"True intuitive expertise is learned

from prolonged experience with good feedback on mistakes."

Daniel Kahneman

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I wrote this master thesis at the chemosensory laboratory of the neuroscience unit, department of psychology. It was truly exciting and challenging, and I am grateful to have had such an opportunity.

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Last but not least....Well, you know who you are!

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Abstract

Of all the senses, the sense of smell is the evolutionary oldest, and all organisms possess an organ that allows for detection and discrimination of chemosensory information. For a wide variety of animal species olfaction influences a diverse set of behaviors, as well as learning and memory. In this study, female heliothine moths were used as model animals to investigate the fiber tracts formed by second order projection neurons carrying olfactory information from the antennal-lobe, the primary olfactory processing center, to higher brain centers. This part of the insect olfactory pathway corresponds to the lateral olfactory tract of mammals consisting of axons projecting from the olfactory bulb to cortical regions in the temporal lobe. Previous studies on heliothine moths have described three main antennal-lobe tracts both in males and females. Since a fourth antennal-lobe tract was recently discovered in male moths, one of the main aims of the current study was to explore whether a corresponding tract is present in the female moth as well. Although the projection pattern of the classic antennal-lobe tracts has been previously mapped, there are still unanswered questions, especially regarding terminal regions of the lateral and the medial antennal-lobe tract. Data in the form of confocal images were obtained using anterograde mass staining of second order neurons originating in the antennal lobe. This labeling technique clearly visualized the antennal-lobe tracts. In some preparations, a double-labeling technique including two fluorescent dyes applied into different regions of the antennal lobe was used in order to investigate whether different categories of antennal-lobe glomeruli give rise to projection neurons targeting distinct regions of the lateral horn. Altogether, the successful stainings confirmed previous findings including visualization of the three classic antennal-lobe tracts. In addition, the transverse tract was identified in the female moth.

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Abbreviations

AL	-	Antennal-lobe
ALT	-	Antennal-lobe tract
CB	-	Central body
GABA	-	Y-Aminobutyric-acid
GPCR	-	G-protein coupled receptor
lALT	-	lateral antennal-lobe tract
LC	-	lateral cell cluster
LFG	-	Large female glomeruli
LH	-	lateral horn
mALT	-	medial antennal-lobe tract
MB	-	Mushroom bodies
mlALT	-	mediolateral antennal-lobe tract
MGC	-	macroglomerular complex
MOE	-	main olfactory epithelium
OB	-	olfactory bulb
OR	-	olfactory receptor
OSN	-	olfactory sensory neuron
PN	-	projection neuron
tALT	-	transverse antennal-lobe tract

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1: Introduction

Of all sensory systems, the sense of smell is considered the evolutionary oldest (Martin et al, 2011). The presence of an organ that facilitates detection and discrimination of chemosensory cues from the external environment is found in all organisms (Vosshall, Amrein, Morozov, Rzhetsky & Axel, 1999). A wide repertoire of behaviours such as mating and feeding, as well as processes related to learning and memory are highly influenced by odorants (Hildebrand & Shepherd, 1997). The crucial importance of olfactory ability is a general phenomenon characterizing all creatures across the animal kingdom (Menini, 2010).

1.1: Using insects as a model organism

The use of animals as so called "animal models" has a long and varied tradition within science. One of the earliest known examples of a model animal was the frog, used by the Dutch biologist Jan Swammerdam (1637-1680). This relatively simple model gave groundbreaking insight into the mechanisms of signal conduction in the nervous system. For several reasons, invertebrates have served as popular model animals since the 19th century (Galizia & Lledo, 2013). The advantage of using insects as model objects was evidently justified by Steven Kuffler in the seminal book from 1976 entitled "*From neuron to brain*". The following expression gives reason for studying so-called simple organisms: "We are convinced that behind each problem that appears extraordinarily complex and insoluble, there lies a simplifying principle that will lead to an unraveling of the events" (Kuffler & Nichols, 1976; cited by Galizia & Lledo, 2013).

Approximately 70% of all identified species are insects, making them a diverse animal group with a wide range of highly adapted behaviors. For many insects, olfactory stimuli are of fundamental importance for behaviors related to foraging, mating, and oviposition. The behavioral repertoire is reflected in a relatively modest nervous system – making insects particularly valuable as model organisms within neuroscience. Insect brains have 10^5 to 10^6 neurons, orders of magnitude less than the 10^{11} of a human brain (Menini, 2010). The accessibility of the insect brain combined with the many similarities in the olfactory system of various organisms have made these small creatures highly regarded as model animals for exploring olfactory mechanisms (Hildebrand & Shepherd, 1997).

1.2: The olfactory system of humans

In mammals, olfactory sensory neurons (OSNs) are found in the main olfactory epithelium (MOE) of the nasal cavity. Humans have about 6 million olfactory sensory neurons (Doty, 2001). Like in other organisms, these neurons are bipolar, with an unmyelinated axon at the basal surface and a single dendritic protrusion at the apical surface. The olfactory receptors (ORs) are located on the membrane of olfactory cilia that extend from the dendritic protrusion. These fingerlike structures are bathed in a mucus layer covering the nasal cavity. (Kandel, Schwartz, Jessel, Siegelbaum & Hudspeth, 2012). Mammalian cilia are smaller but more numerous compared to those of lower vertebrates (Kleene, 2008). Although so-called odorant binding proteins have been found in mammals, they have not been shown to have any functional significance (Hildebrand & Shepherd, 1997).

The ORs are part of the G protein coupled receptor (GPCR) superfamily. They can bind a great number of different odorant molecules, an ability linked to the divergence seen in their third, fourth, and fifth trans-membrane domains (Buck & Axel, 1991). There is also a divergence in the number of different ORs, and the genes coding for them. Humans have approximately 1000 OR genes. However, due to a relative large amount of pseudogenes, the number of functional ORs is 347 (Zozulya, Echeverri & Nguyen, 2001). Interestingly, any given OSN only one specific receptor type (Malnic, Hirono, Sato and Buck, 1999), giving rise to the "one neuron-one receptor" rule.

An OSN expressing a specific OR is restricted to one of four zones in the MOE, with a random topographical intrazonal distribution (Vassar, Ngai & Axel, 1993; cited by Axel, 2004). A specific odor molecule can activate several types of ORs, and a single OR can respond to several different odor molecules. Different combinations of ORs are activated by different odorants, and odorants with slightly different molecular structures activate over-lapping but different OR sets. Taken together, it seems likely that mammalian ORs use a combinatorial coding scheme (Malnic et al, 1999).

Axons from OSNs gather in a bundle of fibers that make up the olfactory nerve (cranial nerve I). In each hemisphere, the sensory axons project through the cribiform plate and terminate in the ipsilateral olfactory bulb (OB), the primary olfactory center of the vertebrate/mammalian brain. The OB is situated on the ventral anterior part of the cerebral hemisphere, just below the frontal cortex.

In the OB, the axon terminals of the OSNs form synapses with second order neurons in characteristic neuropils termed glomeruli (Shepherd, 2004). Mammalian glomeruli are thus

spheroidal structures consisting of dendritic branches of periglomerular, tufted, and mitral cells, in addition to OSNs. The border of each glomerulus is made up of several layers of glia processes and a ring of periglomerular cells. Mammalian glomeruli are typically 50-200 μm in diameter (Hildebrand & Shepherd, 1997). Each OSN axon target one glomerulus in the OB (Shepherd, 2004). Mammals show great variance in the number of glomeruli, with humans having surprisingly many; a low estimate is 5500 (Maresh, Gil, Whitman & Greer). Glomeruli are analogous to the so-called barrels and columns in the cerebral cortex, and a good example of the grouping of neuronal elements and synapses into clearly defined anatomical modules (Shepherd, 2004).

Although there is a large variation in the number of glomeruli, a large degree of convergence seems to be a common feature (Hildebrand & Shepherd, 1997), meaning that a single glomerulus is innervated by numerous OSNs that make synapses with a restricted number of mitral cells, the primary projection neurons (PNs) of the OB (Purves et al, 2012). Also acting as output neurons are the internal, external, and middle tufted cells, which are named according to their position in the external plexiform layer of the OB (Shepherd, 2004). Tufted cells are reported to have a quicker and broader response to odorants than mitral cells (Giessel & Datta, 2014).

There are two main kinds of local circuit neurons in the OB, periglomerular and the granule cells, the former innervating glomeruli, the latter making synapses with the basal dendrites of mitral cells in the external plexiform layer (Purves et al, 2012). Periglomerular cells are inhibitory, releasing dopamine and/or GABA. They are activated by direct glutamatergic input from OSNs and then retrogradely inhibit OSNs (Wilson & Mainen, 2006). Granule cells are believed to form inhibitory circuits with mitral cells, and they are assumed to be important for synaptic plasticity in the OB (Purves et al, 2012).

The previously mentioned combinatorial receptor code is preserved in the OB, in the form of a combinatorial glomerular code. Such a coding system allows for a better discrimination of odorants than what would be possible with a labelled line system. In addition, temporal coding involving spike timing of mitral/tufted cells is suggested to play a role in odor information processing (Rubin & Katz, 1999).

Bundles of mitral and tufted cell axons form the lateral olfactory tract as they project to brain areas known as the olfactory cortex. These regions include the piriform cortex, the cortical amygdala, the olfactory tubercle, the anterior olfactory nucleus, and the lateral enthorinal cortex (Giessel & Datta, 2014). The piriform cortex of humans, found in the ventromedial aspect of the temporal lobe, is a three layered cortical structure (Purves et al,

2012). It is the primary target of olfactory information from the OB. Layer 1a receives afferent axons from the OB, layers 2 and 3 are involved with feedback, and feed-forward inhibition/excitation. The olfactory tubercle is an extension of the striatum, receiving input from other olfactory areas, as well as the hippocampus, prefrontal cortex, and amygdala. It outputs to the pallidum, thalamus, and hypothalamus. Although it does not output to other olfactory areas, it is bi-directionally connected to the ventral tegmental area, which integrates olfactory information with motivational state information received from the basal forebrain (Giessel & Datta, 2014).
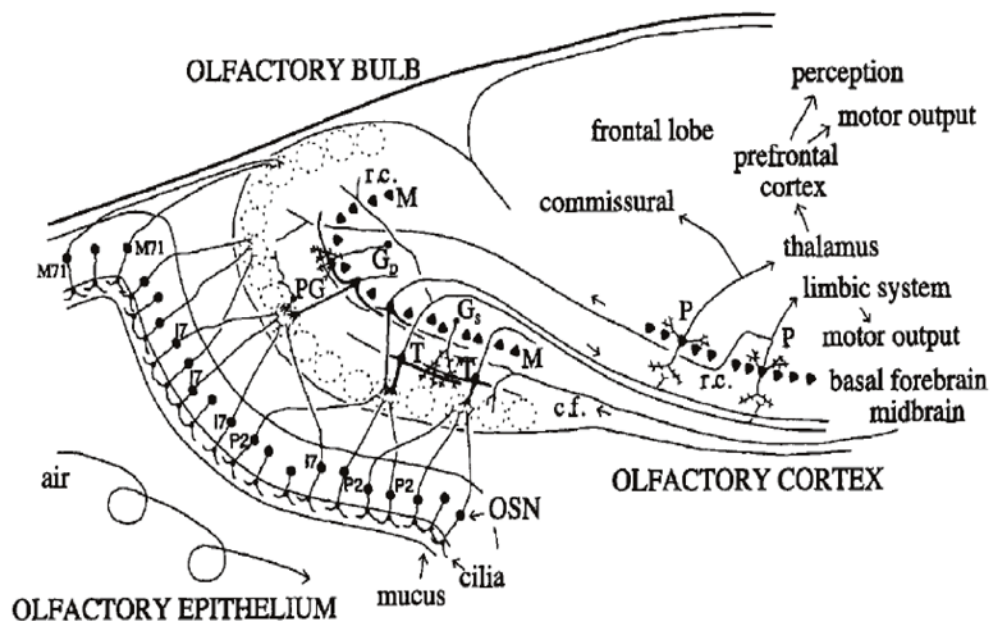

Figure 1.1. Overview of the olfactory pathway. The olfactory bulb receives input from the olfactory sensory neurons in the olfactory epithelium and projects to the olfactory cortex. The diagram indicates some essential aspects of the projection patterns between the regions, as well as the main neural elements within the olfactory bulb. Note that the olfactory epithelium is arranged in overlapping populations of olfactory sensory neurons (e.g., M71,17, P2) which project to individual glomeruli. Some of the central olfactory connections to limbic brain structures are also indicated. Abbreviations: c.f., centrifugal fiber; G_D, deep granule cell; G_S, superficial granule cell; M, mitral cell; OSN, olfactory sensory neuron; P, pyramidal cell; PG, periglomerular cell; r.c., recurrent axon collateral; T, tufted cell. Figure and legend from Shepherd, 2004.

1.3: The olfactory system of insects

1.3.1: The peripheral olfactory system

In insects, the main olfactory organ consists of numerous olfactory sensilla, hair-like structures covering the flagellum, which is the third segment of the antennae. The numerous sensilla consist of various morphological categories and each single sensillum can contain a variable number of OSNs. Similar to human OSNs, those in insects are bipolar, with a single axon at their basal surface and a single dendritic process on their apical side (Kaupp, 2010). The insect OSNs make up approximately one third of the total assembly of cells on the flagellum; the rest are sensillar support cells and glia cells (Vosshall et al, 1999).

When an odorant molecule hits the sensilla, it sticks to the waxy surface before diffusing trough the pores of the cuticular wall. Since the sensillum lymph is aqueous, a large number of odorant-binding proteins transport the hydrophobic odorant molecule to the receptor on the dendritic membrane (Hildebrand & Shepherd, 1997).

As compared to mammals, insects have relatively few genes coding for ORs. However, OR pseudogenes are seemingly non-existent in insects (Kaupp, 2010). The fruit fly, *Drosophila melanogaster*, for example, has 60 OR genes, which trough alternative splicing codes for 62 ORs. Although the number of OR genes vary due to the presence of species-specific receptors, the gene family is highly conserved between insect species (Robertson, Warr & Carlson, 2003). Insect OR are heteromeric ligand-gated ion channels consisting of two-G-protein coupled proteins, one being a conventional OR and the other a highly conserved co-receptor named OR83b (Sato et al. 2008). This dual receptor complex has been compared to the "one neuron, one receptor" in mammals (Kaupp, 2010). Concerning the transduction process, the details are still uncertain (for a review, see Kaupp, 2010). Sensilla housing OSNs appear to be randomly distributed along the proximal-distal and dorsal-ventral axis of the flagellum (Vosshall et al, 1999).

The male moth has established two olfactory sub-systems, one for pheromones and one for plant odors. A number of publications have reported about physiological properties of sensory neurons both in males and females of heliothine moths. In general, the sensory neurons are reported to respond specifically to one or a few odor molecules (Almaas & Mustaparta, 1991; Berg, Almaas, Bjaalie & Mustaparta, 1998; Røstelien, Borg-Karlson, Fäldt, Jacobsson & Mustaparta, 2000a; Røstelien et al, 2000b). This principle seems to apply to the

plant odor-sensitive neurons – which have been investigated via single cell recordings combined with gas chromatography – as well as the pheromone-responding neurons.

1.3.2: The antennal- lobe

The antennal-lobe (AL) is the analogue to the mammalian OB and thus the primary olfactory center of the insect brain. Like in mammals, the OSNs project to the ipsilateral olfactory center. Since the number of OR genes in insects are relatively few compared to that of mammals, a considerably lower number of glomeruli are present in the primary olfactory center, typically 50-300 (Hildebrand & Shepherd, 1997). The heliothine species, *Heliothis virescens,* has approximately 65 glomeruli (Berg, Galizia, Brandt & Mustaparta, 2002; Løfaldli, Kvello & Mustaparta, 2010). In male moths, there is a division between "ordinary" glomeruli and pheromone specific glomeruli. The latter category forms the so-called macroglomerular complex (MGC; Berg et al. 1998). Also, in some species of female moths, two enlarged glomeruli located near the entrance of the antennal nerve has been identified as female-specific, the so-called large female glomeruli (LFG) (Galizia & Rössler, 2010).

In addition to the terminals of OSNs, the AL consists of two main neuron categories, projection neurons and local interneurons. The projection neurons are categorized as uniglomerular, meaning they innervate a single glomerulus, or multiglomerular, meaning that they innervate several glomeruli (Wilson & Mainen, 2006). Also, the projection neurons are often categorized according to the antennal-lobe tract they are confined to. The majority of the local interneurons are GABAergic. Tanaka et al have classified the local neurons in two categories, LN1 and LN2. The former category arborizes in the glomerular core and the latter in the entire glomerulus (Tanaka, Endo & Ito (2012)). Similar to the neural network in mammals, OSNs form synaptic contact mainly with local neurons which then can inhibit PNs trough dendro-dendritic synapses (Wilson & Laurent, 2005).

1.3.3: Antennal-lobe tract nomenclature

Note that the naming of the olfactory tracts of insects follow the guidelines proposed by Ito et al. for a systematic nomenclature of the insect brain, based on *D. melanogaster* (Ito et al, 2014). Previous publications have referred to the three main protecerebral tracts originating from the antennal-lobe as the inner, the middle, and the outer protocerebral tract (PCT). More recently, the terms medial, mediolateral, and lateral PCTs appeared. Also, the

term antennocerebral has occasionally been used instead of protocerebral. According to the suggestion from Ito et al., these three tracts are now referred to as the medial antennal-lobe tract (mALT), the mediolateral antennal-lobe tract (mlALT), and the lateral antennal-lobe tract (lALT).

A fourth ALT recently found in heliothine moths has previously been referred to as the second mediolateral ALT. Due to the similarity between this tract and the so-called transverse ALT (tALT), recently identified in *D. melanogaster*, we have chosen to use this term for the current tract (Tanaka et al, 2012).

1.3.4: The tracts of the antennal-lobe

Although different insect species show a wide variety in which ALTs are present, for moths, the relevant ALTs are the previously mentioned mALT, mlaLT, lALT, the so called main ALTs. These ALTs have been described in many species of moth (Homberg, Montague & Hildebrand, 1998), *H. virescens* included (Rø, Müller & Mustaparta, 2007). Sometimes a fourth tract, the tALT is visualized. The ALTs carry olfactory information to higher order olfactory centers in the moth brain.

The mALT is the most prominent of the ALTs. It has two roots, the ventral and the dorsal (mALT-VR and mALT-DR), respectively) which project medio-ventrally from the lateral cell cluster I (LC) and MGC region the AL. As they turn caudally towards the posterior-lateral part of the central body (CB), the roots fuse together (Homberg et al, 1988). The mALT then projects posteriorly towards the calyces of the mushroom bodies (MB). After innervating the calyces, the mALT projects antero-laterally before terminating in the lateral horn (LH) (Rø et al, 2007). Note that the mALT contains three main types of PN, based on their projection patterns. These are called type *a*, *b* and *c*. Pm*a* indicates a type a PN in the mALT. The most common are Pm*a*, which are uniglomerular PNs. Type *b* includes axons that bypass the calyces before terminating in the LH. The third category, type *c*, consists of multiglomerular PNs terminating in the LH before projecting further to the calyces (Homberg et al, 1988).

Because of the prevalence and similarity of the mALT in a wide range of insect species, it is believed to be of great importance for carrying olfactory information to higher brain centers (Galizia & Rössler, 2010).

The mlALT projects from the AL together with the mALT, but near the CB it turns laterally towards the LH. Since it has contains fewer axons than the mALT it appears as less

prominent. Unlike mALT PNs, mlALT PNs are mainly multiglomerular, with smaller somata located in LC II (Homberg et al, 1988).
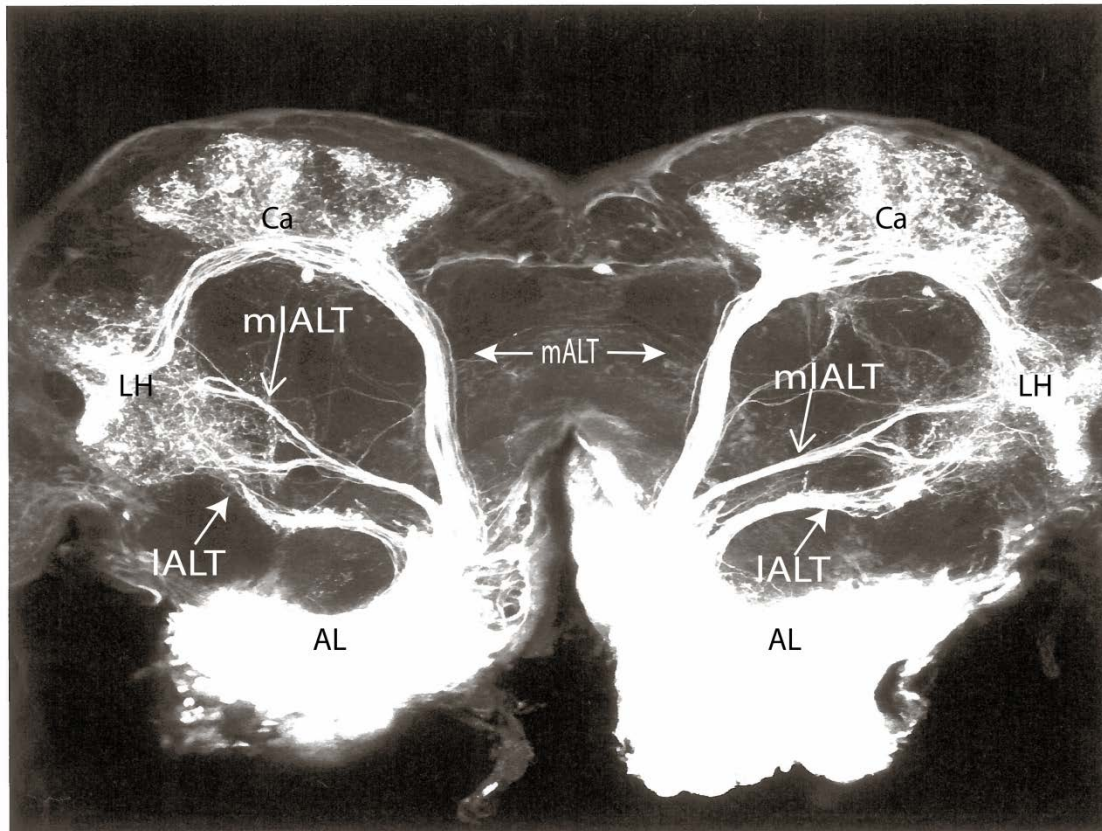

Figure 1.2. Confocal image showing a dorsal view of a moth brain. The medial antennal-lobe tract (mALT) is seen projecting posteriorly from the antennal-lobe (AL) as it innervates the calyces (Ca) before turning antero-laterally before terminating in the lateral horn (LH). Also seen are the lateral ALT (lALT) and the mediolateral ALT (mlALT) as the project directly to the LH. Confocal scan made by Xin-Cheng Zhao, unpublished data.

The lALT consists of very thin axons, which after leaving the AL ventral to the ventral root of the MALT, project directly to the LH. Compared to the mlALT its course is more antero-ventral (Rø et al, 2007). lALT fibers have been described as going in different directions soon after leaving the AL, and being difficult to separate from surrounding tissue, both issues that contribute to the lALT being less prominent and defined compared to the mALT and mlALT (Homberg, 1988). Although some PNs in the lALT have been identified as being uniglomerular, the majority are multiglomerular (Rø et al, 2007), with their somata situated in the LC I (Homberg et al, 1988).

A fourth ALT, whose presence and numbers vary greatly between insect species can sometimes be visualized. Although a study on *D. Melanogaster* found four novel tALTs, with

three innervating the LH exclusively, and the fourth innervating an area in the posterior lateral protocerebrum (Tanaka et al, 2012), details surrounding its presence and morphology in moths remains somewhat elusive. Confirming whether the tALT is present in female *H. virescens* is part of this current investigation.

1.3.5: Olfactory information processing in higher order brain areas

Similar to other insects, moths have two main areas in the protocerebrum for processing olfactory information, the calyces of the mushroom bodies (MB), and the lateral horn (LH) (Galizia & Rössler, 2010). Whereas the calyces receive input from several sensory modalities in many insects, the calyces of moths is dedicated mainly to processing olfactory information. In general, the calyces are innervated by the dendrites of numerous densely packed neurons named Kenyon cells. The axons of Kenyon cells project along the lobes, which function as the MBs output (Galizia & Szyszka, 2008). The calyces, located posterior-dorsally in the protocerebrum, consist of two parts, or cups, the lateral and the medial calyx. At their base, the cups form the tracts known as the peduncle and the Y tract. Both tracts project to the various lobes of the MB (Rø et al, 2007). Although there are uncertainties regarding the processing in the MB, some types of memory and learning are reported to be correlated with specific lobes (Strausfeld, Sinakevitch & Vilinsky, 2003). Interestingly, a recent publication dealing with heliothine moths, has demonstrated that medial-tract PNs originating from the MGC and ordinary glomeruli, respectively, form distinct projection patterns in the calyces (Zhao et al. 2014).

The LH is a collection of neuropil in the lateral protocerebrum that receives multisensory, but mainly olfactory input (Galizia & Rössler, 2010). It has been described as a premotoric area (Rø et al, 2007). However, the majority of third order olfactory neurons receiving input from the antennal-lobe PNs seem not project to the ventral cord (Pramod , 2014). The lateral accessory lobe, on the other hand, which is positioned posteriorly to each AL appears to receive odor information that is relayed directly to the ventral cord (Wada & Kanzaki, 2005).

Due to the MB being associated with learning and memory, the LH has been hypothesized to be involved with more non-associative behavioral responses evoked via olfactory input (Tanaka, Awasaki, Shimada & Ito, 2004). In particular, the LH has been suggested to play a role in odor detection and discrimination (Homberg et al, 1988). Recently, new results on heliothine moths were published showing that different types of olfactory

signals are distinctly represented in the LH. Similar to previous findings in the fruit fly and the silk moth, these data demonstrate that pheromones and plant odors are represented in non-overlapping regions of the LH (Zhao et al. 2014). Also, PNs carrying information about pheromones and interspecific signals, respectively, each of which evokes a particular behavioral response, were reported to target partly distinct areas of the LH (Zhao et al, 2014).

1.6: Main goal of the present study

Principal goal: To explore the anatomical arrangement of the antennal-lobe tracts of the female heliothine moth.

Specific goals:
- To map the general projection pattern of the antennal-lobe tracts in the female moth by means of anterograde mass staining.
- To explore whether the transverse ALT is present in female heliothine moths.
- To investigate whether female-specific antennal-lobe glomeruli give rise to projection neurons targeting distinct regions of the lateral horn as compared to projection neurons originating from the ordinary glomeruli.

2: Method

The method used in this study was *in vivo* fluorescent staining of the brain tissue of moths. In more detail, fluorescent dye applied to specific parts of the brain was carried by the anterograde axonal transport system of the stained neurons.

2.1: Insects and insect culture

This study was carried out using females of the tobacco budworm *H. virescens,* and the cotton budworm, *H. armigera* (Both Lepidoptera: Noctuidae: Heliothis). Some of the moths were reared in the laboratory, and some arrived as pupae. All pupae were sorted by gender, and after hatching they were kept in Plexiglas cylinders in gender specific heating cabinets (Refritherm 6E incubator, Struers). The heating cabinets maintained a constant temperature of 27° C and 70% air humidity. Their day-night cycle was programmed to give 14 hours of light, then 10 hours of darkness. Each Plexiglas cylinder contained a set amount of moths, either five or eight, depending on cylinder size. The source of nutrition was a sucrose solution (100g saccharose per liter of water), with one cup in each cylinder.

Although hatched male and female moths are kept in separate heating cabinets, the antennae were inspected before each experiment to confirm that specimens were female.

2.2 Preparation and dye injection

For this study, 77 moths were stained, all of them female. A stereo microscope (Leica, MZ 12.5) was used for preparation and staining. The moth was put in a small plastic tube, and dental wax (Kerr Corporation, Romulus, MI, USA) was used to immobilize the protruding head. After removing hair, the cuticle was cut in a rectangular shape, using a razorblade knife. To allow better access to the brain, a fine needle was inserted through each eye lobe, keeping them as separate as possible. Then muscle tissue, trachea and the membrane covering the AL were taken away from the exposed brain using a set of fine forceps. For staining of the antennal-lobe tracts, dye crystals were applied into the glomeruli of the antennal lobe using a micro-needle, or a glass pipette. Glass pipettes were made using a Flaming-Brown pipette puller (P97; Sutter instruments, Novarto, CA, USA).

The fluorescent dye used was Dextran tetramethylrodamine/biotin (3000MW; micro-ruby; ext/emis: 490/508nm).). In some experiments, a second dye was used in addition to micro-ruby, i.e Dextran fluorescein/biotin (3000MW; micro-emerald; ext/emis: 550/570nm). In these experiments micro-ruby was applied to the central or ventral region of the antennal lobe, whereas micro-emerald was applied close to the entrance of the antennal nerve, in the region of the LFG. Both dyes were from Life technologies. Dyes were stored in a freezer at -20° C, maintaining the form of the dye crystals. After being taken out of the freezer, the glass containing the dye was kept in room temperature for a short while before being opened, to avoid that the crystals melt.

Before injecting the dye crystals, fluids were removed from the exposed brain using fine paper. After the injection of dye, Ringers solution (Nacl: 150mM, cacl2: 3mM, KCL: 3mM, TES buffer: 10mM, Sucrose ($C_{12}H_{22}O_{11}$): 25mM, ph 7.2) was dripped onto the exposed brain using a suction pipette. The exposed brain was then covered with fine paper soaked in Ringers solution. This helps prevent both dehydration and degeneration of neural tissue. To allow for propagation of the fluorescent dye, moths were kept in darkness either at room temperature for two hours, or overnight in a fridge, at 4° C.

Figure 2.1. Image showing a female *H.virescens* stuck in
Wax, prior to the head being immobilized.

2.3: Dissection

After removing the moths head using fine scissors, the head was mounted in a blob melted wax in a small glass dish, then submerged in Ringers solution. Fine scissors, a scalpel and various forceps were used to remove the head capsule, the outer pigmented layer of the eye lobes, the proboscis, maxilla, as well as various muscles and tracheas.

2.4: Fixation and dehydration

Dissected brains were submerged in a 4% paraformaldehyde solution (Roti, Histofix, Carl Roth, GmbH +Co.KG, Germany), either overnight, refrigerated to 4°, or in room temperature for 2-4 hours. Following fixation, brains were rinsed using a phosphate buffer solution (PBS NaCL: 684mM, KCL: 13mM, Na_2HPO_4: 50.7 mM, KH_2PO_4: 5mM, PH 7.4). Then they were dehydrated in consecutively stronger alcohol series (50%, 70%, 90%, 96%, 100% & 100%), each step taking ten minutes. To ensure that PBS and alcohol properly contacts brain tissue, both rinsing and dehydration was performed on a rotator.

Finally, prepared specimens were placed on a metal slide that fits in microscope holders. The metal slide has a hole, in which the brain is kept between two transparent coverslips. Before the second coverslip, acting as a lid was fastened, the brain was submerged in methylsalicylate, giving the brain itself, but not the stained neurons transparent properties.

2.5: Initial examination and confocal imaging

Examining of preparations was performed in two steps. First they were examined under a light microscope, with fluorescent filters*. Preparations that showed a successful uptake of fluorescent dye were then scanned using a laser confocal microscope (LSM Zeiss 510 Meta Mira 900F, GmbH, Jena, Germany).

While a 10x0.45W objective was used to establish an overview of the brain, all actual scans were performed using a 20x0.45 objective The scan resolution was set to 1024x1024 pixels, with a scan speed of either 6 or 7. All scans were made with a z-slice thickness of 2.1-3.2µm. A helium neon laser (543nm wavelength) was used excite micro-ruby/CY3, and an argon (488nm wavelength) laser was used for micro-emerald/Cy2. While pin hole diameter

was set using the "optimal" function in the scanner software, settings such as detector gain, detector offset and amplification* was set manually according the specific preparation being scanned. Upon completion, scanned image stacks were saved in the .lsm format (Zeiss image files).

2.6: Studying and processing scanned images

The .lsm files were studied using the LSM 510 image browser software (Carl Zeiss microscopy, Jena, Germany version 4.2.0 121). The software allows for exporting single slices, or composites consisting of several optical slices to external graphic editors. The image browser also has a built in feature for exporting 3D images of scanned brains.

Properties such as brightness/contrast and rotation were adjusted in Adobe Photoshop CS5 (Version 12.0, Adobe systems, San Jose, CA, USA). Edited images were then arranged in grids using Adobe illustrator CS6 (version 16.0), also from Adobe systems.

2.7: Ethics regarding moths as research animals

There are regulations regarding the use of animals for research purposes in Norway, "lov om dyrevelferd). However, the animals that are subject to this law include mainly vertebrates, and only a few species of invertebrates, of which there are no species of lepidoptera (https://lovdata.no/dokument/NL/lov/2009-06-19-97). While this means that there are no juridical considerations regarding moths as research animals, we strive to take as good care of them as we can. The insect containers are inspected on a daily basis, and the sucrose containers are re-filled and cleaned as needed. The same goes for papers in the bottom and sides of the cylinder, which provides the moths with a climbable surface, as well as helping with hygiene and waste disposal. There is also a limit on how many moths that are kept in a single cylinder, to avoid unnecessary stress.

3: Results

For this study, a total of 77 moths were stained, 37 *H. armigera*, and 40 *H. virescens*. In most preparations, micro-ruby crystals were applied to both ALs. Totally, seven successfully stained ALs were obtained from these experiments. In addition, four attempts to perform double-labeling with micro-ruby in one AL, and micro-emerald in the same brain preparation resulted in two successful stainings. The figures presented in this chapter are from *H.virescens* only. For simplification, some confocal images have been rotated vertically and/or reflected horizontally, to ensure a uniform layout. The common orientation is demonstrated in the schematic in figure 3.1.
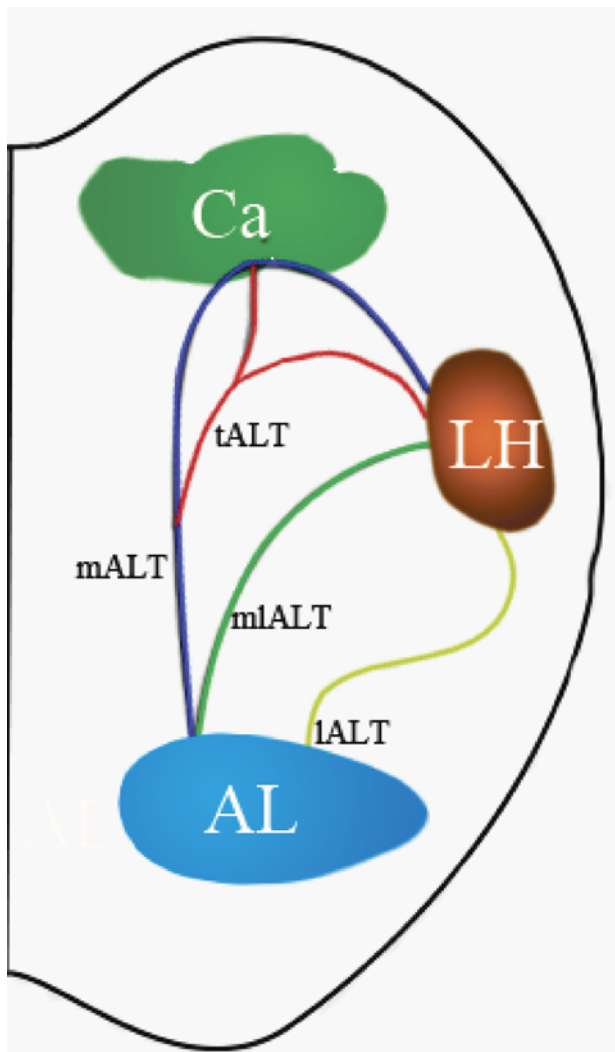

Figure 3.1. Schematic display of one brain hemisphere, the dorsal orientation applies to all confocal images presented in the subsequent figures. Ca Calyces, mALT medial antennal lobe tract (ALT), lALT lateral ALT, mlALT mediolateral ALT, tALT transverse ALT, LH lateral horn, AL antennal lobe.

3.1: Antennal-lobe tracts in the female moth

In most of the successfully stained preparations, the three main ALTs, previously described both in male and female moths, the mALT, the lALT and the mlALT, were visible. Also, the more recently discovered was the tALT was visible in some brains.

3.1.1: The medial antennal-lobe tract

The mALT could be seen as a relatively prominent fiber bundle in all scanned preparations, and it can be argued that visualizing the mALT is a minimum requirement for a successful staining. In the figures shown here (Fig 3.2-3.7), the mALT can be seen projecting from the AL to the LH, but innervating the calyces on its way. The mALT leaves the medial part of the AL, projecting posteriorly. The thick fiber bundle passes ventrally of the central body before it turns in a lateral direction anteriorly of the calyces. After innervating the calyces, the mALT projects anterior-laterally towards the LH, where it terminates.

3.1.2: The lateral antennal lobe tract

The lALT can be seen as a fiber bundle leaving the posterior-lateral side of the AL, to then project to the LH (Fig 3.3 and 3.4). This tract projects directly to the LH. Also, as demonstrated in all figures, it is more ventrally positioned than either of the other ALTs. It appears as thicker than the mlALT.

3.1.3: The mediolateral antennal lobe tract

The mlALT can be seen to be thinner than both the mALT and the lALT. It leaves the AL along with the mALT, but shortly afterwards turn laterally towards the LH, where it terminates (fig 3.2-3.7). Before terminating in the LH, the mlALT splits into several fiber bundles which innervate different areas (fig 3.2. 3.5, 3.6).

3.1.4: The transverse antennal lobe tract

The figures presented here also show the tALT. This tract is seen as a thin fiber bundle that leaves the AL together with the mALT. Like the mlALT, it diverges from the mALT and projects laterally. Compared to the mlALT, the tALT diverges closer to the calyces. This tract spreads into at least three sub-branches, two terminating in different parts of the LH and one in a protocerebral region located anterior to the calyces (Fig. 3.4).

3.2: Projection patterns in the lateral horn

In the two successfully double-stained preparations, micro-emerald was applied to the region near the antennal nerve and micro-ruby to the more central and ventral part of the antennal lobe. As shown in figure 3.6, the lateral horn is extensively innervated by axon terminals labeled by each of the two dyes. Based on the confocal images from the present study, these projections seem to overlap completely.
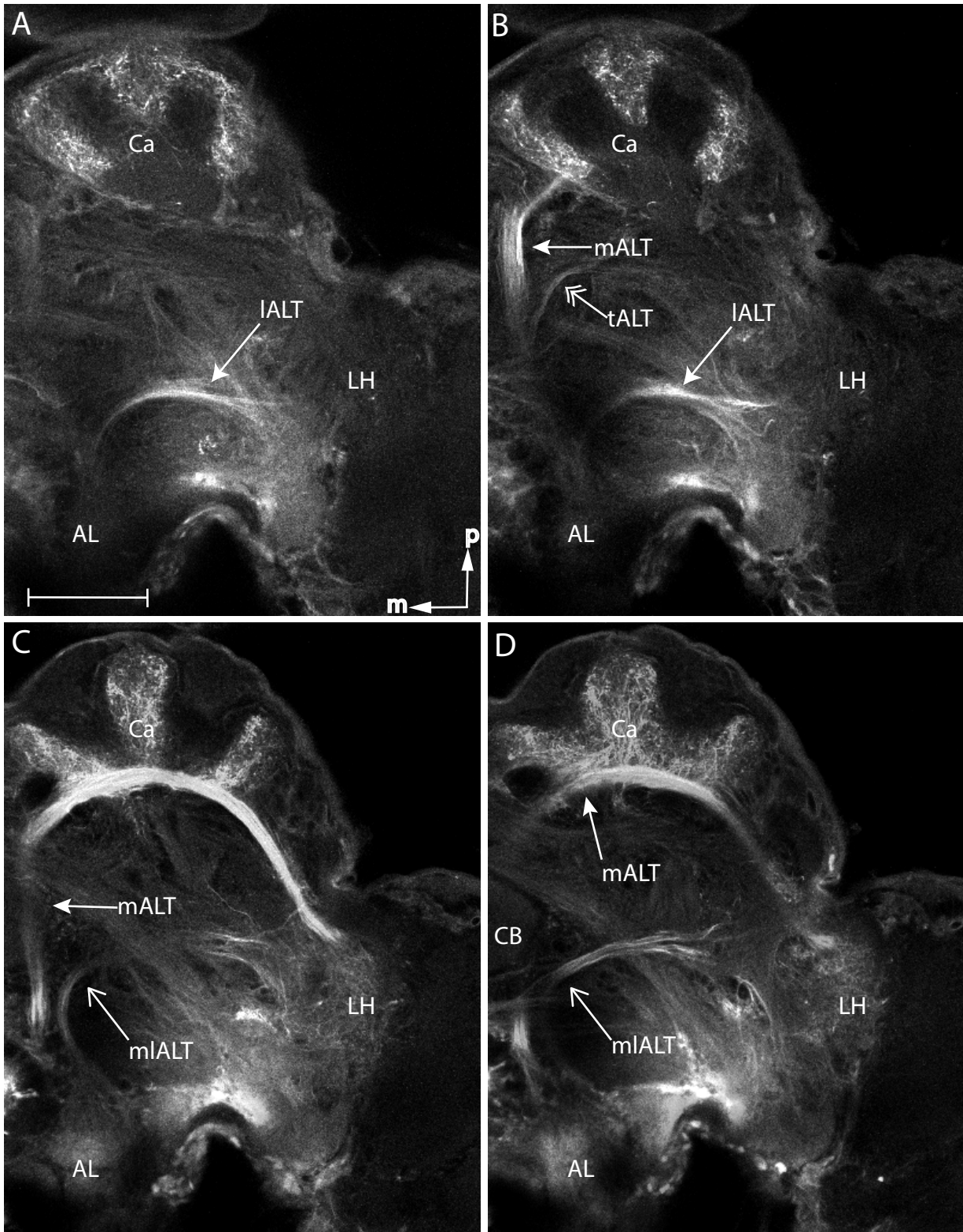
Figure 3.2. Confocal images showing the antennal-lobe tracts (ALTs). The images in (A-D) represent single slices in a ventral to dorsal position. A, Slice showing the lateral ALT (lALT), projecting from the antennal lobe (AL) to the lateral horn (LH). B, Slice showing in addition to the lALT, the medial ALT (mALT) which terminates in the LH after passing via the calyces (Ca). Also, the transverse ALT (tALT), dividing from the mALT can be seen. C-D, Two slices showing the mediolateral ALT (mlALT), passing together with the mALT close to the AL, and then projecting directly to the LH. Scale bar 100 μm. p posterior, m medial. CB central body.
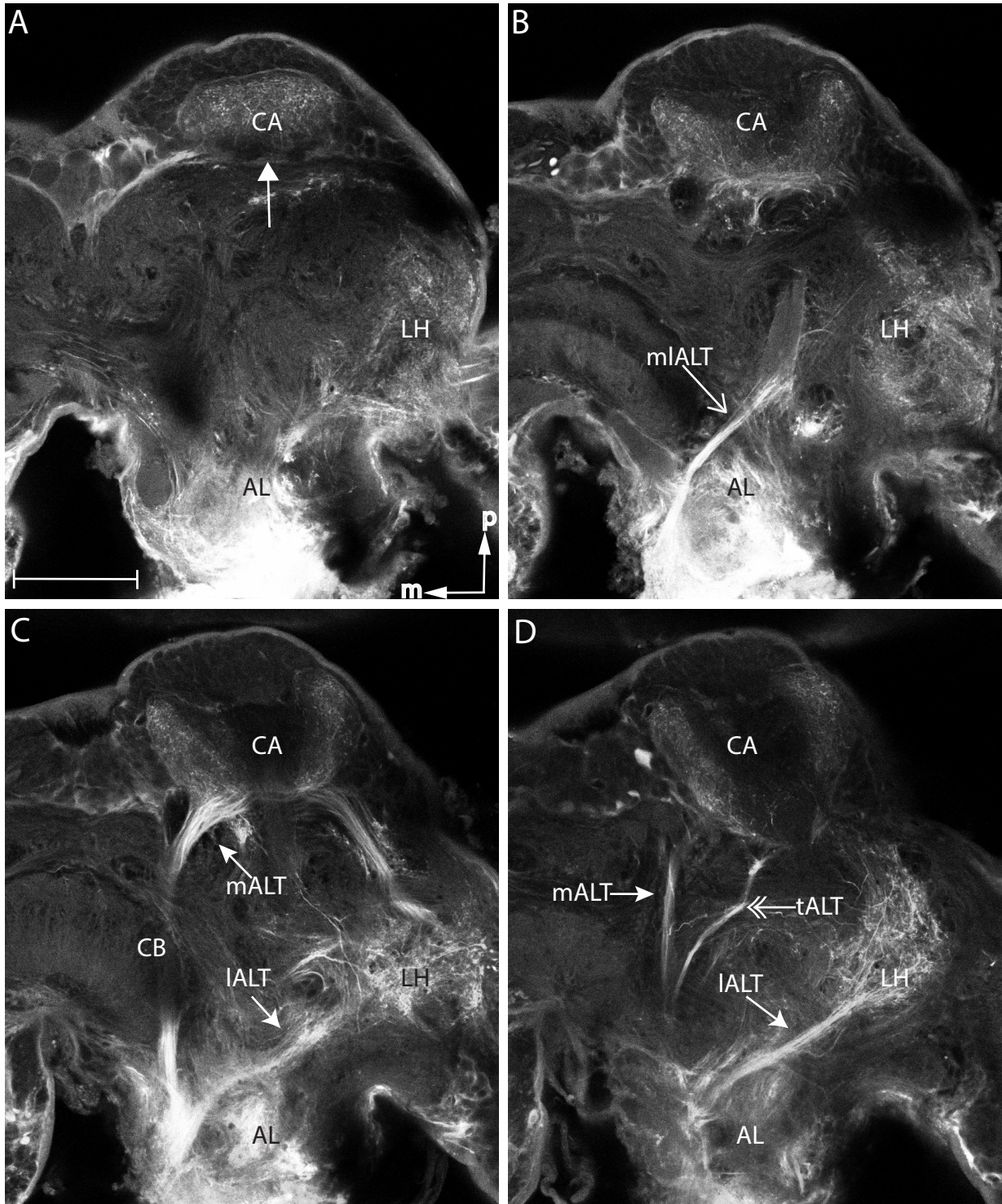
Figure 3.3. Confocal images showing the antennal lobe tracts (ALTs). The images in (A-D) represent single slices in a dorsal to ventral position (As compared to the brain in Fig. 3.2. the preparation shown here is tilted a bit more dorsally). A, Slice showing the calyces of the mushroom bodies (Ca). B, Slice showing the mediolateral ALT (mlALT), which projects directly from the antennal lobe (AL) to the lateral horn (LH). As shown in the confocal image, the mlALT turns laterally nearby the lateral edge of the central body (CB). C, Slice showing the medial ALT (mALT), which terminates in the LH afterpassing via the calyces (Ca).Also, the lateral ALT (lALT), which projects directly from the AL to the LH is visible. D, slice showing the course of the mALT, the lALT and the transverse ALT (tALT). The last mentioned tract pass together with the mALT, and then projectis laterally giving off branches in a region close to the anterior part of the Ca, and to the LH. Scale bar 100 μm. p posterior, m medial.
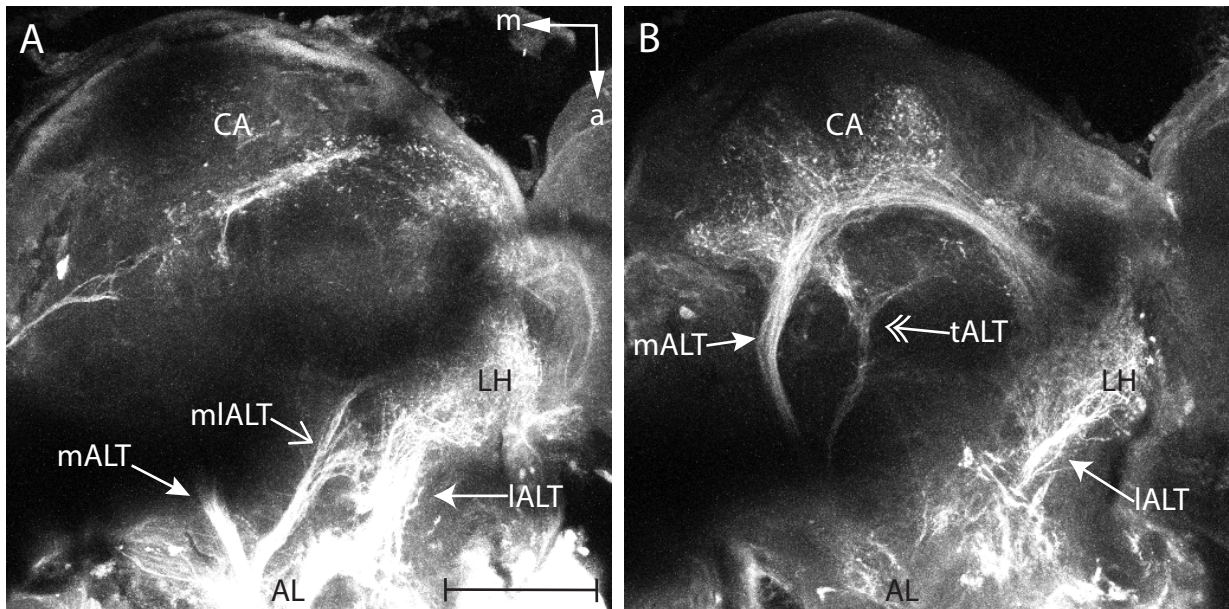
Figure 3.4. Confocal images showing the antennal-lobe tracts (ALTs), in a dorsal to ventral position. A, is a composite consisting of 25 optical slices, showing the course of the three main ALTs nearby the entrance of the antennal lobe (AL). Both the lateral ALT (lALT) as well as the mediolateral ALT (mlALT) project directly from the AL to the lateral horn (LH). Also seen is the mediolateral ALT (mlALT), passing together with the mALT close to the AL, and then projecting directly to the LH. The mALT on the other hand, passes more posteriorly. B, is a composite consisting of 13 images, showing the lALT, the mALT and the transverse ALT (tALT), which divides off from the mALT. Scale bar 100 µm. m medial, a anterior.
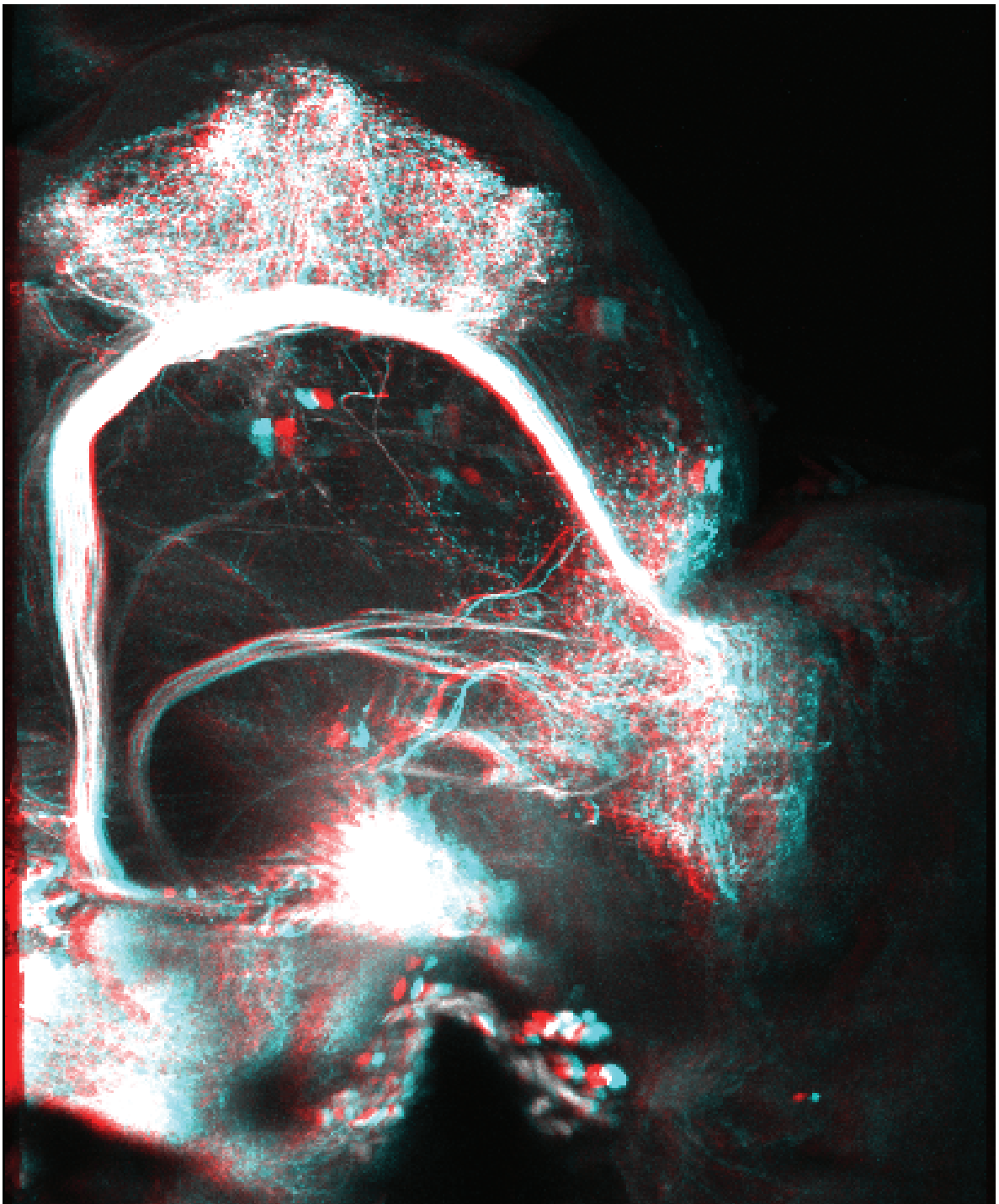
Figure 3.5. A three-dimensional composite of a stack of confocal images showing the routes of the antennal lobe tracts (ALTs) in a dorsally oriented hemisphere. A stereoscopic 3D effect is achieved by using red/green glasses (red for the left eye and green for the right entails a dorsal view). Image is from the same preparation as in figure 3.2.
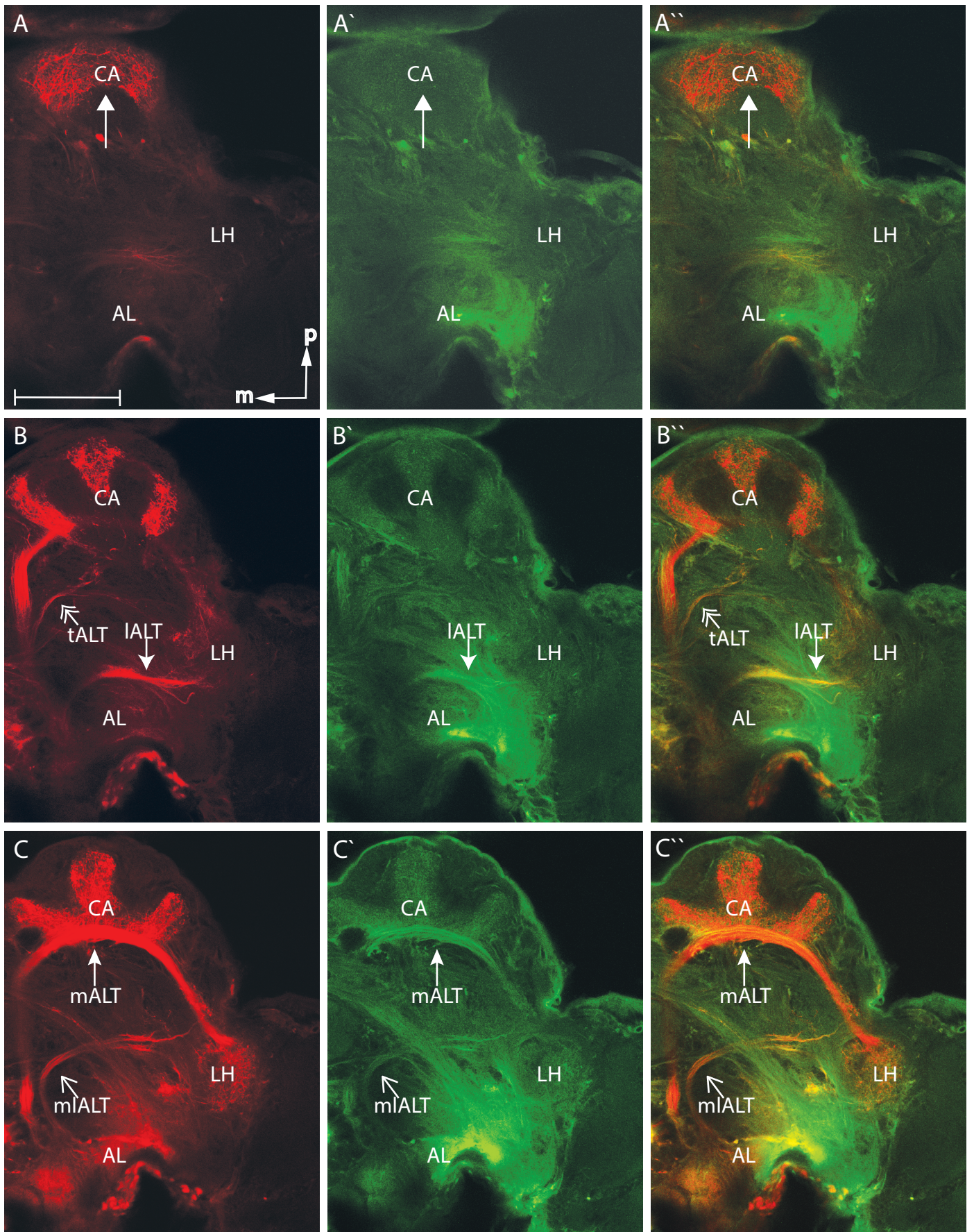
Figure 3.6.

Figure 3.6. Confocal images of a double-labelled preparation showing the antennal-lobe tracts (ALTs). The images in (A-C), (A'-C`) and (A``-C``) represent single slices in a ventral to dorsal position, showing staining from micro-ruby, micro-emerald, and both, respectively (Micro-ruby was applied to the central and ventral part of the antennal lobe (AL) whereas micro-emarald was applied to a small region close to the entrance of the antennal nerve). A-A``, slices showing the calyces (Ca) of the mushroom bodies. B-B``, Slices showing the medial ALT (mALT) which terminates in the LH after passing via the calyces (Ca), and the lateral ALT (lALT), projecting directly from the antennal lobe (AL) to the lateral horn (LH). Also, in B and B`` the transverse ALT (tALT), appears as a thin fiber bundle dividing from the mALT.  C-C``, slices showing the mALT, and the mediolateral ALT (mlALT). As shown in the confocal scan, the mlALT projects together with the mALT near the AL, and then turns laterally passing directly to the LH. Scale bar 100 µm. p posterior, m medial Images are from the same preparation as in figure 3.2.
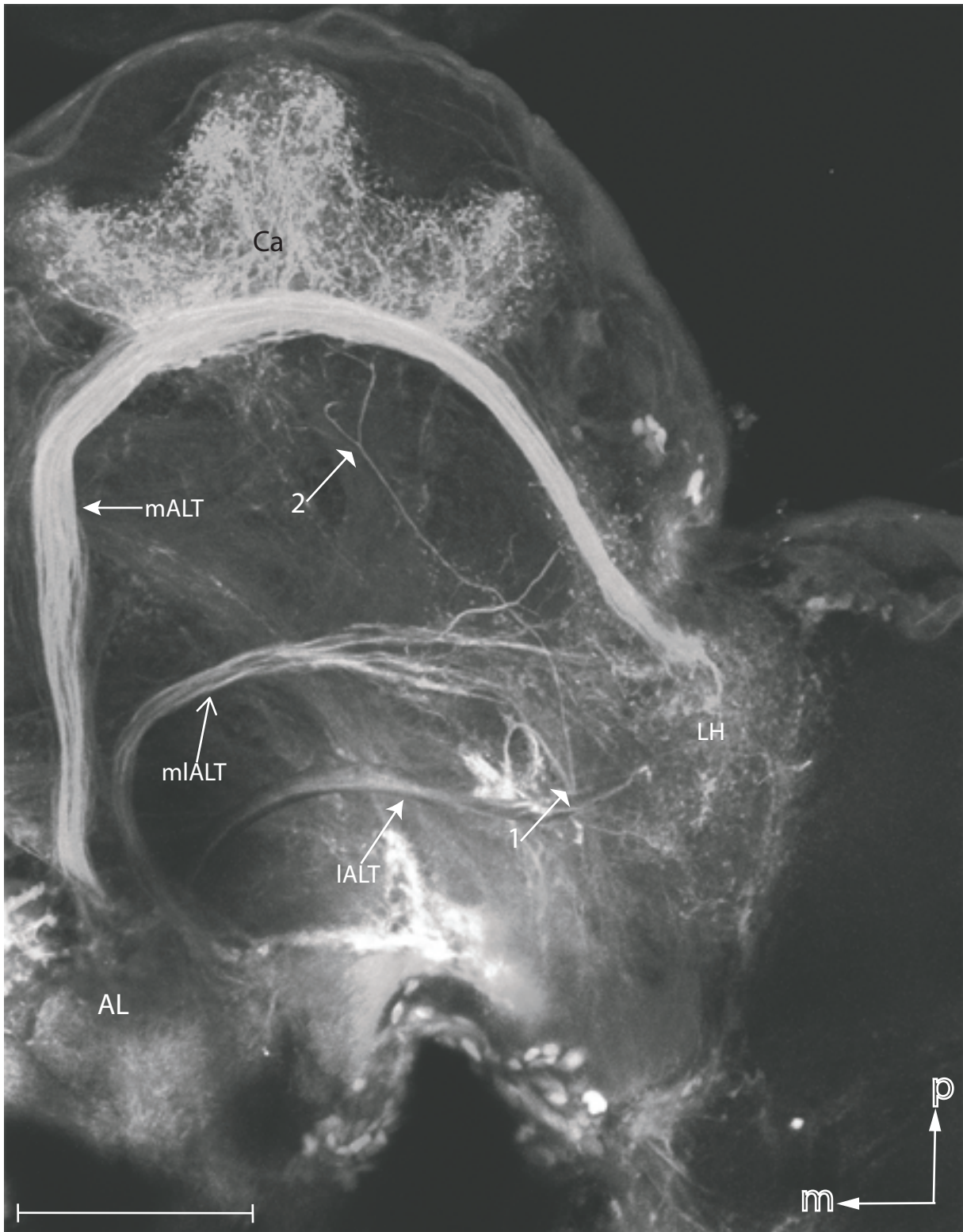
Figure 3.7. Confocal image showing one lateral-tract projection neuron innervating the calyces (Ca). As indicated by arrow 1, one axon branches off from the lateral antennal lobe tract (lALT), and projects postero-medially. As indicated by arrow 2, it divides anteriorly of the calyces and each sub-branch innervates one calyx. The confocal image consists of two stacks of optical slices merged together. AL antennal-lobe, LH lateral horn, Ca calyces, mALT medial ALT, mlALT mediolateral ALT, lALT lateral ALT, p posterior, m medial, scale bar 200 μm.

4: Discussion

The general goal of this project was to study the projection pattern of the ALTs of the heliothine female moth. In particular, we wanted to ascertain whether the antennal-lobe tract recently discovered in heliothine males, the tALT, is present in females. Also, we aimed at investigating the terminal projections in the LH in order to compare the innervation pattern with that previously described in males.

4.1: Summary of the results

Successful staining of antennal-lobe projection neurons visualized the three classic ALTs, the mALT, the lALT, and the mlALT. In addition, a fourth tract, classified as the tALT, was labelled. The confocal images showed that all ALTs terminate in the protocerebral region called the LH. The prominent mALT also innervates the calyces of the mushroom bodies extensively. Interestingly, in one preparation a stained lALT axon sending neural branches into this neuropil was visible. As regards the termination pattern of the medial-tract projection neurons in the LH, no obvious distinctions between neurons originating from different glomerular regions appeared. However, in order to determine the target regions of mALT neurons in the lateral horn of the female, additional staining experiments with the use of two dyes have to be performed.

4.2: Anatomical arrangement of the antennal-lobe tracts

4.2.1: The transverse antennal-lobe tract

The appearance of a thin fiber bundle, which branches off from the medial tract posterior of the central body, confirms the presence of the tALT in the heliothine female. This tract seems to be the thinnest of the ALTs. The tALT was discovered in the *H. virescens* male during a former master project in the lab (Lillevoll, 2012). Actually, it appears to have been labeled in the *H. virescens* female in a previous work (Løfaldli, Kvello, Kirkerud, & Mustaparta 2012). However, it was then identified as the mlALT. Considering the thinness of the tALT, and its frequent absence in mass-stained preparations, it is reasonable to assume that it consists of relatively few neurons innervating a small number of glomeruli.

A previous master study performed at the lab by Ingrid Moe Dahl examined second order PNs from the labial pit organ, that seem to project via the tALT. This could indicate that CO_2 related information is carried through the current tract (Dahl, 2013).

From the confocal images presented here it appears that the tALT innervates an area anterior to the calyces in addition to terminating in the LH. This is consistent with the findings in the former master thesis (Lillevoll, 2013).

4.2.2: The medial antennal-lobe tract

Among the main results of the present study was the finding of the mALT as a prominent fiber bundle projecting posteriorly from the antennal lobe and innervating in the calyces before terminating in the LH. This finding is in support of previous studies on *H. virescens* females (Rø et al, 2007) as well as males (Zhao et al, 2014).

A former anatomical study done on the sphinx moth, *Manduca sexta*, reported about approximately 300 axon fibers in the mALT (Homberg et al. 1988). Interestingly there seems to be a significant difference in the amount number of mALT fibers between the two sexes, since Homberg and his colleagues found a mean number of 317 mALT fibers in males and 280 in females. The number of axons has not been estimated in the heliothine moth; however, according to general features characterizing the olfactory system of different moth families, it is likely to assume that it corresponds to that reported in *M. sexta*. In a previous master study on *H. virescens* males, dealing with retrograde labeling of uniglomerular projection neurons confined to the mALT, Aleksander Berg showed that the neurons originating in the MGC projected in the dorsal root (Berg, 2013). This is in full agreement with the arrangement described in *M. sexta* (Homberg et al. 1988). Also, the additional fibers counted in the sphinx moth male were confined to the dorsal root. Due to the staining technique used in the present study, which included anterograde labeling via dye applied to the antennal lobe, it was impossible to visualize the dorsal and ventral root. That study found the mALT-DR, and the mALT-VR of females to consist of averagely 280 and 153 PNs, respectively, compared to 317 and 164 in males. Note that while the study on *M. sexta* found a small number of Pm*a* in the mALT, it found no Pm*c* neurons, which is contrasted by results from female *H. virescens*, which found no Pm*b*, but a small amount of Pm*c* (Rø et al, 2007).

In all investigations, the mALT is found to be thickest of the ALTs. This is probably due to both the number of axons confined to this tract and their relative thickness. From the results of the present study, the thickness of the mALT appears to be uniform throughout the

entire course, indicating that the majority of PNs in this tract innervates both the calyces and the LH.

There are also gender differences in the projection pattern of the mALT in the LH. In females, the single fiber bundle project does not show a specific termination pattern, but rather one that is continuous with the entire LH. This pattern was also observed in a dual-labeled preparation with micro-ruby applied to the central AL, and micro-emerald applied to a distinct are near the entrance of the antennal nerve, where the large female glomeruli are situated. This is in contrast with males, where PNs carrying pheromone information innervate a distinct area of the LH that does not overlap with the more laterally and posteriorly oriented innervation pattern of PNs carrying information about plant odors (Zhao et al, 2014). However, more studies using dual-labeling of the previously mentioned AL areas are needed to confirm this finding.

4.2.3: The lateral antennal-lobe tract

The finding of the lALT as a tract being thicker than both the mlALT and the tALT, but thinner than the mALT, is consistent with previous studies in moths (Homberg, 1988; Rø, 2007). This tract is not as well described as the mALT. That it projects to the LH is obvious, but details regarding its remaining projection pattern are lacking. Two previous master studies, by Berg and Brekken, have shown that only a very small amount of PNs in the lALT project to the calyces. Therefore, the finding of one particular lALT axon projecting to both the LH and the calyces, as shown in figure 3.7, was indeed interesting. Although such PNs have been described in the heliothine moth before, those studies used either intracellular staining (Rø et al, 2007) or retrograde labeling of AL PNs (Berg, 2013; Brekken 2014). This study is thus the first to label one of the relatively rare morphological types of lALT-projection neurons via the anterograde mass staining technique. Interestingly, the unusual projection pattern of the current axon including division in two sub-branches – one targeting medial calyx and the other the lateral, the is in full agreement with previous data from *H. virescens* females; actually, Rø et al. (2007) report about one category of uniglomerular projection neurons confined to the lALT that innervates the calyces and the lateral horn in a similar manner (Rø et al, 2007).

As mentioned above, previous studies have reported that the lALT terminates in various parts of the protocerebrum, and that its fiber bundle seems to consist of projection neurons being less uniform than those forming the other ALTs. The confocal images obtained

in this study confirm at least parts of the former findings by displaying a projection pattern including terminal regions in various parts of the LH. Whether the number of axon fibers forming the lALT in heliothine females is lower than in males, as reported in *M. sexta*, is an unanswered question. In the sphinx moth, the lALT was shown to consist of significantly fewer fibers in females than in males, 327 compared to 359 (Homberg et al, 1988).

4.2.4: The mediolateral antennal lobe tract

As expected, successful dye application to the AL visualized the mlALT as the thinnest of the three classic ALTs. The course of the mlALT passing together with the mALT before turning laterally at the edge of the central body is in full agreement with previous publications (Homberg et al, 1988, Rø et al, 2007). Also, the pattern of the sub-bundles branching off from the mlALT near the region of the pedunculus is in accordance with the former data. As demonstrated in the confocal images, these sub-bundles project their fibers into slightly different parts of the LH. The number of axons passing in the mlALT is reported to be similar in males and females of *M. sexta* in which the majority of the PNs in the current tract were identified as multiglomerular (Homberg et al, 1988). Approximately 70 out of an estimated total 130 PNs of the mlALT have been identified as GABAergic in *H. virescens*, although the functional significance is not yet clear (Berg et al, 2009).

4.3: Parallel olfactory tracts

While there are still many uncertainties why PNs travel in multiple separate tracts on their way to higher olfactory processing centers, most insects studied so far have been shown to have them, suggesting that this is a common feature (Galizia & Rössler, 2010).

As shown in this study, all ALTs target the LH via a relatively substantial number of axons. This is in correspondence with previous studies on moths, as well as other insects (Galizia & Rössler, 2010). The other protocerebral centre, the calyces, on the other hand, is reported to be innervated mainly by one tract, the mALT (Berg 2013; Brekken 2014). Actually, Brekken counted maximally six axons in the lALT when applying dye into the calyces. In spite of data obtained via intracellular recordings from the AL of *H. virescens* females including three lALT PNs projecting to the calyces (Rø et al, 2007), it is relevant to point out that the connection between the AL and the calyces via the lALT, as indicated by Galizia & Rössler (2010), comprises relatively few neurons.

The LH has been shown here to receive PNs from the three main ALTs, which is in consistency with previous studies on moths (Galizia & Rössler, 2010). In addition, the tALT also terminates in the LH. This means that the Calyces and the LH constitutes the majority of target areas for AL PNs (Rø et al, 2007). Although there are studies that have described the LH arborisation patterns of either individual PNs or entire ALTs for many species of insects, and found some degree of overlap, there has not been any thorough examination on how much overlap there is between the terminals of the ALTs.

In the male moth, the segregation of pheromone and general odorant information along distinct axon fibers is well documented, in both the periphery and at the level of the ALTs (Hildebrand & Shepherd, 1997). This segregation can also be seen in the LH of moths and other insects. In the silk moth, *Bombyx mori,* PNs carrying plant odor information were found to innervate an area of the LH located posteriorly and laterally compared to that innervated by PNs carrying pheromone information (Seki, Aonuma & Kanzaki, 2005). The same pattern was found in other moths, including *H. virescens* and *H. assulta* (Zhao et al, 2014), plus the sphinx moth, *M. sexta* (Homberg et al, 1988), Also, a corresponding arrangement has been reported in the fruit fly, *D. melanogaster* (Jefferis et al, 2007). Noteworthy, however, is the fact that neither the pheromone information nor the plant odor information is projected separately in a single ALT – at least as regards the three classic tracts. Thus, in several species of moth, both projection neurons originating from ordinary glomeruli are reported to project together with pheromone neurons in all the classic ALTs (Homberg et al, 1988; Zhao et al, 2014). If the tALT in moths carries pheromone information or not is still unknown, so further investigation could be warranted. Taken together however, this could indicate that each of the ALTs is related to processing certain stimuli characteristics rather than information originating from qualitatively distinct stimuli.

Another interesting feature regarding the ALTs is that there is a discrepancy in axon diameter in the various tracts, with the mALT having the thickest axons, as previously mentioned. Since thin axons have a relatively slow conduction velocity, it has been hypothesized that the parallel ALTs targeting partly similar regions in the lateral horn and the calyces may operate as a system where the inherent delay is part of a temporal code decoded by downstream targets (Galizia & Rössler, 2010). A study on axon efficiency found that there is a selective pressure for thin axons, since a doubling of axon diameter likely means that volume and energy use is quadrupled. However, a few thicker axons may encode certain features more efficient than many thinner axons, meaning that axon thickness may be a result

of selective pressure for the thinnest diameter that will allow for a certain information rate (Perge, Niven, Mugnaini, Balasubramian & Sterling, 2012).

Another issue that warrants addressing is the presence of diverse neurotransmitters in the ALTs. Although the main neurotransmitter released by PNs in most insects studied is acetylcholine (Wilson & Mainen, 2006), this is yet to be identified in *H. virescens.* The mlALT and lALT has been shown to contain the axons of neurons that release a wide range of neurotransmitters, and as mentioned above, as many as half the PNs in the mlALT could be GABAergic (Berg, Schactner, Utz & Homberg, 2007; Berg, Schactner & Homberg, 2009).This could indicate that information flow through the ALTs is modulated by various neurotransmitters.

4.4: invertebrates compared to humans

When reviewing the many similarities in the olfactory systems of humans and insects, it is easy to understand why one would use insects as model organisms when researching olfaction. If the basic underlying principles of a sensory system are the same, it stands to reason that one chooses to use an organism that is both simpler and readily available when further exploring those principles.

Although there are differences between humans and insects concerning both odorant receptors and olfactory transduction (Sato et al, 2008; Benton et al, 2009; Zozulya et al, 2001), there are also striking similarities. One commonality is the characteristic glomerular organization of the primary olfactory brain center, the olfactory bulb of mammals and the antennal lobe of insects (Hildebrand & Shepherd, 1997). There are also similarities between second order neurons in the different organisms including both projection neurons and local interneurons. Uniglomerular PNs, being the most common in insects (Anton & Homberg, 1999, cited by Wilson & Mainen, 2006), can be compared to the mitral cells of humans. Furthermore, the previously mentioned arrangement of LN1 and LN2 local neurons in the AL has been compared with the periglomerular and granular cells of the olfactory bulb, which suggests that the internal processing in insect glomeruli corresponds with processing in the granule layer of the olfactory bulb (Tanaka et al, 2012). Indeed, periglomerular cells are involved in feed-forward inhibiton, and together with granular cells they are involved in lateral inhibition and sharpening of the signal projected by mitral cells (Shepherd, 2004). Similarly, some GABAergic LNs found in the insect AL are reported to provide feedback

inhibition (Olsen & Wilson, 2008). Other LNs are suspected to be involved with feed-forward inhibition.

Higher order centers for olfactory processing in humans and insects have also been compared. The superior lateral protocerebrum and lateral horn of insects share similarities with the piriform cortex of humans (Strausfeld & Hildebrand, 1999). As mentioned earlier, the mushroom body calyces of insects are known to be involved in olfactory learning and memory (Menzel, 2001, Strausfield et al, 2003). Interestingly, the lobes of the MB are seem to be particularly involved in place-memory functions (Mizunami, Weibrecht & Strausfeld, 1998), much like the hippocampus of humans (Morris, Garru, Raulins & O'Keefe, 1982). Both brain regions are involved in context dependent sensory filtering.

Thus, there appears to be many striking similarities in morphological and physiological arrangements in both primary (Hildebrand and Shepherd, 1997) and secondary olfactory centers in insects and humans (Strausfeld & Hildebrand, 1999). This may suggest that even with uncommon origins and millions of years of evolution separating humans and insects, both have evolved somewhat similar effective solutions.

4.5: Methodological considerations

For an experimental design such as this, the fact that one cannot reliably control the amount of dye that is injected into neuronal tissue during mass staining is a factor to be considered. Variation in dye amount and its transport could explain the variation seen in the lALT and the tALT of the labeled preparations in the present study. Some experiments including application of two fluorescent dyes, micro-ruby and micro-emerald, into distinct regions of the same antennal lobe were also performed. Considering the small size of the current brain region, i.e. ca. 300 µm in diameter, these kinds of staining experiments require a relatively advanced precision level. This could affect not only if any ALTs are stained, but which, depending on the glomerular innervation patter of a given tract. Also, the fact that the moth brain is somewhat auto-fluorescent, with emitted wavelengths falling within the bandwidth spectrum of micro-emerald, means that even unstained structures will be detected by a confocal laser scanner, making micro-emerald less distinct as compared to micro-ruby.

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5.Conlusion

- In addition to the three classic antennal-lobe tracts − the mALT, the mlALT, and the lALT – a fourth tract identified as the transverse ALT was found in the heliothine female moth.
- As previously described in the heliothine male, the transverse tract in the female branches off from the medial ALT posteriorly of the central body and terminates in the lateral horn, plus a region adjacent to the calyces.
- From the present results, which were obtained from females only, no distinct compartmentalization could be observed in the lateral horn.
- Among the significant findings of the present study was the visualization of one lateral-tract projection neuron innervating the calyces.
- In general, no female-specific characteristics of the antennal-lobe tracts could be observed.

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