Many animals, both vertebrates and invertebrates, have specialized receptor organs exhibiting great accuracy for pheromone detection, with humans being one of the exceptions lacking such a structure. For instance, the male silkworm moth can detect the pheromones excreted by a female moth from kilometers away (Reece et al., 2011), and use this information to effectively navigate to her.

Similarities across phyla

Through the study of animal development and evolution the ability of chemosensation has proven rudimentary, as it together with the sense of touch is the phylogenetically oldest sense, present in some form even in all unicellular organisms (Roth, 2013). The preservation of this sense illustrates that the invisible, but rich, dimension provided by olfactory stimuli has been, and continues to be, of vital importance throughout the animal kingdom (Haupt et al., 2010).

Research on the olfactory sensory system has uncovered that several striking similarities exist between different species when it comes to both the organization and the encoding mechanisms (Bear et al., 2007; Kandel et al., 2013). Within the field of neuroscience, insects, and especially moths, have proven to be highly valuable as model organisms for understanding the fundamental mechanisms of olfaction. The insect sense of smell is particularly well developed, and the relatively low number of neurons in the insect brain (10^5-10^6) compared to that of the humans brain (10^{11}) makes the neural circuitry much easier to decipher and study (Haupt et al., 2010). In addition to the moderate complexity of their brain, insects are also highly attractive model organisms because of the accessibility of their nervous system, their rich behavioral repertoire, and the low maintenance cost.

The moth olfactory system

The antennae and olfactory sensory neurons

The antennae are the olfactory sensory organs of insects. On them, the odorants are detected by the dendrites of specialized olfactory sensory neurons (OSNs), bipolar nerve cells with specialized chemosensory receptors for odorant detection, encased within cuticular hairs called sensillum. The antennae can carry up to 200,000 olfactory sensilla, where each sensillum houses dendrites from 1-30 OSNs (Finger et al., 2000; Roth, 2013). The cuticular hairs are filled with sensillar fluid/ lumen, which contains odorant-binding proteins and odorant-degrading enzymes believed to be involved in "presenting" the odorants to the odorant receptors, and in clearing already processed odorants from the receptor cite, respectively (Finger et al., 2000). Once an odor signal is detected by an olfactory receptor, the information is transduced into an electrical signal by the OSN and transmitted via its axon to the primary olfactory center of the insect brain, namely the antennal lobe (Haupt et al., 2010). The descending axons from all the OSNs in the antenna create a neuronal bundle termed the antennal nerve (Finger et al., 2000).

The antennal-lobe

The antennal-lobe (AL) in insects represents the discrete brain region where olfactory information from the periphery is first processed. In the AL, the terminal branches of OSN axons make their first synapses in conspicuous, spherical, neuropilic structures called glomeruli (Haupt et al., 2010; Kandel et al., 2013). In each glomerulus, the axonal branches of the OSNs make synaptic connections with neurites from several second-order olfactory neurons (Rø et al., 2007). In male moths, the AL is divided in two glomerular subregions: the macroglomerular complex (MGC), which is devoted to the processing of pheromone information, and the ordinary glomeruli (also present in females), which is devoted to processing information about general odors (Anton & Homberg, 1999). In the heliothine moth H. *virescens,* there is a total number of 66 glomeruli in the AL, including 62 ordinary and 4 MGC glomeruli (Berg et al., 1998, 2002). The females of this particular species contain, in addition to a similar number of ordinary glomeruli, two sexually dimorphic glomeruli called the large female glomeruli (LFG) (Berg et al., 2002).

Antennal-lobe circuitry

In the AL of insects, three distinct types of second-order olfactory neurons converge on the glomeruli; namely local interneurons, projection neurons, and centrifugal neurons (Homberg et al., 1988; Rø et al., 2007). Local interneurons are confined to the AL, and are typically multiglomerular, connecting many or all glomeruli, or a smaller group of glomeruli. In moths, most local interneurons are global and GABAergic (inhibitory) (Berg et al., 2009; Hoskins et al., 1986; Marin et al., 2011; Seki & Kanzaki, 2008), and thought to mediate lateral inhibition that sharpens the contrast between active and silent glomeruli (Christensen et al., 2001; Haupt et al., 2010; Mustaparta, 2002). Projection neurons innervate one or more glomeruli with their dendrites and send their axons to various sites in the higher brain areas of the ipsilateral protocerebrum (Homberg et al., 1988). In most species of moths, the AL projection neurons send their axons through one of three distinct antennal lobe trackts (ALTs) (Homberg et al., 1988; Rø et al., 2007), the medial-, mediolateral-, and the lateral antennal lobe tract (terminology adopted from Ito et al., 2014), which will be addressed in the next section. Centrifugal neurons are thought to provide modulatory feedback, affecting the AL function with regard to appetitive, circadian, or associative information from other brain areas (Martin et al., 2011). While centrifugal neurons usually have their dendrites and cell bodies located outside the AL, local interneurons and projection neurons have their somata located in one of two cell clusters confined to the AL, namely in the lateral cell cluster (LC), or in the medial cell cluster (MC) (Fig. 1). In certain moth species (H. *virescens* and *Manduca Sexta)* a third cell cluster has been found, namely the small anterior cell cluster (AC), which also holds somata of a few projection neurons (Homberg et al., 1989; master thesis of Alexander Berg, 2014).

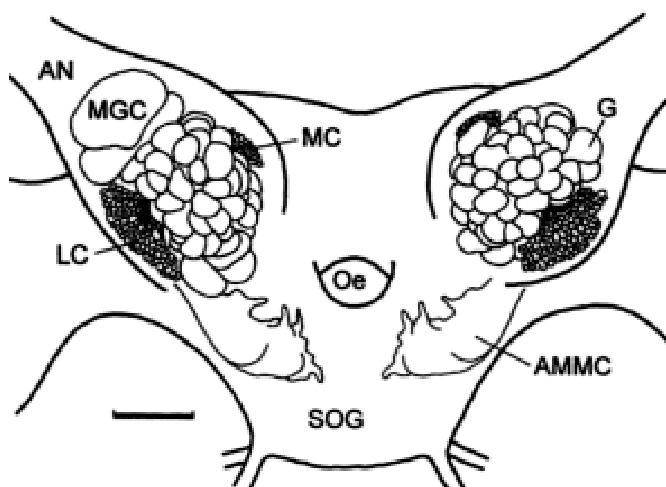

Fig 1. Schematic drawing of the moth antennal lobes in a frontal view (male right, female left), showing the antennal nerve (AN), the macroglomerular complex (MGC), the lateral cell cluster (LC), the medial cell cluster (MC), a glomerulus (G), the antennal mechanosensory and motor center (AMMC), the suboesophageal ganglion (SOG), and the oesophageal canal (Oe) (from Anton & Homberg, 1999).

The antenna- lobe tracts

The AL projection neurons send information to several district higher brain centers in the protocerebrum, most notably to the mushroom body calyces and to the lateral horn (LH) (Finger et al., 2000; Homberg et al., 1988; Rø et al., 2007). The information sent via these projection neurons follows, as mentioned, three distinct tracks in most moth species, which end up in various sites within these two higher brain areas (Fig. 2). Projection neurons that send their axons through the medial antennal-lobe tracts (mALT) are primarily uniglomerular in their dendritic arborizations, and exit the AL in either the dorsal- or ventral root (Homberg et al., 1988; Rø et al., 2007). Neurons exiting through the dorsal root have cell bodies located in the MC and the AC, while those exiting through the ventral root has somata located in the LC. From the AL, projection neurons of the mALT exit dorsomedially, run posteriorly in the protocerebrum, and pass the lateral edge of the central body before turning laterally towards the mushroom body calyces (Homberg et al., 1988). Here they send some innervating branches into the calyxes' neuropils, and continue laterally until they reach and terminate in the LH (Rø et al., 2007). The projection neurons sending their axons through the mediolateral antennal-lobe tract (mlALT) are primarily multiglomerular, and have their cell bodies located in the LC (Homberg et al., 1988; Rø et al., 2007). The mlALT is a considerably smaller tract, with fewer neurons than the prominent mALT. It leaves the AL dorsomedially, where it coincides and follows the mALT trajectory until it turns laterally at the level of the central body. From there it projects directly into the lateral protocerebrum, where it divides into smaller branches, each innervating slightly different areas of the LH. Projection neurons that send their axons through the lateral antennal-lobe tract (lALT) are both uni- and multiglomerular, and have their cell bodies located in the LC (Homberg et al., 1988; Rø et al., 2007). The fibers in this tract exit the AL more ventrally than the two other tracts, and turn laterally towards the lateral protocerebrum, where they branch out and terminate in the LH. Some fibers from this tract have also been shown to continue dorsomedially from the LH and innervate the calyces (Rø et al., 2007).

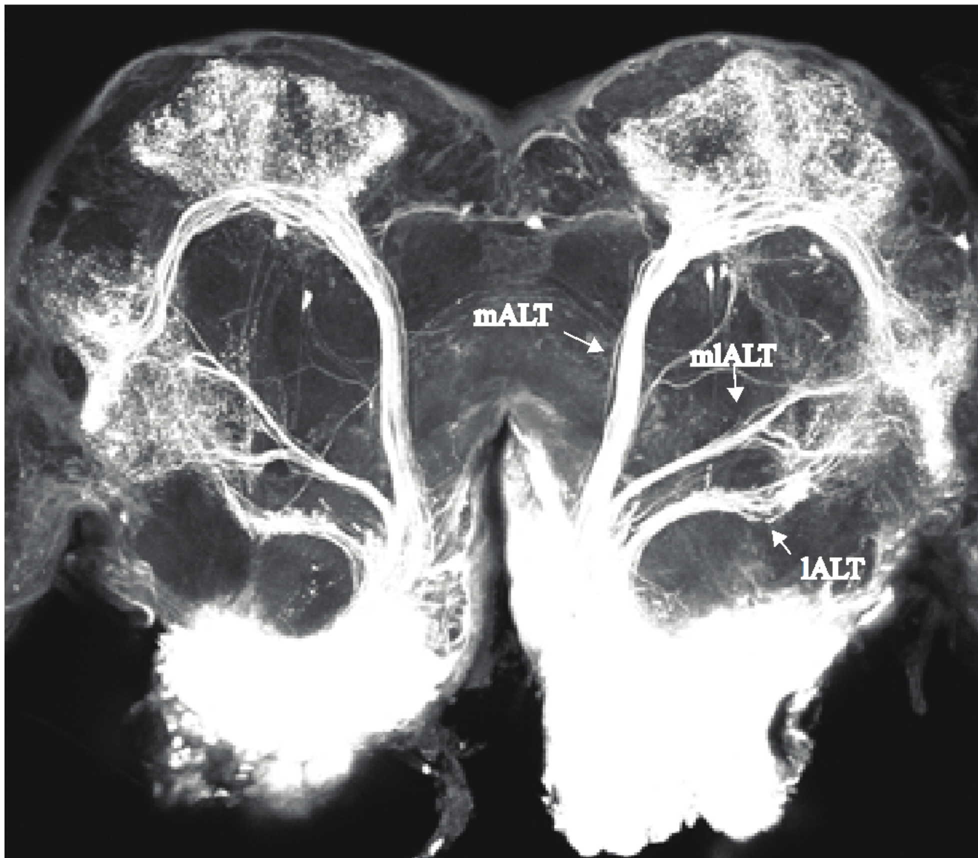

Fig 2. Displaying the three antennal lobe tracts (mALT, lALT, and mlALT) in the moth *Heliothis virescens* (Unpublished data from Xin-Cheng Zhao).

The mushroom body calyces and the lateral horn

The main target areas of the neurons in each ALT are the mushroom body calyces and the LH (Fig.3). The mushroom body calyces are prominent neuropilic structures located posteriorly in the protocerebrum, and consist of Kenyon cells dendrites arranged in two cup-shaped structures, namely the medial and the lateral calyx (Roth, 2013), plus incoming terminal branches form the AL projection neurons. The calyx structures are regarded as one of the highest centers in the insect brain, and are widely known for being essential for short-term memory in odor discrimination tasks and in multimodal associative learning (Haupt et al., 2010; Heisenberg, 1998; Rø et al., 2007). The cell bodies of the Kenyon cells are tightly packed around the calyces, while their axonal branches form the peduncle and lobes that connect the mushroom bodies to other higher order brain areas. The LH is defined as the area in the lateral protocerebrum that receives terminal projections from AL projection neurons (Fig. 3) (Ito et al., 2014). This area is presumed to be more closely connected to the motor system than the mushroom body calyces, and is regarded as an important multimodal

integration center (Christensen & Hildebrand, 1987). However, due to a lack of distinct neuropilic structures in this area, its purpose and function is still under debate.

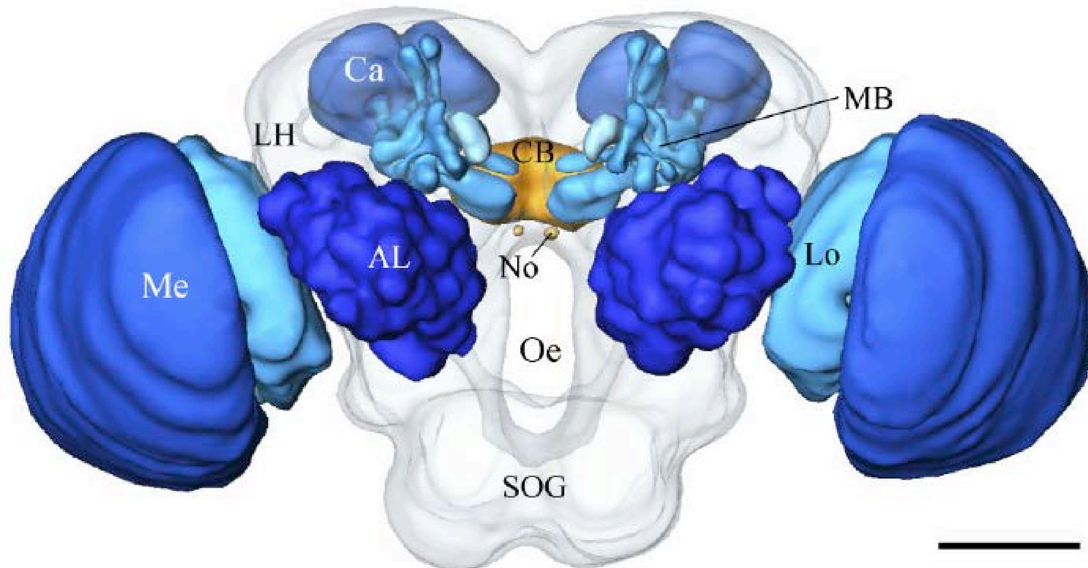

Fig 3. Neuropil atlas of the moth brain of *Heliothis virescens,* frontal view (from Rø et al., 2007).

The human olfactory system

The moth olfactory system shows striking similarities to that of the humans when it comes to both functional organization and physiology, suggesting that the olfactory information is processed through similar neural mechanisms (Hildebrand, 1996). This makes it possible to draw important parallels between the two, and to transfer knowledge about one system to the other.

The olfactory epithelium and olfactory sensory neurons

In the olfactory system of vertebrates odorants are sensed through OSNs embedded in the olfactory epithelium in the nasal cavity (Bear et al., 2007; Brodal, 2009; Kandel et al., 2013). The olfactory receptors of the OSNs are placed on tiny hair-like structures called cilia, which extend from the OSNs dendrite throughout the mucous-covered epithelium to serve as a binding site for incoming odor molecules. The odorants are dissolved in the mucous layer, which enables them to connect with odorant-binding proteins that carry the molecules to the olfactory receptors on the cilia. At the basal end of the OSN an axon is extended through perforations in the scull (the cribriform plate), where they form synapses in the first relay

station of the olfactory pathway (an area equivalent to the AL in insects), namely in the olfactory bulb (Fig. 4). The axons of the OSNs projecting to the olfactory bulb collectively form cranial nerve 1 (the olfactory nerve).

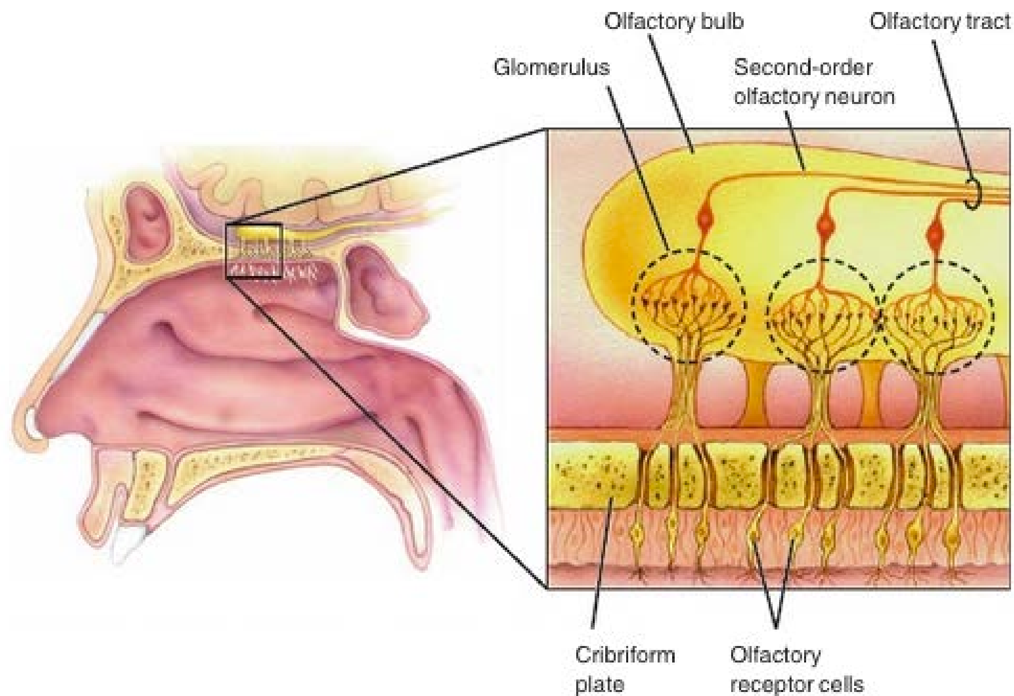

Fig. 4: Schematic drawing of the location and structure of the olfactory bulb and olfactory epithelium in humans (from Bear et al., 2007, p 270).

The olfactory bulb

In the olfactory bulb the terminal branches of axons make synapses in similar structural units as in insects, namely in glomeruli. In mice there are about 2000 glomeruli in each olfactory bulb (human beings are expected to have fewer), where each glomerulus contains and get information from the output terminals of about 25,000 OSN axons (Bear et al., 2007). In the glomerulus, these OSN output areas make synaptic connections with the dendrites of about 50-100 second-order olfactory neurons. These second-order neurons consist of the same three neuronal categories that are found in the insect AL, namely of local interneurons (periglomerular- and granular cells), which are confined to the olfactory bulb; projection neurons (mitral and tufted cells), which project their axons through the olfactory tract (the human equivalent of the moth ALTs) to relay the olfactory information to higher brain areas, and modulatory centrifugal neurons.

The olfactory cortex

From the olfactory bulb, mitral- and tufted cells relay the incoming olfactory information to higher brain areas in the temporal lobe through the olfactory tract. These areas primarily consist of the anterior olfactory nucleus, the olfactory tubercle, the piriform cortex, the amygdala, and the entorhinal cortex. From there the information is further mediated to several other brain areas, i.e. the hypothalamus, the hippocampus, the thalamus, and the orbitofrontal cortex (Brodal, 2009; Kandel et al., 2013). The piriform cortex is regarded as the major olfactory cortical area, which holds synaptic connections between mitral- and tufted cells and pyramidal neurons, the primary projection neurons of the brain. From the piriform cortex, pyramidal neurons project to subcortical and neocortical areas, as well as receiving and mediating modulatory input through centrifugal neurons (Kandel et al., 2013).

Neural coding and the odotopic map

Several similar key features of neural coding can be distinguished in the olfactory system of both humans and moths. In both systems, each OSN expresses only one or sometimes two functional odorant receptor genes, and therefore each OSN has only one olfactory receptor type embedded in its membrane (Kandel et al., 2013; Mustaparta, 2002). Each of these distinct olfactory receptor types recognizes several different odorants, and each odorant is recognized and elicits a response in several different olfactory receptor types, although not with the same intensity. Furthermore, the axons from OSNs that express the same type of odorant receptor converge on the same glomerulus in the AL, or on a few glomeruli closely located in the olfactory bulb, such that each glomerulus is characterized by the chemoprofile of one receptor type (Kandel et al., 2013; Roth, 2013). This means that the recognition of an odor is based on a distinct constellation/set of glomeruli being activated in both systems, which again produce a distinctive signaling pattern that is further relayed to higher brain areas. The astonishing level of accuracy and information segregation displayed in both the olfactory bulb and the AL (called the odotopic map) is perhaps the most unique and intriguing aspect of the olfactory sensory system (Kandel et al., 2013).

Gathering more specific information about this system has proven difficult since it requires the identification of biologically relevant odor stimuli. In humans, most of these odor substances are not known, making it difficult to elicit a response from specific receptor proteins, and thus to describe the system at such a level of detail. In heliotine moths however, a number of biologically relevant odor substances have been identified (both pheromones,

interspecific signals, and plant odors), and can thus be used to characterize OSNs according to their response to specific stimuli. Electrophysiological registrations using biologically relevant stimuli in heliotine moths have provided valuable data indicating that each OSN in the antenna mainly responds to one particular key substance (Berg et al., 2014; Mustaparta et al., 2002). This points towards a higher level of specificity in the olfactory system than what has been uncovered while studying other organisms, and highlights the advantage of using heliotine moths as a means to study the sense of smell.

Expanding knowledge by using model organisms

The fact that so many underlying properties are shared between the olfactory sensory system of vertebrates and invertebrates, humans and moths included, is quite astonishing. Even though the high uniformity and conservatism within these systems is most likely a result of independent adaptation within different phyla (Roth, 2013), important aspects can be discovered about the basic and underlying properties of the olfactory sensory system through the study of model organism such as moths. Any new discovered knowledge, no matter how small or seemingly insignificant, is an important piece of the puzzle and will eventually lead to a greater understanding of this essential sensory system. By the use of heliothine moths, the part of the central olfactory pathway forming connections between the primary brain centers and higher processing regions will be studied in this thesis.

Main aim of the thesis

To be able to uncover the principles underlying the olfactory sensory system, it is imperative to know the morphology of principle second-order neurons relaying the information from the primary olfactory center to higher order processing areas. Therefore, the main aim of this thesis is to investigate the morphological characteristics typifying different antennal-lobe projection neurons in heliothine moths.

Specific goals of this study:

- To map individual projection neurons confined within the main antennal-lobe tracts.
- To investigate the glomerular arborizations and innervations in higher order olfactory centers of different antennal-lobe projection neurons.
- Establishing a new method for labeling projection neurons that are underrepresented in current documentations of the moth olfactory system, by targeting the somata in the lateral cell cluster.

Materials and Method

Insects

Both males and females of the two moth species *Heliothis virescens* (Lepidoptera; Noctuidae, Heliothinae) and *Helicoverpa armigera* (Lepidoptera; Noctuidae, Heliothinae) were used for the purpose of this experimental study. The *H. virescens* moths were obtained from our own lab culture, where the very first larvae were sent from Bayern (Crop science, Germany). They were kept in a climate incubator (BINDER, GmbH, Tuttlingen, Germany) at 27°C and 66% air humidity, and fed with an artificial diet until pupation. H. *armigera* was sent from China and delivered to our lab as pupae. Pupas from both species were sorted by gender and kept in separate hatching chambers (18 x 12 x 17cm) in two climate cabinets (Refitherem 200, Struers-Kebolab, Albertsund, Denmark). The climate cabinets were set at a temperature of 22-24°C and a reversed night and day photoperiod cycle of 14h light and 10h dark. Fully hatched the moths were sorted by gender, age, and species into cylindrical Plexiglas containers (18 x 10cm) with perforated lids and fed a 10% sucrose solution.

Ethics

According to the Norwegian law of animal welfare (Dyrevernsloven) there are no restrictions regarding experimental use of Lepidoptera. The law includes animals such as reptiles, amphibians, decapods, and honeybees, but the particular order of insect used in this experimental study is not subject to any limitations. Nevertheless, lawful restrictions are not the only factors to be considered when working with live subjects, however low in order. The animal welfare act also states that: "all animals have intrinsic value independent of the utility value they may have for humans" (http://www.nspca.no/egenweb/lov.htm). In thread with these regulations and ones own consciousness, the insects were well cared for and protected from any unnecessary harm or stress to the best of our ability. The environment in which they were kept was checked upon daily, kept clean, and stocked with sucrose solution for food. In addition each chamber was equipped with sheaths of paper for the moths to climb on. Also, the number of individuals kept in each chamber was restricted to reduce any environmental stress related to overcrowding.

Preparation of model organisms

To sedate and relax the insect, it was first put in a refrigerator (4°C) for 5-10 minutes prior to any preparatory handling. It was then immobilized in a plastic tube with its head and antennas protruding. Malleable dental wax was placed around its head, further fixating the insect, before fur and cephalic scales between the antennas were removed with forceps and wet paper. Once placed under a stereomicroscope (Leica; MZ 12.5) an incision was made using a fine razor knife to loosen and remove the cuticle between the eyes, exposing the brain in a dorsal view. The proboscis, palps, esophagus, intracranial muscles, and some of the trachea were then removed to further expose the antennal lobes and eliminate movements. A thin wolfram alpha needle was then thread into each of the eyes preventing them from collapsing inwards and obstructing the visibility of the brain. Using fine forceps, the antennal lobes were desheated to ease insertion of the glass electrode. Ringer solution with sucrose (NaCl: 150mM, KCl: 3mM, TES buffer: 10MM, $CaCl^2$: 3mM, 25mM sucrose, pH: 6.9) was administered through a plastic pipette continuously throughout the procedure to keep the neural tissue alive and the brain hydrated.

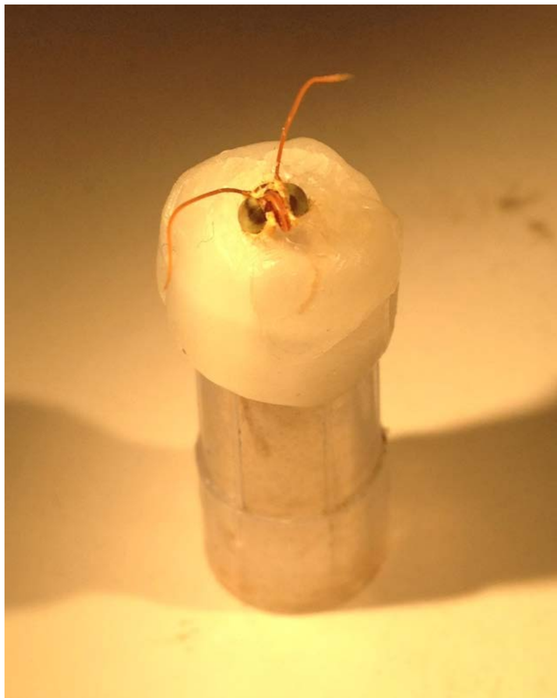

Fig. 5: Showing the insect immobilized in a plastic tube and how the brain is exposed through preparation.

Preparation for intracellular recording and electrophoretic staining

A flaming horizontal puller (Sutter Instruments Co, USA) was used to make the conducting glass electrodes. A preprogrammed setting was used, ensuring that all electrode tips had the same measurements (Program 47, Heat: 330, Pull: 80, Velocity: 50, Time: 250) prior to insertion. The electrode was dipped in the fluorescent dye dextran tetramethylrhodamine-biotin (micro-Ruby, Invitrogen, Germany) or dextran fluorescein/biotin (micro-Emerald, Invitrogen, Germany) and back-filled with 0.2M K^+-acetate. The electrodes had a resistance of 100-350MΩ. A reference electrode was inserted through the eye into the hemolymph, ensuring a full current circuit. Together with an oscilloscope (Tektronix 5111A, Oregon, USA), a computer software program (Spike 2, version 7, program 5, CED) was used to view the fluctuations and to initiate the actual staining procedure, delivering the electrical pulses through an amplifier (Axon Instruments, AXOPROBE-1A) in a setup for ionophoretic staining. This setup also consists a floating table (TMC, USA), a microscope (LEICA, MC APO), and a speaker (Monacor, MAB-30AK). Two different staining methods were used; one aiming for the PN cell bodies located in the lateral cell cluster, and one aiming for the PN dendrites within the antennal lobe. Each preparation was stained at least twice (once in each antennal-lobe) and up to four times when both microruby and microemerald was used.

Staining from the lateral cell cluster

The electrode was inserted as accurately as possible using a mechanical micromanipulator (Leica) into the lateral outer rim of the antennal lobe, the area known to hold the lateral cell cluster. This cell cluster contains somata from all the different projection neurons in the antennal lobe. An oscilloscope was used to guide the insertion and ensure that the electrode penetrated the brain tissue. If there were no recognizable fluctuations in electrical activity a current of 10nA was applied and delivered through the amplifier in 200ms pulses at 1Hz for 20 minutes. If recognizable, depolarizing electrical fluctuations were observed, indicating that the electrode had penetrated a neuronal membrane, a current of 0.7nA was delivered in 200ms pulses at 1Hz for 20 minutes.

Staining from the dendrites of antennal lobe neurons

Guided by microscopy and oscilloscope readings, the electrode was inserted into the medial or upper part of the AL using a micromanipulator. When the oscilloscope showed spiking activity, the current was set to 0.7nA and delivered through the amplifier in 200ms pulses at

1Hz for 20 minutes. If the oscilloscope showed hyperpolarized spikes, indicating that the electrode was situated outside the neuronal membrane, a buzz delivering a small depolarizing current or a manual knock on the table was administered to break through the membrane before staining.

Dissection

After the staining procedure, the insects were kept in a moist and dark container for either two hours in room temperature, or overnight in a refrigerator (4°C), to allow for the dye to move from the cell body to the distal parts of the neuron by anterograde transportation. Following this, the antennas were removed using micro-scissors before decapitation. The head with the intact brain was then fixed in wax in a small glass dissection bowl and covered with Ringer solution. The retinas, leftover trachea, and cuticles were removed with a fine razor knife and fine forceps. Fully dissected with intact eye lobes the brain was submerged in 4% paraformaldehyde (Roti, histofix) in a small glass bottle, where it was left for 2 hours in room temperature on a rotator, or over night in a refrigerator, to fixate the brain structures and to prevent neuronal degradation.

Dehydration and mounting

After fixation the brain was washed in phosphate buffered saline (PBS; NaCl: 684mM, KCl: 13mM, Na2HPO2: 50.7mM, KH2PO4: 5mM, pH 7.4) for 10 minutes to remove any leftover fix solution. Furthermore, the brain was dehydrated in an increasing ethanol series (50%, 70%, 90%, 96%, and 2 x 100%), where it was submerged for 10 minutes during each step. The brain was then cleared in methyl salicylate (methyl 2-hydroxybenzonate) and mounted as a whole in between thin glass slides tightly sealing a compartment in a metal-plate.

Confocal microscopy

Preliminary viewings of the mounted brains were done using a light microscope (Leitz Aristoplan, Wetzlar, Germany). However, weakly stained neurons or very thin neurons were often not visible through the light microscope. Thus, all of the mounted brains were also checked using a confocal laser-scanning microscope (LSM 510 Meta Mira Zeiss 900F, Jena, Germany). The successfully stained preparations were then scanned as a whole using dry-lens objectives (10 x 0.3 or 20 x 0.5; Plan-Neofluar). The brains were illuminated by a helium neon laser (543nm) and/or an argon laser (488nm), exciting the tetramethylrodamine and the dextran fluorescein, respectively. A resolution of 1024 x 1024 pixels, a pinhole size of 1 airy

unit, and a section distance of 2µm were used in scanning the preparations. The level of excitation used depended on the strength of each individual staining (40-90%).

Intensification and Immunohistochemistry

Intensification. The preparations containing successfully stained antennal-lobe projection neurons were put through a process to intensify the staining. The preparations were first rehydrated through a decreasing ethanol series (2 x 100%, 96%, 90%, 70% and 50%) for 10 minutes each, before they were rinsed with PBS for 10 minutes and incubated for 2 hours in room temperature in streptavidin-Cy3 (1:200, in 0.1M PBS Ph 7.2). Streptavidin binds to biotin, which is a constituent of both microruby and microemarald. The preparations were washed again in PBS for 10 minutes, before they were dehydrated again in an increasing ethanol series (50%, 70%, 90%, 96% and 2x 100%) for 10 minutes each. Finally they were cleared and embedded in methyl salicylate.

Immunostaining. For better visualization of brain structures surrounding the stained neurons some preparations were put through the process of synapsin immunostaining. This kind of immunostaining visualizes the glomeruli and surrounding neuropiles by acting with antibodies on the antigens present in the neuronal tissue, specifically on the presynaptic terminal protein synapsin. First, the preparations were rehydrated through a decreasing ethanol series and washed in PBS for 10 minutes. They were then dehydrated through an increasing ethanol series and washed with Xylol for 5 minutes to remove fat. Another series of rehydration was applied before washing the preparations in PBS for 10 minutes. To minimize the non-specific dying the preparations were pre-incubated in normal goat serum (NGS;Sigma, ST. Louis, MO, USA; 10%) diluted in PBS added triton (OBS oBS) x PBS (PBStx; 1%) for 30 minutes. Triton increases the permeability of the cell membrane and thus facilitates the entrance of intracellular antigens. The preparations were then incubated in SYNORF 1 diluted in PBSxt (containing 0.1% triton and 10% NGS) with the concentration of 1:30 for 48 hours in 4°C. SYNORF 1 is a monoclonal mouse antibody, while NGS effectively blocks the binding of unspecific proteins. The preparations were then washed in PBS for 6 x 20 minutes and incubated in Cy5-conjugated goat anti-mouse secondary antibody (Jackson Immunorsearch) diluted in PBStx for 48 hours in 4°C (1µL Cy5 + 500µL PBS). Cy5 binds to the primary antibody and is a hydrophilic fluorescent dye. Again the preparations are washed in PBS for 6 x 1 hours, dehydrated in an increasing ethanol series and finally cleared and mounted in methyl salicylate.

Image processing

The confocal microscope scans were further processed using a LSM 510 projection tool and ImageJ 1.48, where the stacks of optical sections could be analyzed further, reconstructed, and manipulated into showing 3D projections. Adobe Photoshop CS6 was used to adjust brightness, contrast, and color before the images were edited in Adobe Illustrator CS6 to produce the final figures.

Results

Of 177 attempted antennal-lobe stainings, 7 were successful. As can be seen in Table 1, these included a total of 15 stained antennal-lobe neurons, ten projection neurons and five local interneurons. Of the 27 preparations showing labeled neurons, 20 displayed neurons originating outside the AL and were thus not relevant to this study. Only the results relating to the AL projection neurons will be presented in detail and discussed further in this thesis.

Table 1: Summary of experimental results

	H. armigera	*H. virescens*	Total
Insects used (male/female)	38 (32/6)	39 (32/7)	77 (64/13)
Average age of the insects (days)	5.6	10.8	8.2
Attempted stainings	93	84	177
Preparations showing labeled neurons	12	15	27
Successfully stained AL projection neurons*	9	1	10
AL Projection neurons in lALT*	4	0	4
AL Projection neurons in mALT*	4	1	5
AL Projection neurons in mlALT*	1	0	1
Successfully stained AL local interneurons	5	0	5
Sucessfully stained neurons using microruby	14	1	15
Sucessfully stained neurons using microemerald	0	0	0

Note: (*) AL projection neurons included in the results
Table representing the results from the cooperative experimental effort of miss Sveum and myself

Cataloguing

To keep track of all of the preparations that were stained, each preparation was catalogued according to the name corresponding to the date they were stained (nameday). The ten successfully stained AL projection neurons in this study were obtained in six brains owned by moths consequently named Margunn, Noralf, Sølve, Bastian, Marius, and Svein. The collection of stained projection neurons consists of four neurons confined to the lateral antennal lobe tract (lALT), five to the medial antennal lobe tract (mALT), and one to the mediolateral antennal lobe tract (mlALT). One preparation, Margunn, contained four of the stained projection neuros (two in the mALT, one in the lALT, and one in the mlALT).

Staining from somata in the lateral cell cluster versus dendrites in the glomerular region

Generally, the method for targeting dendrites within the ALs often implied measurement of spiking activity. Here, a 0.7nA current pulse was used for staining the neuron. When targeting the cell bodies in the LC, on the other hand, no recognizable electrical fluctuations were recorded. Here, a 10nA current pulse was therefore used to maximize the probability of labeling the somata. However, separating the results with respect to the two different approaches used has not been possible, since both methods were used in each preparation. This dual approach will be addressed and elaborated on in the Discussion section.

Arborization and innervation patterns of the successfully stained projection neurons in the three ALTs

Projection neurons in the lALT

The four successfully stained projection neurons running through the lALT all showed distinct morphological characteristics:

The lALT projection neuron displayed in Margunn (Fig. 6) exhibited a single, thick axon and a cell body localized in the LC. Its projection exited the AL medioventrally and turned dorsolaterally into the protocerebrum. Along its projection pathway it sent off several small fibers that innervated a medially located area in the protocerebrum, before splitting and ramifying within two areas of the LH. Its general innervation area in the LH was distinct from that of co-stained sister neurons passing in the mALT. These non-overlapping innervations can be observed in the stereo image of the preparation (Fig. 7).

The two lALT neurons co-stained in the AL of Marius (Fig. 8) displayed distinctly different morphologies from each other. One projection neuron (denoted PN1 in the figure) displayed a single prominent axon leaving the AL medioventrally and turning sharply in a lateral direction. It continued as a single axon without any visible fiber branching until it reached the LH, where it split into several collaterals and displays one very large, club-like terminal. The other projection neuron (denoted PN2 in the figure) followed the projection of PN1 medioventrally from the AL. However, after turning laterally it crossed PN1 and continued slightly more frontally, where it bent off and projected like a peak superiorly in the medial protocerebrum (visible in the stereo image in Fig. 9). Small fibers extended from the projection neuron along its path from the AL. Three cell bodies, all localized in the LC, were

visible in the preparation, out of which two probably belonged to the two lALT projection neurons and one to a co-stained local interneuron.

The fourth lALT projection neuron, displayed in Svein (Fig. 10), exhibited a single, prominent axon leaving the AL medioventrally. Right after exiting the AL, it sent off one prominent branch in the medial protocerebrum in a more ventral and inferior direction (better visualized in the immunostained preparation, Fig. 14). The main projection continued dorsolaterally to the LH, where it branched in two. The most prominent branch (denoted BR1 in the figure) terminated ventrolaterally in the LH, while the second branch (denoted BR2 in the figure) projected more superiorly and terminated anterior of the mushroom body calyces. The cell body of the lALT projection neuron probably belonged to one of several stained somata, all located in the LC.

The glomerular arborization patterns exhibited by these lALT neurons were impossible to describe, as they were all co-stained with other neurons (either mALT neurons or local interneurons).

Projection neurons in the mALT

The five AL projection neurons sending their axons through the mALT displayed several typical morphological characteristics. As demonstrated in figure 6, 7, 11, and 12, all neurons exhibited projections leaving the AL mediodorsally and continuing posteriorly past the central body without splitting into any innervating processes along the way. Posteriorly of the central body the projections turned laterally and continued in an elaborate bow towards the calyces of the mushroom bodies. From here, four out of the five stained axons sent off several processes with extensive synaptic terminal blobs into both the medial- and lateral calyces, before continuing ventrolaterally and splitting into several terminal branches in the LH (Fig. 6,7,11, 12D). The fifth mALT neuron, obtained in Bastian, showed a unique projection pattern by lacking innervation in the calyces, but rather projecting directly to the LH (Fig. 12A-C). This neuron also displayed a biglomerular arborization pattern in the AL, while the other four were either uniglomerular or difficult to determine due to co-staining of other principal AL neurons. The cell body of Bastian’s neuron was located in the MC. The cell bodies of the four other neurons were not visible.

Projection neurons in the mlALT

The single mlALT projection neuron successfully stained also displayed several typical morphological traits. As can be seen in figure 13, the projection left the AL posterior-medially

before running past the central body. At the ventral edge of the central body, the axon took a sharp lateral turn and projected directly to the LH, bypassing the mushroom body calyces entirely. At this point the neuron divided into two distinct branches and ramified within various regions of the LH. The cell body of the mlALT projection neuron was located in the LC and the dendritic arborizations branched within several glomeruli.

Immunohistochemisty of preparations

Two preparations, namely Margunn and Svein, were put through the process of immunostaining for labeling relevant brain structures surrounding the projection neurons. In spite of the similar protocol used on the two brain preparations, the outcome differed significantly. The immunostaining of Svein was highly successful and resulted in distinct visualization of the surrounding neuropils and brain structures, which can be seen in figure 14. The immunostaining of Margunn however, only resulted in visualization of the AL glomeruli associated with the stained dendritic arborizations (Fig. 15).

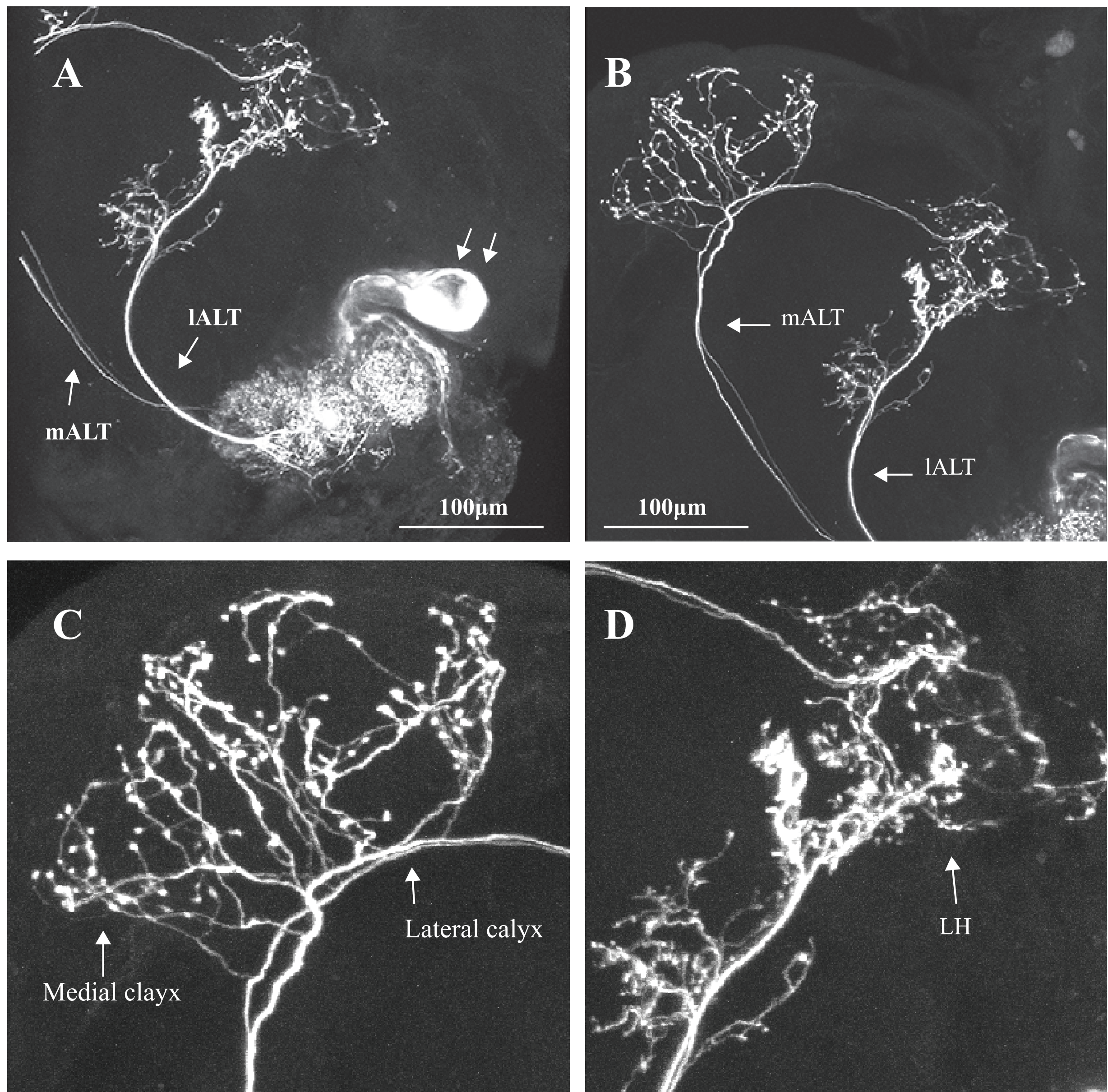

Figure 6: Confocal images of the preparation called Margunn, displaying two sister neurons running in the medial antennal lobe tract (mALT), and one neuron in the lateral antennal lobe tract (lALT) (20X objective). **A:** dorsal visualization of the entire lALT neuron with a single prominent axon leaving the antennal lobe (AL) medioventrally and turning dorsolaterally before ramifying in the lateral horn (LH). Several processes are sent off along the projection and innervate medially in the hemisphere. The double arrows indicate the cell body. The dense multiglomerular arborizations display dendrites from both the mALT neurons and the lALT neuron. **B:** visualization of the two sister neurons following the mALT, running mediodorsally from the AL and continuing posteriorly in the protocerebrum, where it turns laterally into the calyces of the mushroom bodies and the LH. **C:** image displaying terminal branches of the mALT neurons densely ramifying in the lateral and medial cup-shaped calyx (enlarged image, 20X objective) **D:** visualization of the innervation area of both the lALT and mALT neurons in the LH (enlarged image, 20X objective).

Note: The cell body seems to have “popped out” during the staining procedure.

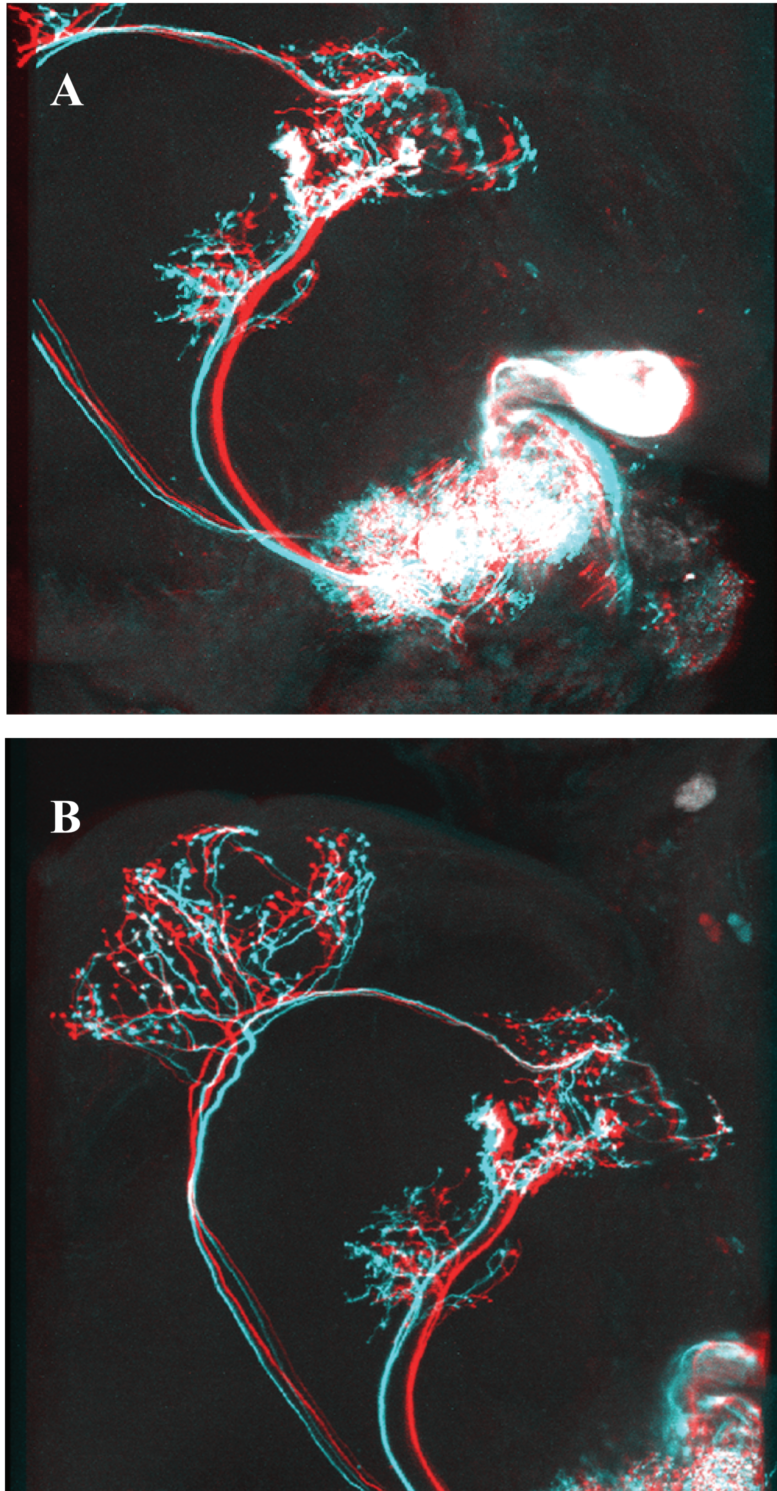

Figure 7: Stereo projection image of the preparation called Margunn, displaying both the lateral antennal lobe tract (lALT) neuron, and the medial antennal lobe tract (mALT) neurons from figure 6. *Viewing requires 3D bifocals enclosed in the thesis (green filter on right eye, red filter on the left)* **A,B:** both figures are presented in a ventral view, and clearly illustrate that the lALT neuron runs much more ventrally than the mALT neurons. The mALT neurons ramify in a more dorsal area of the lateral horn, and the projection clearly shows that there is no overlap between the two tracts in area of innervation.

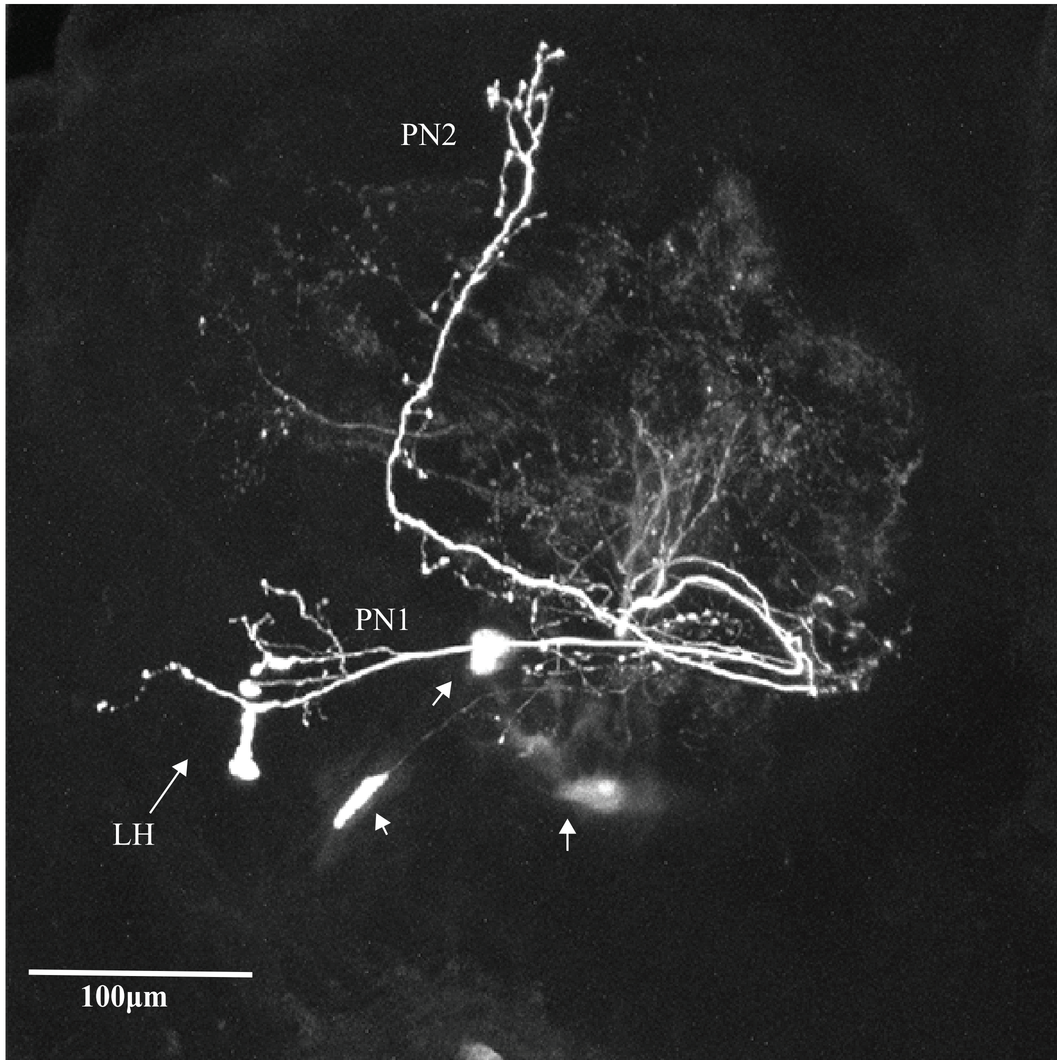

Figure 8: Confocal image of the preparation called Marius, visualizing two lateral antennal lobe tract (lALT) neurons and one local interneuron in the same antennal lobe (AL) (frontal view). All stained cell bodies are localized in the lateral cell cluster (LC) and are indicated by the arrowheads. The neuron denoted PN1 exits medioventrally and continues laterally towards the lateral protocerebrum. In the lateral horn (LH) it splits into several collaterals and terminal branches, with one prominent club-like terminal ending. The neuron denoted PN2 follows the PN1 projection while exiting the AL, before crossing and continuing superiorly in the medial protocerebrum.

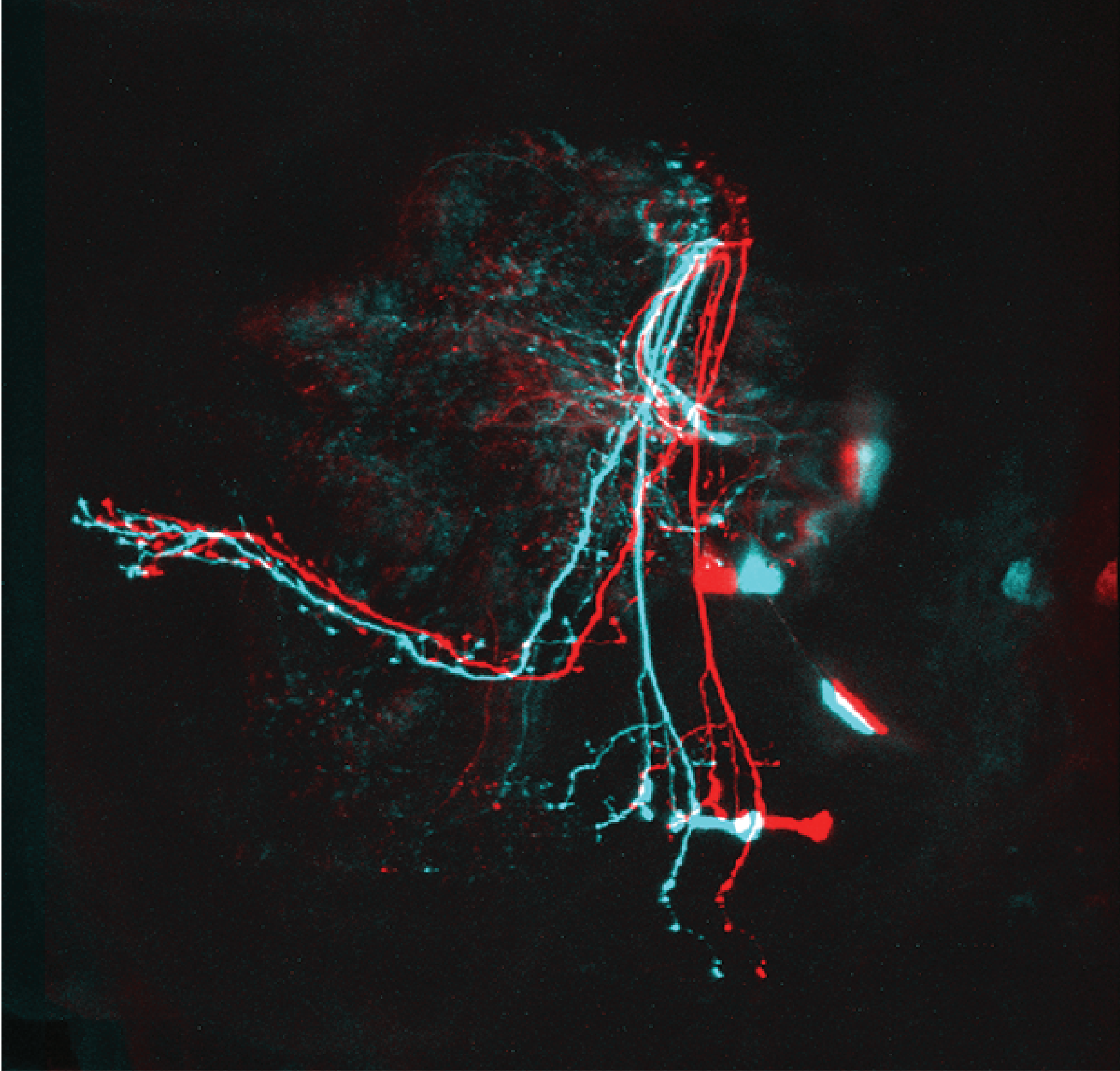

Figure 9: Stereo projection image of the preparation called Marius, displaying the two lateral antennal lobe tract (lALT) neurons, and the co-stained local interneuron from figure 8 in a posterior view. *Viewing requires 3D bifocals enclosed in the thesis (green filter on right eye, red filter on the left). Unfortunately the image is rotated 90º to the left. Aligning it the right way removes the 3D effect.* The stereo image clearly illustrates that the two lALT neurons have distinct projection patterns (20X objective).

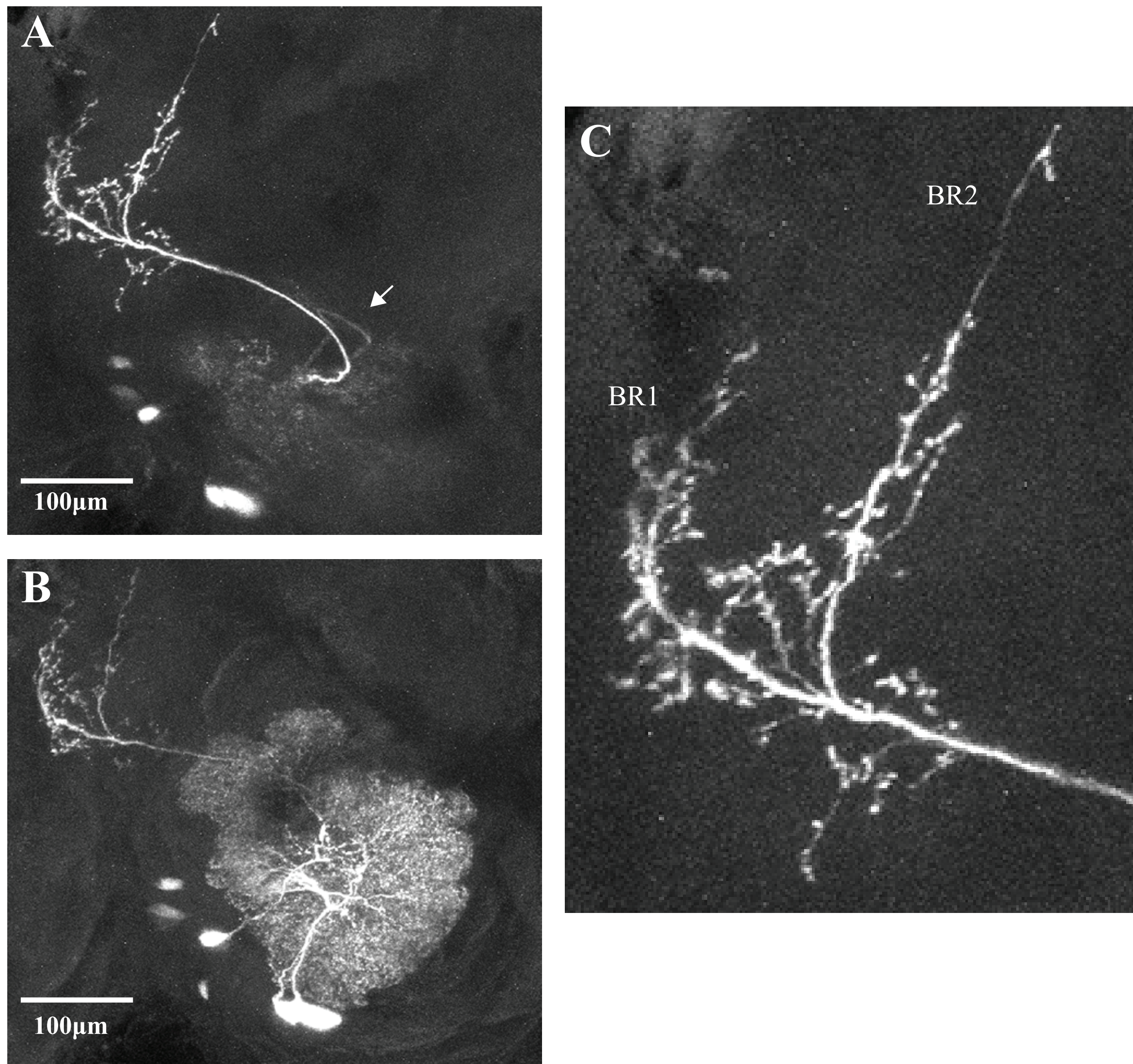

Figure 10: Confocal images of the preparation named Svein, visualizing a lateral antennal lobe tract (lALT) neuron and a co-stained local interneuron (20X objective) **A:** dorsal visualization of the lALT projection neuron, exiting medioventrally from the antennal lobe (AL), before sending off a branch in the medial protocerebrum (indicated by an arrow). The main projection continues dorsolaterally before branching in two. **B:** flipped scan of same neuron, better visualizing the co-stained local interneuron and the glomerular arborizations. Several cell bodies in the lateral cell cluster (LC) have been stained. **C:** dorsal display of the projection neuron's two terminal branches; innervating the vental part of the lateral horn (LH) (denoted BR1), and more superior region in the protocerebrum (denoted BR2), respectively (20X objective, magnified image).

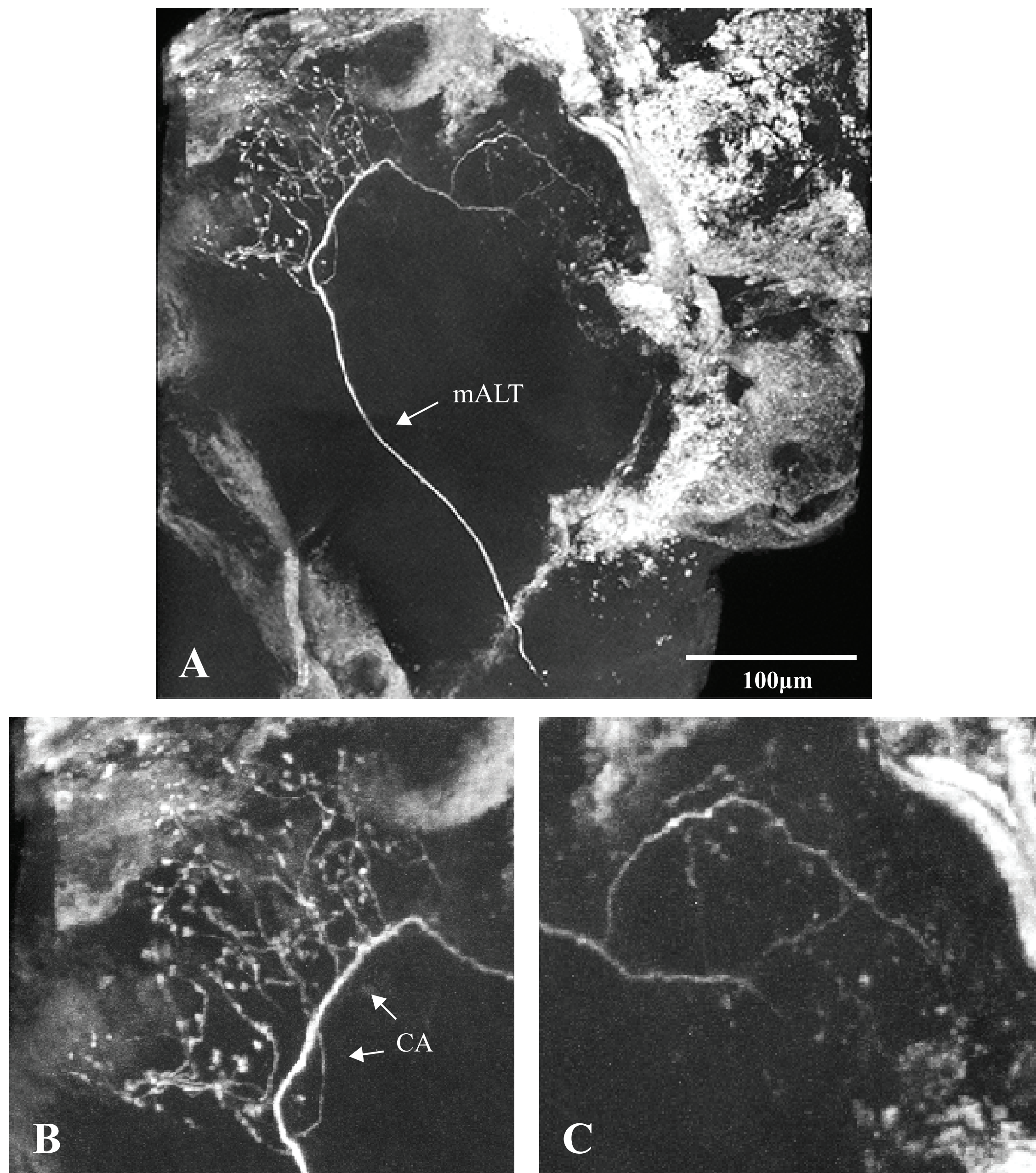

Figure 11: Confocal images of the preparation named Noralf, displaying a single medial antennal lobe tract (mALT) neuron in a dorsal view. **A:** visualization of the entire hemisphere with the full projection pathway. The mALT neuron exits mediodorsally from the antennal lobe (AL) and runs posteriorly in the protocerebrum, before turning laterally and ramifying extensively in both the lateral and medial calyx (CA) of the mushroom bodies. Projecting further, the neuron continues laterally into the superior part of the lateral protocerebrum, where it divides in two distinct terminal branches in the lateral horn (LH) (10X objective). **B:** visualization of the extensive ramifications with multiple terminal blobs in the cup-shaped calycal structures in greater detail (enlarged image, 10X objective). **C:** visualization of the two distinct innervation branches with terminal blobs in the LH (enlarged image, 10X objective).

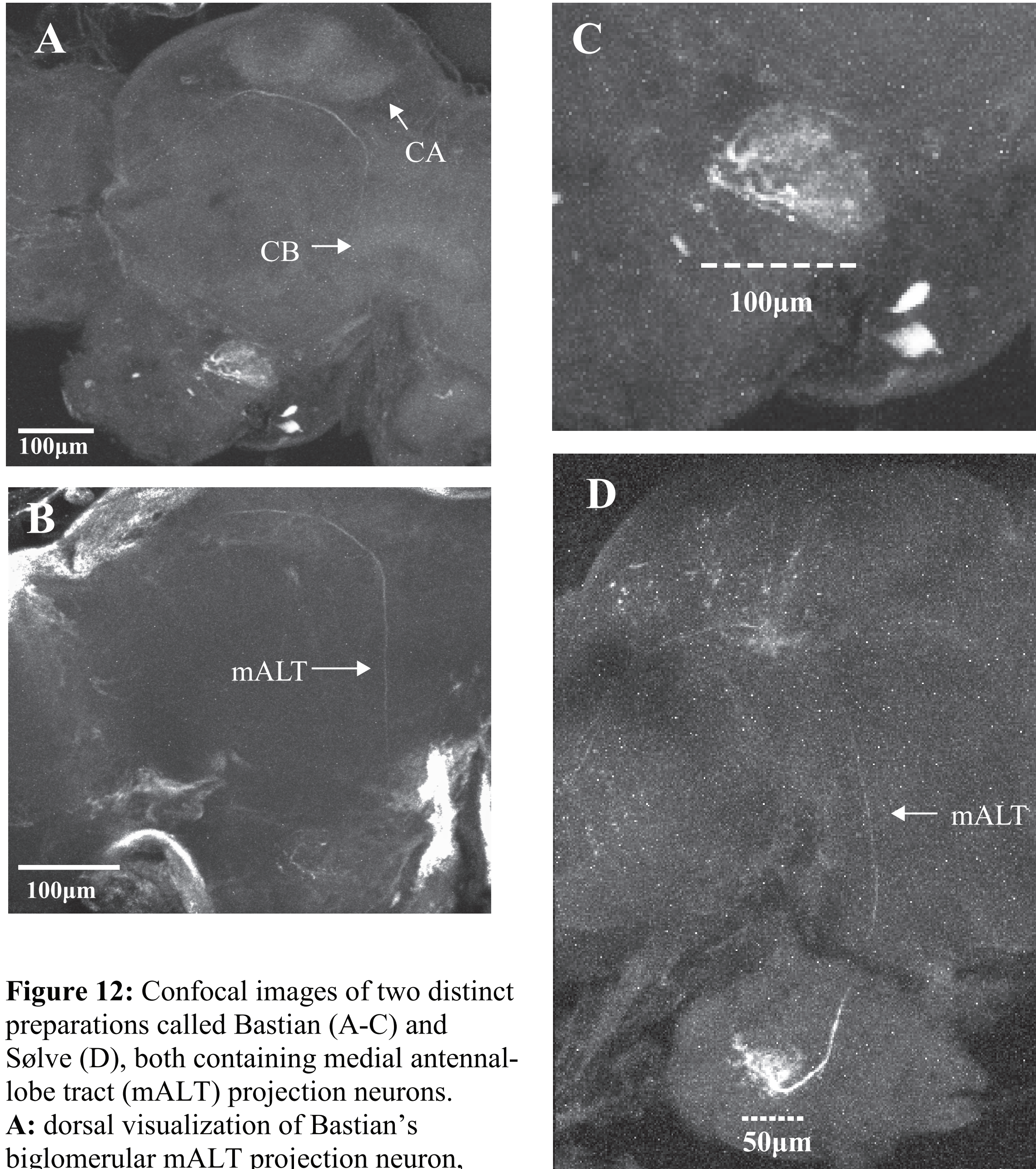

Figure 12: Confocal images of two distinct preparations called Bastian (A-C) and Sølve (D), both containing medial antennal-lobe tract (mALT) projection neurons. **A:** dorsal visualization of Bastian's biglomerular mALT projection neuron, running mediodorsally from the antennal-lobe (AL) and posterior past the central body (CB). Past the CB it turns laterally and bypasses the calyces (CA) of the mushroom bodies, and continues straight to the lateral protocerebrum where it splits in two and terminates in the lateral horn (LH) (10X objective). **B:** intensified image better visualizing the middle part of the projection (20X objective). **C:** visualization of the glomerular arborization in the AL and the two medially located cell bodies that were stained (enlarged image, 10X objective). **D:** dorsal visualization of Sølve's uniglomerular mALT projection neuron in a slightly damaged preparation, with extensive ramifications in the CA and LH (10X objective).

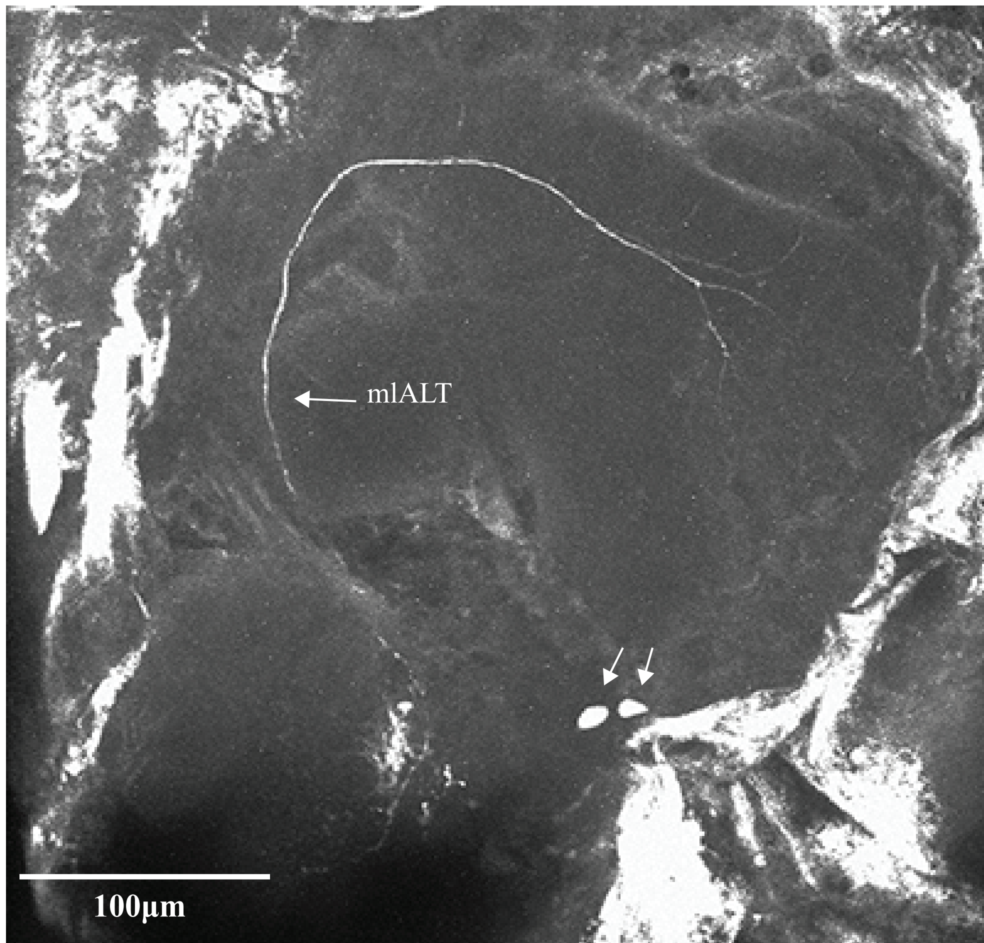

Figure 13: Confocal image of the preparation called Margunn, displaying a single projection neuron in the medial antennal lobe tract (mlALT) in the left hemisphere (20X objective). The neuron projects posterior-medially and turns laterally, through the medial protocerebrum, before splitting into two distinct branches and terminating in the lateral horn. The cell bodies are indicated by arrows and are localized in the lateral cell cluster.

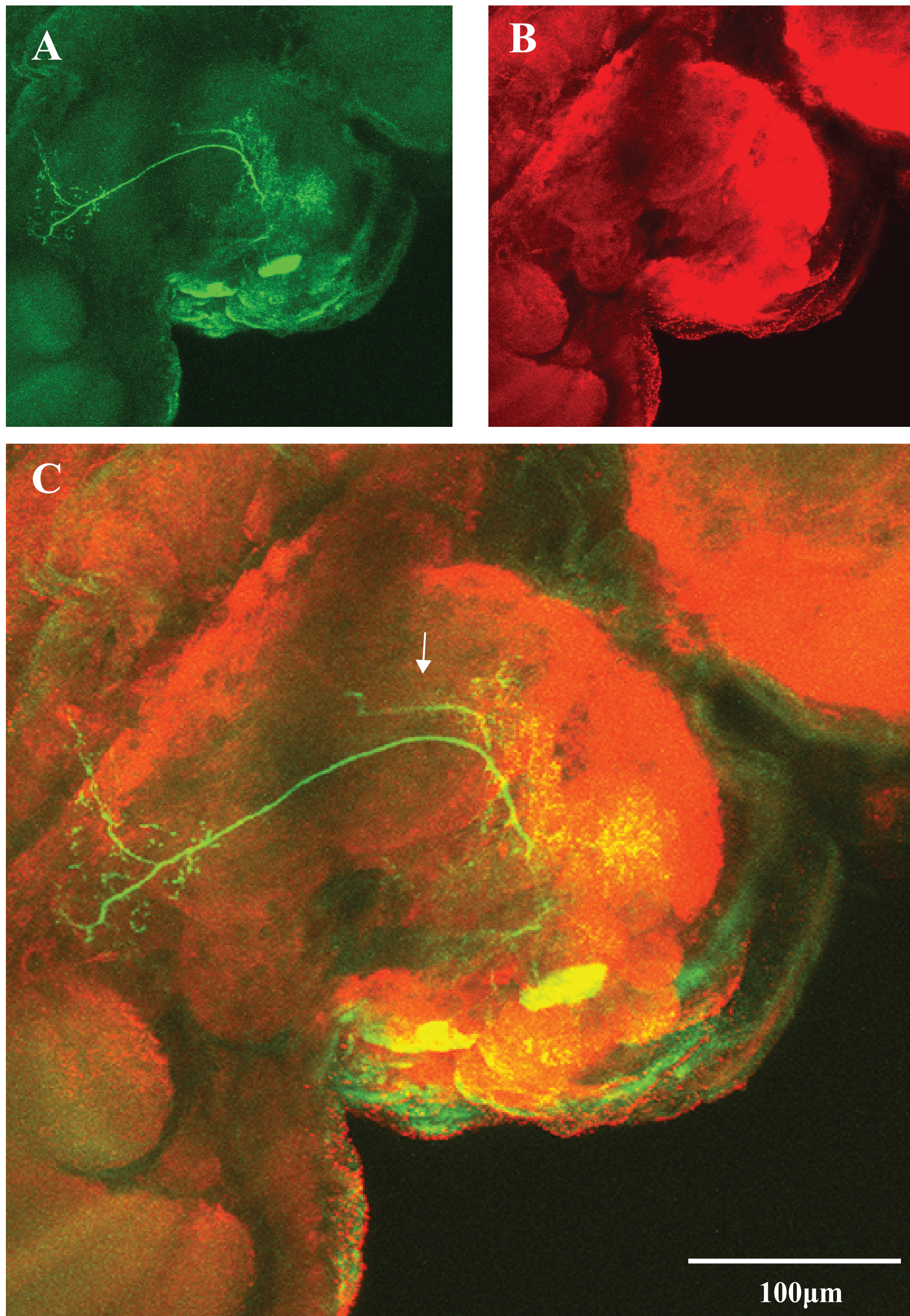

Figure 14: Two-track confocal images of the preparation called Svein after immunostaining, displaying one later antennal lobe tract (lALT) neuron, and one local interneuron (20X objective). **A:** showing the preparation excited by a helium neon lazer (543nm), visualizing the microruby used for the intracellular iontophoretic staining. **B:** displaying the antisynapsin immunostaining, excited by a helium neon lazer (643nm), visualizing the surrounding neuropiles and brain structures. **C:** showing the two-track image of **A** and **B** merged, visualizing the lALT neuron with some of its glomerular arborizations and surrounding brain structures in a dorsal view. The arrow points towards a branch of the lALT neuron splitting off and projecting medially into the hemisphere.

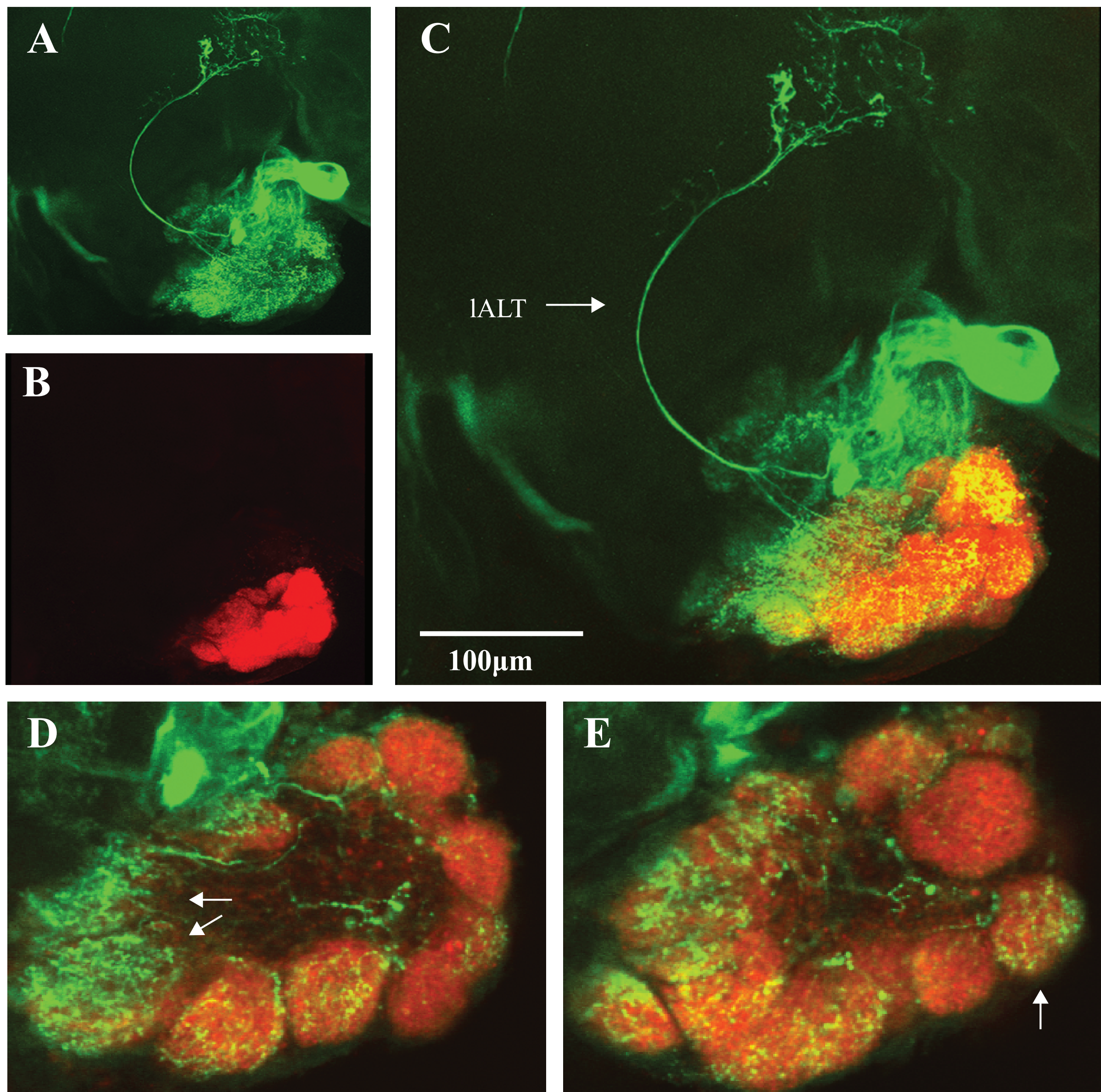

Figure 15: Two-track confocal images of the preparation named Margunn after immunostaining, displaying the lateral antennal lobe tract (lALT) neuron, and its glomerular innervations (20X objective). **A:** showing the preparation excited by a helium neon lazer (543nm), visualizing the microruby used for the intracellular iontophoretic staining. As a result of series of scans and thus degradation of the tetramethylrodamine, the weaker stained sister medial antennal lobe tract neurons shown in figure 6 of the same preparation does not appear in this scan. **B:** displaying the synapsin immunostaining, excited by a helium neon lazer (643nm), visualizing the surrounding neuropiles and glomeruli. **C:** displaying the two-track image of **A** and **B** merged, visualizing the lALT neurons projection pathway and its dendritic arboirizations in the antennal lobe (AL) glomeruli. **D:** displaying a dorsal serial slice of the AL (enlarged image, 20X objective), showing that the dendrites envelopes each innervated glomeruli from the outside. The arrows points towards the glomeruli fully enveloped in this slice. **E:** displaying another more ventral serial slice of the AL, with the arrowhead pointing towards one of the enveloped glomeruli.

Discussion

In order to uncover the mechanisms underlying the sense of smell it is imperative to know the morphology of principal second-order olfactory neurons that relay the information gathered from the external world to more complex processing centers. Antennal-lobe projection neurons transmit the information gathered by ORNs into higher brain areas, making their anatomical organization into distinct tracts, and arborizaions in secondary olfactory centers, particularly interesting. The results gathered in this thesis include morphological characterization of ten AL projection neurons distributed among the three main olfactory tracts in the brain of the heliotine moths H. *virescens* and H. *armigera*. These ten AL projection neurons consist of five mALT neurons, four lALT neurons, and one mlALT neuron. The results were obtained through the use of a dual staining approach, targeting both the somata in the LC, and the projection neuron dendrites within the AL through separate methods. In addition to complementing previous reports on morphological characteristics typifying antennal-lobe projection neurons in heliothine moths, the present study contribute new findings previously not described.

The finding of three distinct morphological types of AL projection neurons in this study is in full compliance with existing data, where all previous investigations have found three main ALTs to be present in the olfactory systems of moths (Haupt et al., 2010; Homberg et al., 1988; Kanzaki et al., 2003; Løfaldi et al., 2010; Rø et al., 2007). In the following discussion, the particular features characterizing each of these projection neuron types will be elaborated upon, as well as the possible underlying functions of such and organization into parallel tracts.

Morphology of mALT projection neurons

In the present study, the largest number (5) of successfully stained AL projection neurons sent their axons through the mALT, the most prominent of the ALTs. A characteristic feature typifying the stained projection neurons confined to this tract was their morphological similarity. All five neurons projected posteriorly and then turned laterally, running along the anterior edge of the calyces before terminating in the lateral horn. The majority of these projection neurons also displayed innervations in the calyces (Fig. 6, 11, 12D), and thus

corresponds to the description of uniglomerular mALT projection neurons portrayed in all previous studies of both *Manduca sexta* and H. *virescens* (Homberg et al., 1988; Rø et al., 2007; Kanzaki et al., 2003; Berg et al., 1998; Løfaldi et al., 2010; Schachtner et al., 2005; Zhao et al. 2014). This neuronal category has been found in several insect species, and appears to be relatively abundant and highly homogeneous in morphological appearance. The glomerular arborization patterns in most of the preparations containing mALT projection neurons could not be discerned due to either co-staining if other neurons or probable damage to the AL, however, they most likely consist of uniglomerular innervations. According to Homberg et al. (1988), these neurons typically fill a single glomerulus with dense dendritic arborizations, and sometimes send out short neurites that approach neighboring glomeruli, which is visible in one particular preparation here (Fig. 12D). As a side note, the morphology displayed by these neurons is strikingly similar to the mitral projection neurons found in the human olfactory system, which also carry odor information gathered from uniglomerular dendritic arborizations to higher brain areas (Kandel et al., 2013). The cell bodies of the stained mALT projection neurons could not be identified due to simultaneous labeling of other neurons. However, all mALT neurons have been reported to have their somata located in the cell cluster located closest to the glomerulus they innervate (Homberg et al., 1988).

One of the five mALT neurons exhibited a unique projection pattern, distinguishing it from the others by entirely bypassing the calyces along its course to the LH (Fig. 12ABC). In the AL, it innervated loosely an area with a diameter of about 100µm, indicating that it has arborizations in at least two glomeruli, as a single ordinary glomerulus in the AL of heliotine moths (both H.*virescens* and *Helicoverpa assulta*) has been shown to have a diameter of 40-60µm (Berg et al., 2002). Medial ALT neurons with similar projection patterns have been described in in a few previous studies (Homberg et al., 1988; Rø et al., 2006; Vaagsland Stav, 2014). In Rø et al. (2007) and Homberg et al. (1988) the neurons displaying such projection patterns have been termed “PIc”. These PIc neurons were reported not only to bypass the calyces to innervate the LH, but also to send several processes back into the calyces. However, no such processes extending into the calyces can be observed in the present preparation.

Morphology of lALT projection neurons

The second largest group of stained AL projection neurons in this study sent their axons through the lALT. In general, relatively few neurons confined to this tract have been identified in previous studies. This is probably due to the fact that the lALT contain thinner axons (fiber diameter < 1µm) than those in the mALT (Homberg et al., 1988). In this study however, a relatively large portion of all obtained neurons (4/10) were confined to the lALT. In addition, they all exhibited relatively thick projections, conflicting with the previously mentioned accounts of lALT neurons as rather thin. Furthermore, previous studies have reported that lALT neurons often send several branches to both the lateral and medial calyces, while none of the lALT neurons found in this study innervate the calyces. In contrast to the homogenous morphology exhibited by the aforementioned mALT neurons, all the lALT neurons found in this study displayed unique projection patterns by innervating dissimilar regions of the protocerebrum (Fig. 6, 8, 10). These distinct morphological characteristics suggest that the different neurons projecting in the lALT might serve different functions. Like the preparations containing mALT neurons, the glomerular arborization patterns of the lALT neurons were difficult to discern due to co-staining. In general however, the different types of AL projection neurons in the lALT have been shown to be both uni- and multiglomerular in nature, and to have their somata located in the LC (Rø et al., 2007; Homberg et al., 1988). The localization of the somata belonging to the lALT neurons found in this study concur with these accounts, as all stained cell bodies were confined to the LC.

One particular preparation containing one stained lALT projection neuron allowed for investigation of the glomerular arborizations (Fig. 6). This neuron exhibited a unique innervation pattern by completely enveloping the glomeruli it innervated. This can be best observed in the immunostained visualization of the preparation (Fig. 14), where several individual glomeruli are visible and clearly enveloped from the outside by the dendritic innervations. This is rather special, as most dendritic arborizations in the AL from projection neurons have been shown to innervate the glomeruli from the inside, while ORN axons are reported to terminate in the peripheral layers (Hansson & Anton, 2000). The current lALT neuron was however co-stained with sister neurons confined to the mALT. These mALT neurons exhibited projection patterns typical for mALT neurons with uniglomerular dendritic innervations, making it reasonable to assume that at least two of the glomeruli innervated in the preparation are connected to these two neurons, and not to the lALT neuron.

Although all of the lALT neurons displayed unique projection patterns, many of the features separating them from each other have been encountered before. In figure 6, the only lALT neuron in this study displaying thin fibers bifurcating medially in the ipsilateral hemisphere can be viewed. Although this bifurcation pattern represents a unique trait in the present set of stained neurons, some lALT neurons have been reported to have similar branching fibers in the medial protocerebrum in the previous investigation by Homberg et al. (1988). Interestingly, as was suggested in the same study, these fibers might be responsible for mediating input to the lALT neuron from other sensory modalities. Furthermore, the large, club-like terminal endings displayed in the outermost part of the LH, as found in another neuron (PN1 in Fig. 8), also represents a very unique feature, separating the current neuron from all other stained AL projection neurons. However, a corresponding termination pattern was encountered in neurons classified as "POb" both by Homberg et al. (1988) and Rø et al. (2007). In contrast to the other lALT neurons, this neuronal type does not display any fiber branching along its projection; it targets the most lateral border of the LH (almost entering the optic lobe), and is the only neuronal ALT type that displays such unique, club-like terminal endings. Why they exhibit this special kind of innervation pattern, however, is unknown. Another unique neuron found in this study featured the only encountered branch splitting from the main lALT projection and innervating the dorsomedial protocerebrum (figures 10 and 14). Although this represent an exceptional feature among the set of stained neurons, this region has been reported to receive occasional innervations from lALT neurons by both Homberg et al. (1988) and Rø et al. (2007).

The last lALT projection neuron, denoted PN2 in figure 8, also exhibited a distinct projection pathway by presenting itself like a peak into the superior part of the medial hemisphere. Similar characteristics have also been described in Rø et al (2007), where a so-called POa neuron in the lALT exhibited a columnar shaped target region in the superior protocerebrum. This particular projection pathway has also been described in the master theses of Ingrid Moe Dahl (2013) and Siri Corneliussen Lillevold (2013), where a distinct "columnar" or "pillar-like" structure has been described as projecting from the lALT and into the superior part of the medial protocerebrum. Furthermore, the lALT neurons encountered by Dahl (2013) were linked to the labial pit organ glomerulus, which receives input from CO_2 –sensory neurons situated on the palps. Thus the lALT projection neurons do not only carry information gathered from the antennae, but also information gathered from the palps. Also, previous studies have shown that the lALT contains fibers originating from both the antennal

mechanosensory and motor center (AMMC) and the suboesophageal ganglion (SOG). As mentioned before, it has been suggested that the branching fibers exhibited by some lALT projection neurons might receive input from other sensory modalities (Homberg et al., 1988). Taken together, this might suggest that the lALT is exceptional compared to the other ALTs by allowing for information to be integrated from several different sensory modalities.

Morphology of the singe mlALT neuron

One single AL projection neuron sent its axon through the mlALT (Fig. 13), and displayed morphological traits typical for neurons confined to this tract. Like other reports of mlALT projection neuron pathways, it projected posterior-medially past the central body, where it turned laterally and split into two branches innervating the more medial part of the LH (Løfaldli et al., 2012; Homberg et al., 1988; Hoskins et al., 1986; Rø et al., 2007). That the cell bodies were located in the LC, and that the dendritic branches seemed to ramify loosely within several glomeruli, is also in concurrence with previous reports. Interestingly, in a study done by Berg et al. (2009), it was established that a substantial proportion of neurons in the mlALT of H.*virescens* were GABAergic. This feature separates the mlALT neurons from the assumed excitatory nature of neurons in the two other ALTs. The GABAergic, and thus inhibitory, transmission from these mlALT neurons is suggested to produce an odor-dependent inhibition of target brain areas. GABAergic inhibitory input from these neurons have actually been suggested to converge on excitatory input from the uniglomerular mALT neurons carrying pheromone information, and the uniglomerular mALT neurons carrying plant odor information in this area, and by this, possibly enhancing odor discrimination and sensitivity (Homberg et al., 1988).

None-overlapping innervations in the lateral horn

A particularly interesting feature was found in the preparation called Margunn, where two sister mALT neurons and one lALT neuron were co-stained (Fig. 6 and 7). Three-dimensional reconstruction of the confocal serial slices revealed that the two neuronal categories displayed in the preparation projected to completely separate areas of the LH. The two sister mALT neurons terminate dorsally compared to the more ventrally situated target area of the lALT neuron. In H. *virescens* it has been suggested that the terminal fields of the different ALTs are partially separated within the LH, and that most branches of mALT

neurons project dorsally in this area, while lALT neurons follow a more ventral pathway (Løfladli et al., 2010;, 2012; Rø et al., 2007). Several studies have revealed distinct termination zones, and in such, discrete processing areas, as for example in the fly *Drosophila melanogaster* (Galiza & Rössler, 2010; Jefferis et al., 2007), however, such an explicit none-overlap in the termination areas of mALT and lALT neurons has not previously been demonstrated in moths. The segregation demonstrated in this study shows that at least some mALT neurons and lALT neurons in heliothine moths have discrete termination zones in the LH, and that they in turn might connect to distinct third order neurons in this area.

Parallel tracts organization

As can bee seen from the results presented in this study, the olfactory system of heliothine moths clearly consists of three distinct and separate tracts for relaying information to higher brain areas. This sort of parallel organization constitutes an important processing feature found in most biological sensory systems. Commonly, such parallel pathways either mediate different aspects related to one and the same stimulus, or it constitutes a division in stimuli which elicit functionally distinct response profiles. This heightens both the processing speed and accuracy of the system. For instance, by encoding different features of a stimulus in different sets of neurons, all of the available features can be simultaneously relayed to higher brain areas, where they in turn can be integrated and analyzed, and elicit behavioral responses and learning. In the human visual system, two separate neuronal pathways carry different elemental properties of vision, where the so-called magno- and parvocellular pathways mainly carry information about the movement of objects in space and the identity of objects, respectively (Kandel et al., 2013). In the same regard, the olfactory information gathered in AL glomeruli of heliothine moths is relayed through three separate neuronal pathways, the three ALTs, which may carry different elemental qualities of smell to higher order processing areas.

The information that is relayed through these three tracts is not completely naïve, as some of it has already been preprocessed and categorized at the level of the AL. In male moths, the AL is separated into two discrete odor-processing subsystems, namely the MGC and the ordinary glomeruli (OG). The MGC consists of a specific set of glomeruli devoted to processing pheromone information, while the rest of the glomeruli in the AL, the OGs, are

devoted to the predominant processing of plant odorants (Galizia & Rössler, 2010). This segregation in itself constitutes a clear parallel processing system, where the information gathered from specific OSNs end up in specific and separate glomerular subunits. Specific subsets of different glomeruli are in turn connected to the different ALT projection neurons, where both pheromone- and plant information has been shown to be present in all three ALTs (Homberg et al., 1988; Zhao et al., 2014). Keeping these two types of odor information separate is evolutionary adaptive, as the ability to react quickly to mating opportunities is what enables the sustainment of species with relatively short lifespans, such as moths. The opportunity for procreation thus needs the power to override all other instincts, taking precedence over both feeding opportunities and possible dangers. Consequently, it comes as no surprise that the termination area of ALTs originating from the MCG is spatially separate from the termination area of ALTs originating from OGs, both in the LH and in the mushroom body calyces (Zhao et al., 2014). However, such an anatomical segregation in termination areas does not necessarily mean that the pheromone information and plant odor information is processed completely independent of each other in this area.

In fish, it has been demonstrated that the parallel projection bundles from the primary olfactory center each conveys specific information about a particular functional group of odors: one bundle mediates information about social cues, one about pheromones, and one about food odors, each eliciting sets of characteristic behavior as a response (Døving, 2007). In moths however, we know that both pheromone and plant odor information is relayed in all three ALTs, and thus that there is no such division of functionally distinct odor groups. What then, might be the underlying function of relaying olfactory information in such parallel tracts? Needless to say, it would constitute an ineffective design if the separation into parallel tracts had no meaning, since separating them requires more space than bundling them together. One possible function of such a separation might be that each tract conveys information about specific aspects related to one and the same stimulus. In the honeybee for instance, it has been demonstrated that the two main ALTs both respond to all tested odors, as well as having widely overlapping response profiles. Furthermore, the response profiles also indicated that one tract carried information about generalized odor profiles, while the other carried more odor specific information (Brill et al., 2013; Müller et al., 2002), which might be the case in moths as well. Another possibility is that each tract conveys the same information about the same stimulus, but that the different lengths of their projection pathways are most meaningful, creating different and important temporal delays in signal

arrival into functionally different higher processing areas. In addition to being anatomically separated, the primary innervation areas of the three ALTs, the calyces of the mushroom bodies and the LH, are believed to serve such different functions. It has been proposed that the LH primarily determines odor quality and concentration, while the calyces of the mushroom body serves as the main seat for learning and memory (Homberg et al., 1988). It has also been shown in several studies that the calyces receive innervations both from neurons confined to the mALT, and from neurons confined to the lALT, while the LH receives input from different neurons confined to all three main ALTs (Homberg et al., 1988; Galizia & Rössler, 2010; Rø et al., 2007). The termination areas of the ALTs in these two higher brain areas are partially overlapping. Consequently, there is likely to be such a difference in arrival time both in the calyces and the LH, but whether it is of functional importance remains to be seen. In sum, further experimental investigations are needed to determine the functional importance of the parallel organization of the ALTs in moths.

Methodological considerations

As described in the method section, two different staining strategies were used to obtain the results presented in this study: one approach targeting somata in the LC, and one targeting the dendrites in the AL. This dual approach proved fruitful as it resulted in individually stained AL projection neurons in all three ALTs, whereas other studies targeting only the AL dendrites alone most often have resulted in staining of projection neurons confined to the most prominent of the tracts, the mALT. A particularly positive aspect of staining from the LC seems to be that it allowed for the successful staining of several lALT neurons, which accounts for a large number of all obtained projection neurons in this study. In addition, the high pulse currents (10nA) used during this particular approach resulted in strongly stained neurons with clear morphological features. Consequently, this method gave rise to detailed data for further analysis.

However, several different reasons make it difficult to say, with absolute certainty, if the stained neurons actually resulted from effective targeting of the somata in the LC. The main reason for this difficulty relates to the fact that the LC was not visible through a microscope. This means that targeting the LC relied solely on a personal and visual assessment of where the cluster was supposed to be situated. This assessment was of course based on knowledge

from existing studies and anatomical schematics; however, it was impossible to be completely sure of whether the glass electrode was inside the LC during the attempted staining or not. In this regard, targeting dendrites in the AL was easier, as the entire AL is clearly visible through the microscope and comprises a much greater area. Furthermore, there were no recognizable fluctuations noted during any of the attempts to stain from the LC, so telling whether the electrode had penetrated a soma membrane or not was futile. The complicating lack of spiking activity in this area was expected to some degree, as neuronal cell bodies differ from axons and dendrites in their electrical properties (Kandel et al., 2013). However, spiking activity has been reported in current-clamp studies targeting the somata of AL projection neurons in *Manduca sexta* (Kloppenburg et al., 1999), so the fact that they were completely absent in this study was not anticipated. For comparison, targeting the AL dendrites resulted in spiking activity during most staining attempts, and therefore provided a clear clue as to weather the glass electrode had penetrated a membrane or not, as apposed to the more “blind” approach taken through staining from the LC.

Nevertheless, the two approaches taken resulted in valuable data making it possible to accurately describe morphological characteristics of individual projection neurons confined to each of the three main ALTs in the heliothine brain.

Conclusion

- Morphological characteristics typifying the individual antennal-lobe projection neurons confined to all three main antennal-lobe tracts (mALT, lALT, and mlALT) were described.
- Targeting the LC allowed for strongly stained neurons with clear morphological characteristics, especially in the lALT where previous documentations have been scarce.
- The projection neurons confined to the lALT all exhibited relatively thick axons, conflicting with previous accounts of projection neurons running through this tract.
- The projection neurons confined to the mALT exhibited highly homogenous characteristics, while the projection neurons confined to the lALT all exhibited unique projection patterns.
- Explicit none-overlapping innervations were demonstrated in the LH between a projection neuron confined to the lALT, and two projection neurons confined to the mALT in the same preparation.
- A unique glomerular arborization pattern was demonstrated in a preparation with co-stained mALT and lALT neurons, where the dendrites enveloped the innervated glomeruli from the outside.

Abbrevations

AC	Anterior cell cluser
AL	Antennal-lobe
ALT	Antennal-lobe tract
AMMC	Antennal mechanosensory and motor center
GABA	γ-aminobutyric acid
lALT	Lateral antennal-lobe tract
LC	Lateral cell cluster
LFG	Large female glomeruli
LH	Lateral horn
mALT	Medial antennal-lobe tract
MC	Medial cell clusert
mlALT	Mediolateral antennal-lobe tract
MGC	Macroglomerular complex
OG	Ordinary glomeruli
OSN	Olfactory sensory neuron
SOG	Suboesophageal ganglion

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