

Anatomical organization of the central olfactory pathways – mapping the transverse tract in the moth brain

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Abstract

The sense of smell is regarded as the oldest and best conserved of all the senses. The fact that every organism has established the ability to detect chemicals in the external environment implies the importance of chemosensation. Due to their well-developed sense of smell and easily accessible nervous system, moths have served as suitable model organisms for researchers exploring general principles underlying odor information processing. Like in other insects, moths perceive odorants via olfactory sensory neurons located mainly on the antennae. The chemical stimuli, including plant odorants and pheromones, initiate different instinctive behaviors associated with mating and foraging. In this study, one distinctive level of the olfactory pathway that connects the primary olfactory center, the antennal lobe, to higher regions in the moth brain, was anatomically characterized. This path is formed by several parallel antennal-lobe tracts, including one newly discovered fiber bundle named the transverse tract. The last mentioned tract was especially explored in the study presented here. Generally, the neural pathway formed by the antennal-lobe tracts corresponds to the human olfactory tract conveying olfactory information from the olfactory bulb to cortical areas. The data in this study was obtained by performing anterograde mass labeling combined with confocal microscopy. Successfully stained preparations visualized the tree classical antennal-lobe tracts as well as the transverse tract.

Abbreviations

AL	-	Antennal-lobe
ALT	-	Antennal-lobe tract
dmALT	-	dorsomedial antennal-lobe tract
KC	-	Kenyon cells
lALT	-	lateral antennal-lobe tract
LPOG	-	Labial pit organ glomerulus
LH	-	Lateral horn
mALT	-	medial antennal-lobe tract
MB	-	Mushroom bodies
MGC	-	Macroglomerular complex
mlALT	-	mediolateral antennal-lobe tract
MOE	-	Main olfactory epithelium
mPN	-	multiglomerular projection neuron
OBP	-	Odorant binding protein
OR	-	Odorant receptor
ORN	-	Olfactory receptor neuron
OSN	-	Olfactory sensory neuron
PBP	-	Pheromone binding protein
PN	-	Projection neuron
tALT	-	transverse antennal-lobe tract
uPN	-	uniglomerular projection neuron

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Introduction

Nature has evolved a variety of sensory modalities that give external input an internal interpretation. Some have shown to be more prevalent than others across organisms. The fact that all organisms are capable of responding to chemicals in the environment says something profoundly. In general, the chemical signals comprise internally released cues, like hormones and neurotransmitters regulating physiological processes, as well as external odors and pheromones which induce innate and learned behaviors such as eating, mating and identifying potential predators (Axel, 2004). The olfactory system has the ability to detect and recognize numerous airborne molecules. From an evolutionary perspective the chemical sense is considered to be the oldest and thus the most primitive of the sensory modalities. Notably, the term *primitive* is somewhat ambiguous, giving it a special connotation. On the one hand the meaning of the term may be interpreted as simple and rudimentary. On the other, it can be understood as complex and well developed, due to the duration of the evolutionary timeframe (Purves et al, 2012). Insects, for example, have refined their olfactory pathways during a period of 400 million years. Despite the phylogenetical age of the chemosensory system, it is generally regarded as the best conserved of all sensory arrangements (Dethier, 1990). Therefore, the moths exquisite and relatively simple olfactory system, make them attractive models for experimental research. Their easily accessible central nervous system gives insight into general neural coding mechanisms. A comparative view of the olfactory pathways in humans and insects for example, reveals many striking commonalities from the periphery to higher brain areas.

The olfactory pathway in moths

The sensory organ

Situated on the moth's head capsule, the antennae are deemed the most vital organ in odor detection. The antennae resemble a comb-like structure that filters molecules from the airstream. Located on the antennae are countless mini-organules known as sensilla with specialized ability to detect various sensory modalities such as odorants, taste stimuli, humidity, vibrations, and changes in temperature (Stengl, Hatt & Breer, 1992; Schneider, 1992). The absolute majority of sensilla are devoted to recognizing odor stimuli. Furthermore, many moths show gender-divergences in the antennae, with males having sex-specific sensilla. This is well exemplified in the noctuid moth, *Heliothis virescens*, where only males can detect lure pheromones released by females. This gives in turn navigational instructions.

Excited males thus fly upstream in a characteristic zig-zag pattern alongside the pheromone trail. This behavioral response is based on activation of pheromone-sensitive neurons housed by the male-specific sensilla. It can be argued from an evolutionary standpoint that the quantity and length of these long sensilla, named sensilla trichodea can be advantageous when detecting sex pheromones (Stengl, 2010). In addition to the male-specific sensilla, numerous sensilla housing plant-sensitive neurons cover the antenna. These isomorphic sensilla are present both in males and females.

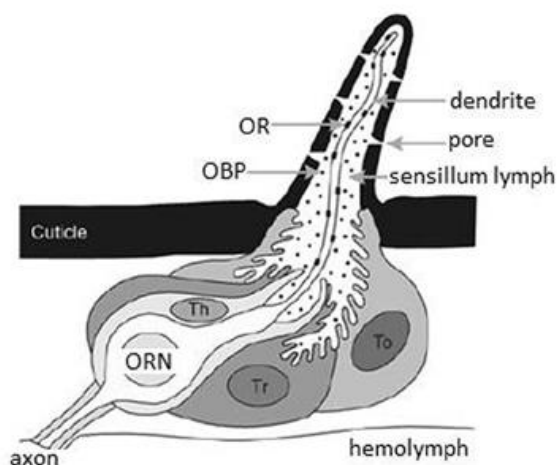


Figure 1: Schematic overview of the general insect olfactory sensillum housing one olfactory receptor neuron (ORN). OR: odorant receptor, OBP: odorant binding protein, Th: thecogencell, To: tormogencell, Tr: trichogencell (Schematic by Jacquin-Joly & Merlin, 2004)

In general, each olfactory sensillum consists of a cuticular structure, with the outer layer functioning as a flexible membrane covered with minute pores that allow odorants to enter (Fig. 1). The olfactory neuron within the sensillum trichodeum is usually surrounded by three non-neural cells called the trichogen-, the tormogen-, and thecogencell. Furthermore, at least two olfactory sensory neurons are present. The bipolar sensory neuron consists of a dendrite that stretches inside the aqueous receptor lymph and an axon that projects directly into the primary olfactory center of the brain, called the antennal lobe. The antennal lobe is equivalent to the olfactory bulb in vertebrates (Stengl, 2010). How hydrophobic odorants pass through the receptor lymph is not fully understood. However, the presence of water-soluble proteins in the receptor lymph, with the ability to bind pheromones and other odor molecules, has been thoroughly demonstrated in insects (reviewed by Pelosi, Zhou, Ban & Calvello, 2006). The pheromone-binding proteins (PBP), for example, are shown to interact with distinct pheromones forming PBP complexes. It is thought that odorant-binding proteins (OBPs), as well PBPs are able to traverse between the pore to the receptive areas at the dendritic membrane, functioning as carriers (Stengl, 2010; Grosse-Wilde, Svatos & Krieger, 2006).

The puzzling transduction process, whereby the odorant signal transforms into an electric signal is one of the issues separating vertebrates and insects. In vertebrates, the chemosensory receptors belong to the G-protein-coupled family that generates action potentials via intracellular events (Buck & Axel, 1991). Insect odor receptors are however structurally, and genetically unrelated to their vertebrate counterparts (reviewed by Kaup, 2010). Most odor receptor in insects is served by a complex of two distinct molecules, known as a heteromeric complex (Sato et al, 2008). Since this complex functions both as a receptor for the odorant, as well as an ion channel, this specific receptor is referred to as ionotropic. The heteromeric complex consists of one odorant-binding unit and a highly conserved, but broadly expressed co-receptor known as OR83b (Kaup, 2010; Sato et al, 2008).

The antennal lobe

The antennal lobe receives input, via the antennal nerve, from olfactory sensory neurons located on the ipsilateral antenna. Each sensory axon targets one condensed neuropil structure better known as a glomerulus. Each of these sphere-shaped structures receives input from many sensory axons. In addition to the sensory terminals, there are three types of antennal-lobe neurons making up the glomeruli: local interneurons, projection neurons, and centrifugal neurons (reviewed by Galizia & Rössler, 2010). The two main types of antennal-lobe neurons, i.e. local interneurons and projection neurons, have their cell bodies located in one of three cell clusters enveloping the antennal lobe glomeruli (Homborg et al, 1988). Both local neurons and projection neurons receive direct input from the ORNs. Projection neurons receive simultaneously indirect input from OSNs through local neurons, before relaying olfactory information from the antennal-lobe to higher brain centers (reviewed by Hanson & Anton, 2000; Galizia & Rössler, 2010). The local interneurons branch exclusively within the antennal-lobe, and many of them innervate the majority glomeruli. The multiglomerular local interneurons are distinguished by displaying symmetric and asymmetric arborization patterns in the glomeruli. A third and minor group of antennal-lobe neurons are the centrifugal neurons, having their dendritic branches localized outside the antennal lobe and are assumed to serve various modulatory functions (reviewed by Hansson & Anton, 2000).

Generally speaking, in lepidopterans the number of glomeruli is reported to average about 60 (reviewed by Galizia & Rössler, 2010). The number and arrangement of glomeruli is not only species specific, but even gender specific. In *H. virescens*, for example, there are 64 glomeruli in the male and 60 in the female (Berg, Galizia, Brandt & Mustaparta, 2002; Løfaldli, Kvello & Mustaparta, 2010). The four additional glomeruli found in males as compared to females,

constitute a group of enlarged glomeruli positioned at the entrance of the antennal nerve, the so-called macroglomerular complex (MGC; Berg, Almaa, Bjallie & Mustaparta, 1998; Vickers, Christensen & Hildebrand, 1998). This male-specific structure is devoted to process information about female-produced substances, i.e. lure pheromones

Interestingly, in the sub-family of heliothine moths consisting of numerous related species some of which are sympatric, the male-specific olfactory system has evolved a system not only for detecting pheromones from conspecific females, but also an arrangement for detecting signals from sympatric females of related species (reviewed by Berg, Zhao & Wang, 2014). Two of the four MGC units in *H. virescens*, for example, are dedicated to process pheromone information whereas two remaining units encode interspecific information (Berg et al, 1998). The numerous ordinary glomeruli, which are present in both sexes, receive input from plant-sensitive OSNs. Among these relatively numerous glomeruli is one very big unit, which is located most ventrally in the antennal lobe. This glomerulus, the so-called labial pit organ glomerulus (LPOG), receives input from sensory neurons located in the labial pit organ (Kent, Harrow, Quartararo & Hildebran, 1986; Zhao et al, 2013).

The antennal-lobe tracts

The olfactory information is carried from the antennal lobe to higher brains centers in the protocerebrum via numerous projection neurons. The projection neuron axons are bundled together in distinct output tracts. As in most other insects, the moth has three main tracts: the medial antennal-lobe tract (m-ALT), the mediolateral antennal-lobe tract (ml-ALT), and the lateral antennal-lobe tract (l-ALT; for naming, see Ito et al, 2014; Fig 2). Generally, the ALTs target two main regions in the protocerebrum, the calyces of the mushroom bodies and the lateral horn (Homberg, Montague & Hildebrand, 1988; Rø et al, 2007). The m-ALT has a prominent appearance due to the sheer quantity and diameter of its axonal bundle (Homberg et al, 1988). The axonal bundle forming the m-ALT runs posteriorly in the protocerebrum, edging by the central body before turning laterally and innervating the calyx of the mushroom bodies (MB) before turning anteriorly and terminating in the lateral horn (LH) (Homberg et al, 1988; Rø et al, 2007). The considerably thinner ml-ALT follows the m-ALT from the antennal-lobe, but turns laterally halfway from the calyx, close to the central body. From here, it projects directly to the LH. On its lateral course, it splits into two smaller sub-branches, which innervate in slightly different areas of the LH. The ml-ALT does not innervate the calyces. The final main ALT, namely the l-ALT, bends off laterally of the ventral m-ALT root in the antennal lobe and projects directly to the LH, however, in a more ventrally positioned

pathway then that of the ml-ALT. The l-ALT appears rather thick in some preparations (Homberg et al, 1988; Rø et al, 2007).

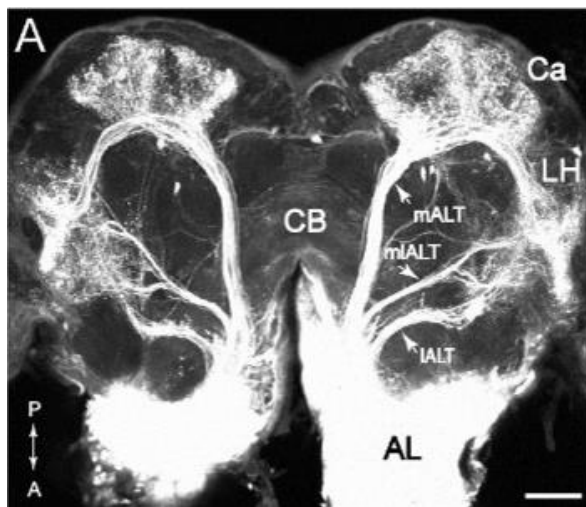


Figure 2: Confocal image showing the three main tracts connecting the antennal lobe (AL) to the calyces (Ca) and lateral horn (LH). The prominent medial antennal lobe tract (m-ALT) projects posteriorly innervating the Ca before terminating in the LH. The medio-lateral antennal lobe tract (ml-ALT) and the lateral antennal lobe tract (l-ALT) bend of laterally innervating the LH directly. Unpublished data by Xin-Cheng Zhao.

In addition to the main ALTs, some minor tracts have been reported. The dorso-medial antennal-lobe tract (dm-ALT), consisting of relatively few axons leaves the antennal-lobe dorso-medially, and projects posteriorly, alongside the dorsal surface of the protocerebrum, close to the brain midline towards the calyces (Homberg, et al 1988; Rø et al, 2007). Also, a new tract was recently discovered in the heliothine moth. In the master thesis of Lillevoll (2013), first reporting about this particular tract, it was named secondary mediolateral antennal-lobe tract. However, due to its similarity to a newly identified antennal-lobe tract in *Drosophila melanogaster*, named the transverse ALT (t-ALT; Tanaka et al, 2012), it has been called the t-ALT also in the moth (Ian et al, 2015; submitted manuscript). Similarly to the ml-ALT which follows the thick m-ALT before bending off and projecting laterally, the t-ALT deviates from the m-ALT and passes laterally. However, it bends off from the medial tract more posteriorly (Fig. 2).

Generally, the antennal-lobe output neurons may be classified in uniglomerular and multiglomerular projection neurons (uPNs and mPNs). The uPNs have dendritic branches in a single glomerulus while the mPN have branches in numerous, if not all glomeruli within the antennal-lobe (Galizia & Rössler, 2010). The absolute majority of axons confined to the prominent m-ALT in moths include uPNs. The ml-ALT, on the other hand, consists of mPNs. The third classical tract, the l-ALT, is reported to comprise both uPNs and mPNs (Rø et al, 2007; Galizia & Rössler, 2010).

Higher olfactory centers

From the antennal-lobes, projection neurons travel through the described tracts as they target two main areas in the protocerebrum, namely the calyces and lateral horn. The prominent neuropil, the calyces of the MB (*corpora pedunculata*) is located posteriorly in the protocerebrum. It's assumed to work mainly as an integrative center of odor learning and memory. Within its structure, numerous uPN from the m-ALT en route to the LH, form synapses with intrinsic MB neurons known as Kenyon cells (KCs) (Strausfeld, Hansen, Li, Gomez & Ito, 1998). The dendrites of the KCs form the cup-shaped calyces whereas their axons make up distinct lobes (Heisenberg, 1998; Galizia & Rössler, 2010). Its structure and function, like any other organism reflects the evolutionary course the species has faced in respect to sensory exposure and behavioral adaptations. Contemporary understanding holds that its main function is olfactory learning and memory. An alternative view suggests it may support various functionalities such as sensory discrimination, integration with other sensory modalities, spatial orientation and the control of behavioral repertoires (Strausfeld, Hansen, Li, Gomez & Ito, 1998). In heliothine moths, the calyces are reported to receive input almost exclusively from the m-ALT (Ian et al, submitted).

The second olfactory center in the protocerebrum, the LH, is reported to lack a clear finite structure. This region is nonetheless regarded as a main integration center for olfactory signals (Rø et al, 2007; Zhao, Pfohl, Surlykke, Tro & Berg, 2014). In *H. virescens* it has been reported that plant odorant and pheromones are represented in segregated areas in the LH (Zhao et al, 2014). This further supports the notion that this region plays a role in hard-wired behavioral responses. Both the calyces and the LH represent two major targets for the antennal-lobe PNs. This is well grounded across insect taxa. A comparative approach across insect species, in terms of structure organization, size, and physiology may give further insight on the function of these areas. Recent studies of heliothine moths have reported that the LH receives input from all the ALTs (Ian et al, submitted). Generally, the calyces and the LH constitute higher order olfactory centers in most insect taxa. A comparative approach across various insect species, in terms of structure, organization, size, and physiology may give further insight on the function of these areas.

The human olfactory pathway

The human olfactory system shares many commonalities with that of insects. Generally, olfaction is categorized as microsomatic in humans: meaning that the sense of smell is less

sensitive and not as vital for survival compared to other modalities. Insects, on the other hand, are classified as macrosomatic beings, critically dependent on their highly sensitive olfactory system (Goldstein, 2001). Interestingly, the largest gene family identified in mammals is that of olfactory genes, including ca. 1000 genes. Whereas the majority of these are normally expressing elements in most mammals, the number of functional olfactory genes sharply decreases in primates (Zhang & Firestein, 2002). In humans, the number of functional olfactory genes is reduced to about 350 (Crasto, Marenco, Miller & Shepherd, 2002). This decline has been attributed to olfaction being a less acute sense. However, the number of olfactory genes does not directly translate to acuity. Though dogs are superior with 850 functional genes, it may be argued that humans counterbalance this decline of functional genes by their processing capabilities in higher brain areas. Traditionally, olfaction in humans has been viewed as an aesthetic sense, being able to induce memories and emotions. From this perspective, it can be argued that we are equipped with a robust ability for analysis and complex processing forming our behavioral responses. Furthermore, an underestimated role of human olfaction is its crucial importance for our capability of flavor perception. During retronasal olfaction, food volatiles travel from back of the mouth through the nasopharynx and stimulate OSNs in the main olfactory organ. A simultaneous stimulation of taste cells and OSNs induce activity in higher brain regions covering an area larger than that including the total area activated via the two modalities alone (Shepherd, 2006; Axel, 2004).

The olfactory sensory organ, the olfactory epithelium

In mammals, odorants in the airstream enter the nose by sniffing. While inside the nasal cavity, they are processed by the main olfactory epithelium (MOE). The epithelium is covered in a mucus layer that serves as a net, catching the odorants. The MOE consists of three cell types, olfactory sensory neurons (OSNs), supporting cells, and stem cells. Like in insects, the OSNs are bipolar neurons (Fig. 3). They have a single dendrite forming a knob-like protrusion whereby 5-20 olfactory cilia further extend into the mucus layer whereas an unmyelinated axon projects directly to the brain. The odor transduction process taking place in the cilia is initiated by receptor proteins interacting with specific odor stimuli according to their binding site. The G-coupled receptors activate a second messenger, cAMP, which initiates a depolarization via opening of distinct ion channels and action potential generation (Purves et al, 2012). The electrical signals target the olfactory bulb located above the nasal cavity and the bony cribriform plate (Purves et al, 2012). As OSN axons leave the MOE, they converge

into axonal bundles thereby forming the olfactory nerve. Like in insects, the OSNs project to the ipsilateral brain hemisphere.

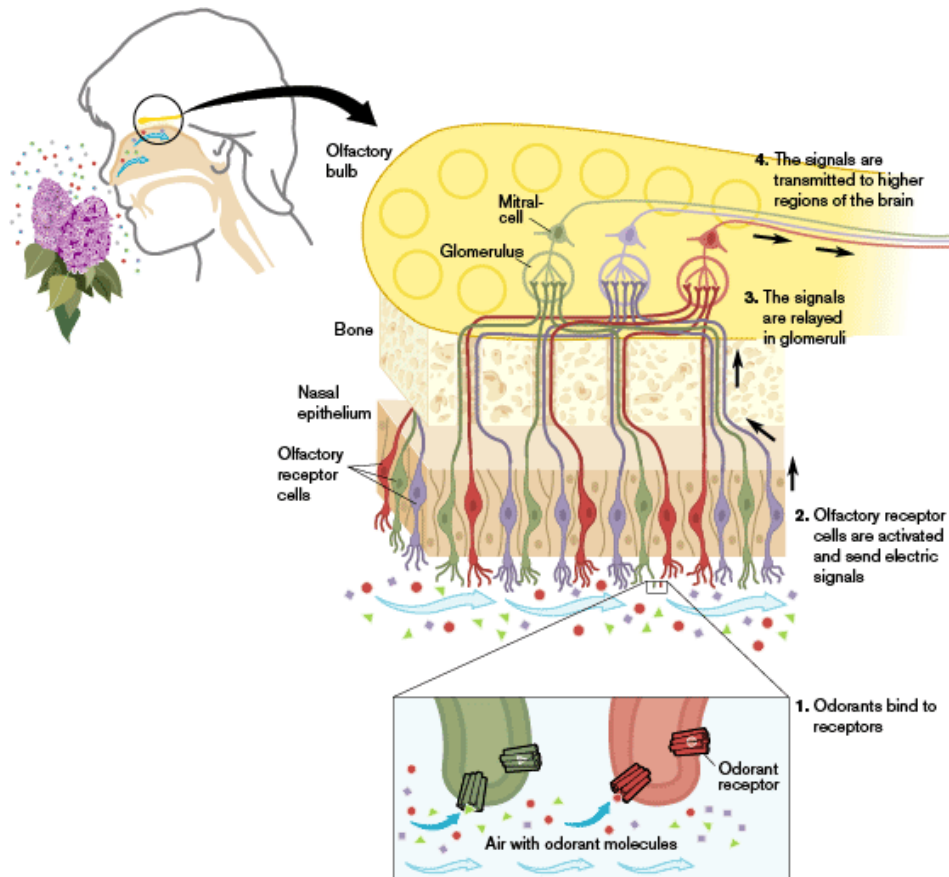


Figure 3: Schematic overview of human olfactory organization. 1. Odorants bind to receptors. 2. Olfactory receptor cells are activated and send electrical signal. 3. The Signals are relayed in glomeruli. 4. The signals are transmitted to higher brain regions.

The primary olfactory center, the olfactory bulb

All olfactory sensory axons project to the olfactory bulb, being the equivalent of the insect antennal lobe. This first synaptic relay of the olfactory pathway in the two groups of organisms share many striking similarities. Firstly, the axons of OSNs form synapses with second order neurons within the glomeruli. Secondly, the main categories of second order neurons comprise, in both groups of organisms partially similar local interneurons and projection neurons. For example, both insects and mammals include several types of local interneurons, many of which are GABAergic (Kay & Stopfer, 2006). Concerning the output neurons, the mammalian mitral cells are quite similar to the uPNs of insects. Both neuron types innervate one glomerulus in the primary olfactory center and project their axon to

higher brain regions located in the ipsilateral hemisphere. In mammals, a main portion of the PNs from the olfactory bulb targets phylogenetically old regions of the temporal lobe (Purves, et al, 2012; Kandel, Schwartz, Jessell, Siegelbaum & Hudspeth 2012). More specifically, the olfactory tract – which is the path formed by the projection neuron axons – terminates in the anterior olfactory nucleus, the piriform cortex, parts of amygdala, the olfactory tubercle, and the entorhinal cortex. The later four areas transmit information to the orbitofrontal cortex, also called the secondary olfactory cortex, located in the frontal lobe. These connections include both direct paths and paths projecting via thalamus. Even though higher-order olfactory processing is poorly understood, a few notable connections may share some insight. It is well known that odor information targets the amygdala, a limbic structure closely linked to emotions. In addition, the olfactory neurons in entorhinal cortex are known to target the hippocampus, a brain region important for establishment of memory. These particular pathways are thus thought to reflect odor induced emotions and memories (Kandel et al, 2012; Purves et al, 2012) Moreover, fact that odor information initially bypasses the thalamus and is conveyed directly to the primary cortical regions, differs from all other sensory pathways. This direct connection of the olfactory pathway is an arrangement giving the external world a “hotline” to the primary olfactory cortex (Shepherd, 2007).

Heliothine moths as model organisms for chemosensory studies

Due to its exquisite sense of smell and its relatively simple nervous system, which is easily accessible for experimental research, insects are suitable model organism for investigations of basic neural principles underlying odor perception. By investigating the organization and function of the olfactory system in moths, knowledge about general principles will contribute to increase our understanding on the encoding mechanisms characterizing this sensory system. In particular, studies aiming at exploring processing principles residing within the pathway conveying olfactory information from the primary olfactory center to higher brain areas may be illuminated. In the research project presented here, one of these connections in the moth will be especially investigated. Differently from the three classical ALTs, which are relatively thoroughly mapped, the recently discovered t-ALT still remains elusively described. Further investigating of this particular tract is therefore highly relevant. Furthermore, as formerly mentioned, the collection of ALTs in insects corresponds to the mammalian olfactory tract carrying information underlying for example emotional response and memory establishments.

Aim of this master thesis

Principal issue: To trace the central olfactory path in a small model brain for the special purpose of mapping a specific part included in the 2nd order level of the sensory pathway.

Specific goals:

- To characterize the transverse antennal-lobe tract anatomically
- To investigate the putative origin of the transverse antennal-lobe tract
- To establish a method for labeling selected antennal-lobe glomeruli

Materials and Methods

Insects

The experiments conducted in this study included the use of a model organism, i.e. the heliothine moth (Lepidoptera; Noctuidae). Both the Oriental cotton budworm *Helicoverpa armigera*, and the American tobacco budworm, *Heliothis virescens*, were used. Newly arrived insect pupae from China and Germany were sorted by gender and kept separated in two heating cabinets (Refitherem 6E incubator, Struers) at 23 °C and 70% humidity. To accommodate a natural day and night cycle, lights were kept on for 14 hours and turned off for 10 hours, opposite of the day-night cycle. Shortly after hatching, the male and female insects, still being separated in different cabinets, were put in Plexiglas cylinders with up to 5-8 moths in each tube. The insects were nourished with a 10% sucrose solution.

Preparation

The moth was placed inside a small plastic tube with its head protruding. The insect was then stabilized by means of dental wax, molding it as a collar around the head. For this procedure, the preparation was placed under a stereomicroscope (Leica; MZ 12.5). Initially, external hairs located on the head cuticle of the moth were removed using fine forceps. Thereafter, the moth's head was cleaned gently with damp paper. Using a razorblade knife, a rectangular incision was made in the cuticle between the antennae, continuing alongside the eyes. The loose cuticle was then removed so that the brain was exposed. Fine needles were placed in the eyelobes, gently pushing them outwards making the access to the brain more available. Ringer's solution with sucrose (NaCl: 150mM, KCl: 3mM, TES buffer: 10mM, CaCl₂: 3mM, and 25 mM, pH 7.2 sucrose) was continuously applied to the brain to add nourishment and avoid any desiccation. The composition of the solution is similar to the insect's bodily fluid. Finally, muscle tissue, trachea and the thin membrane covering the brain were removed making the antennal lobes accessible for dye injection.

Dye injection

For the following experiments, the fluorescent dye Dextran tetramethylrodamine/biotin (3000 MW; micro-Ruby; ex/em: 490/508 nm) was used. This dye was supplied from Life Technologies and stored at -20°C in a freezer to maintain its crystalized quality. Initially, micro-Ruby crystals were applied randomly in the antennal-lobes using the tip of a fine micro-needle. Over the course of the experiments, crystals were injected ventrally in the antennal-lobes, an area suspected to include glomeruli being connected with the transverse

tract. The methodological approach in this study consisted of *in vivo* anterograde staining, meaning that the antennal lobe projection neurons transport the dye from dendritic to the terminal areas. Before injecting micro-Ruby, the bottle containing the dye was kept at room temperature for a couple minutes before it was opened in order to prevent the crystals from melting due to condensation. Before applying the crystals, fine paper was used to absorb liquids surrounding the antennal-lobe to avoid crystals from diluting. After staining the antennal-lobes, the brain was soaked with Ringer's solution and covered with moisturized paper (soaked with Ringer). To allow adequate propagation of the dye within the moth's brain, the preparation was left in room temperature for two hours covered from light, or in a refrigerator overnight at 4°C, before dissection.

Dissection

The maxillary palps, the proboscis, and both antennae were removed. A 180° incision was made at the upper part of both retinas making it easier to grasp with forceps during dissection. The needles placed in the eyes were removed before the head was decapitated and laid in a dissection bowl filled with Ringer's solution. The brain was carefully extracted from its head-capsule using a pair of forceps. The trachea, muscle tissue, and cuticle surrounding the brain were removed. The dissected brain with both antennal lobes and eye-lobes intact was transferred with a pipette to a small glass bottle containing 4% formaldehyde solution (Roti Histofix). The duration of the fixation was either by 2-4 hours in room temperature, or alternatively overnight in the fridge, at 4°C, ideally between 12-18 hours.

Dehydration

To rinse the brain after fixation, a phosphate-buffered saline solution (PBS NaCL: 684mM, KCL: 13mM, Na₂HPO₄: 50.7 mM, KH₂PO₄: 5mM, PH 7.4) was applied to the preparation. Then the brain was dehydrated through a series of continuously potent alcohol solutions, ranging from 50%, 70%, 90%, 96%, and 100%. Each consecutive solution was put on a rotator for 10 minutes, including the PBS solution prior to the alcohol series. The 100% solution was applied twice to ensure complete dehydration. Finally, the brain was carefully mounted on a metallic plate in methylsalicylate which makes the neural tissue transparent.

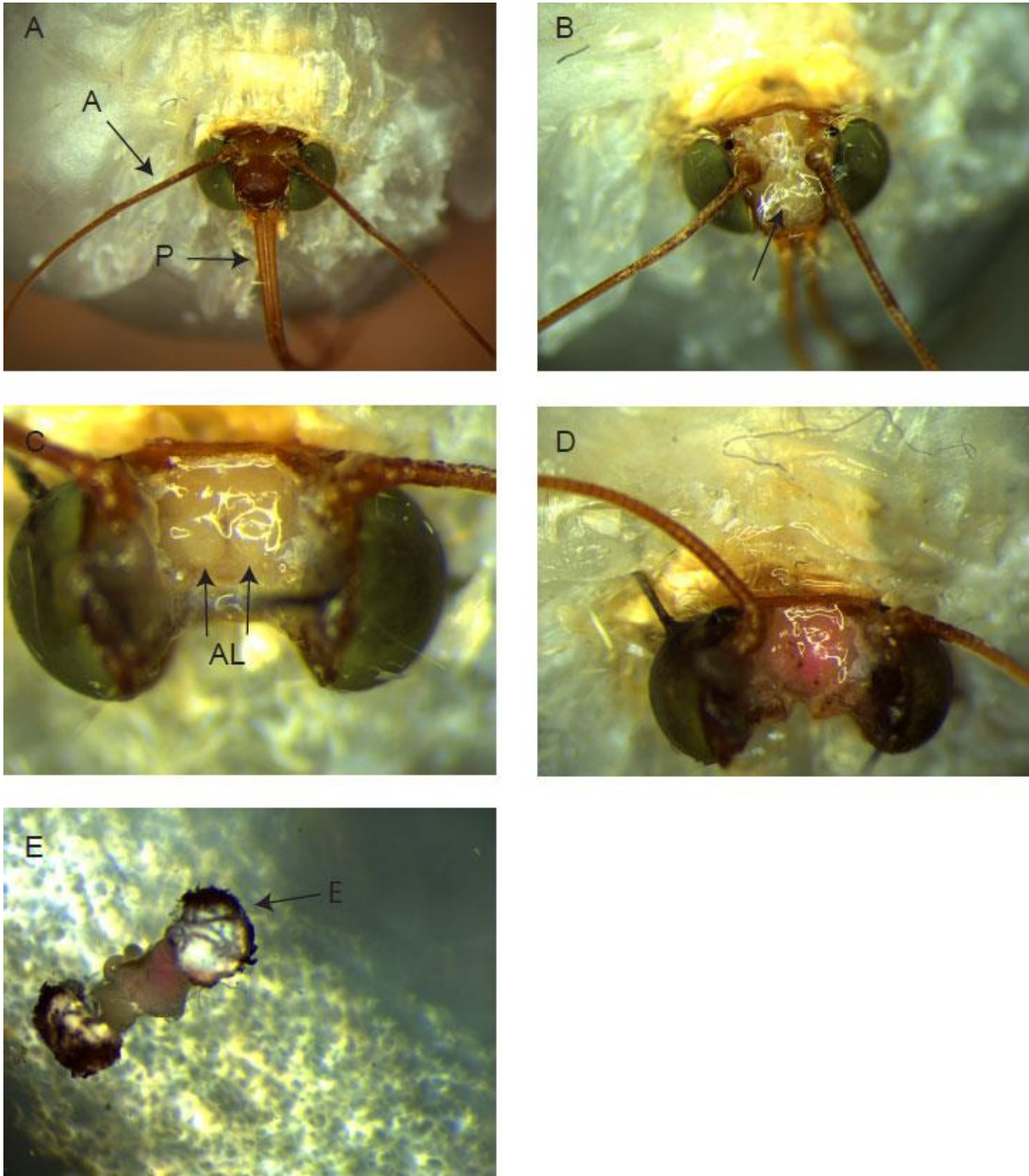


Figure 4: A series of images illustrating the process from preparation to dissection. **A:** The moth's head can be seen, protruding from the plastic tube. The two arrows point to one antenna (A) and to the proboscis (P). **B:** The cuticular structure has been removed, exposing muscle tissue and trachea as indicated by the arrow. **C:** The muscle tissue and trachea covering the brain has been removed. The arrows pinpoint the location of two antennal-lobes (AL). **D:** Micro-ruby crystals have been injected into the in the antennal-lobes. **E:** The brain has been dissected, E Eye-lobe.

Confocal microscopy

Initial examinations of preparations were done under a light microscope (Leitz Aristoplan, Wetzlar, Germany) using fluorescent filters. This indicated whether the preparations had been successfully stained. Preparations with sufficient dye and sensible staining patterns were further probed, exposing them for lasers using a confocal microscope imaging system (LSM Zeiss 510 Meta Mira 900F, GmbH, Jena, Germany). The brains were initially inspected using a dry objective 10x0.3; plan-neofluar to establish an overview, while the actual scan was done with a 20x0.45; plan-neofluar objective. The pinhole eye was set to an optimal value. Furthermore scan resolution was set to 1024x1024 pixels in the x-y direction with scan speed up to 6-7. The confocal image stacks were done in z-axis with a slice thickness of 2,1-3,1µm. A helium neon laser (HeNe, wavelength; 543nm) was applied in order to excite the injected dye, micro-ruby. Several options within the software were manually adjusted such as detector gain, detector offset and amplification to optimize individual preparations. Completed scans were then saved in .lsm format (Zeiss image files).

Image processing

Confocal data: The LSM 510 image browser software (Carl Zeiss microscopy, Jena, Germany version 4.2.0 121) was used to view, and export specific optical slices that best represent results from the stained preparations. This visualization software is capable of illustrating several optic slices in 3D projection views. Both images of single optical slices and those containing projection views were imported to Adobe Photoshop CS5 (Version 12.0, Adobe systems, San Jose, CA, USA) for adjustment of brightness/contrast and rotation (if convenient). The final figures were then made by importing the relevant images, stored as Photoshop files, Adobe Illustrator CS6 (version 16.0).

Images from the stereomicroscope: Images were obtained while preparing and dissecting, by an integrated digital microscope camera (Leica DMC45000), using the stereomicroscope (Leica M60). These pictures were stored at the image acquisition software LAS (version 4.7.1, Leica Microsystems, Switzerland) before being exported and managed as described above.

Ethical considerations regarding use of moths as model objects

When using animals for research purposes in Norway one has to abide the rigid law concerning animal wellbeing, “Dyrevelferdsloven”. The Norwegian law mainly applies to

vertebrates; however, some invertebrates, including for example honey bees comprise under this law (<http://lovdata.no/dokument/NL/lov/2009-06-19-97>). Lepidopterans, on the other hand, do not subject to this law. Using them for experimental research does therefor not conflict with the law in any way. Nevertheless, in our lab we emphasize on their wellbeing by cleaning the residing chambers and providing sucrose nutrition in cups. A healthy hygiene is maintained by disposing paper sheets alongside, and in the bottom of the chamber cylinder. Vertical paper sheets are meant to provide a climbable surface. Dead moths are removed and stored in a box inside a freezer. This inspection is done daily. Additionally, the eclosed insects are held in a non-crowded environment with maximally 5-8 moths inside each Plexiglas cylinder to avoid stress.

Results

In this thesis a total of 81 moths were prepared for mass staining, 68 *H. armigera* and 14 *H. virescens*. Of these preparations, 16 were further analyzed by means of confocal microscopy. In total, 5 of the scanned brains turned out to include successful staining. The appearance of the prominent m-ALT was set as the minimum requirement as a successfully stained preparation. Initially, the micro-ruby was injected at random locations in the antennal lobe; 52 preparations were attempted stained according to this approach. Later in the experimental period, the dye was injected more ventrally in the antennal lobe; 29 preparations were treated in this manner. Both approaches did yield relevant results. The former approach, with random injections resulted in one successfully stained preparation, showing the three classical ALTs, i.e. the medial, the mediolateral and the lateral tract. The t-ALT was absent in this particular preparation. The latter approach provided four successfully stained brains, all of which visualized the three classic ALTs plus the t-ALT. Based on the most frequent orientation in the confocal scans, a simplified scheme of the four ALTs in a dorsal orientation is provided in figure 4. All four tracts project mainly ipsilaterally.

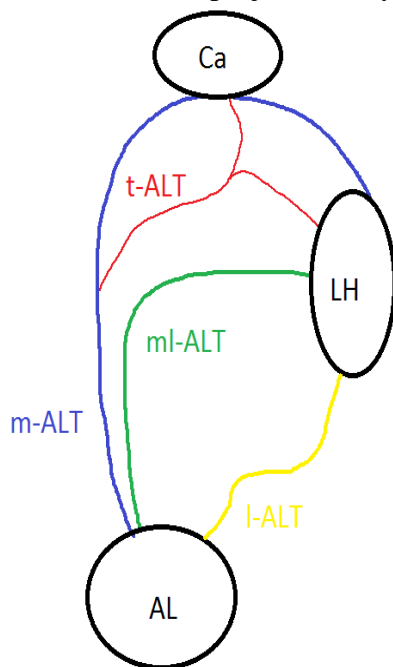


Figure 4: Schematic drawing of the antennal lobe tracts in the left brain hemisphere (dorsal orientation). m-ALT medial antennal-lobe tract, ml-ALT mediolateral antennal-lobe tract, l-ALT lateral antennal-lobe tract, t-ALT transverse antennal-lobe tract, AL antennal lobe, Ca calyces, LH lateral horn.

The transverse antennal-lobe tract

The t-ALT was stained in four of the five successfully labelled brains. In all of these preparations the dye was attempted applied into the ventral part of the antennal lobe. The t-ALT splits off from the m-ALT approximately halfway between the central body and the calyces, appearing rather thin relative to the classical ALTs. The t-ALT projects laterally in a

similar fashion as the ml-ALT, however more posteriorly (Figs. 5, 6). On its lateral path, the t-ALT diverges into at least two fibers, as shown in all the relevant preparations (Figs. 5D, 6D, 7B, 8F). These fibers terminate in two different areas, one in a region anterior to the calyces (Figs. 5D, 8F) and the other in the lateral protocerebrum (Figs. 5D, 7B). The t-ALT was however not always visible in stained preparations. Three of the four brains that included staining of the t-ALT were oriented dorsally (Figs. 5, 6, 7) and one frontally (Fig. 8).

The three classical ALTs

The three classical ALTs which have been previously well described, (Homberg, et al 1988; Rø et al, 2007) were identified in all the five successfully stained preparations. These preparations thus included the first successfully stained brain, which did not visualize the transverse tract (Fig. 9.).

The medial antennal-lobe tract: The most prominent tract in the stained preparations was the m-ALT, as shown in all figures 5-9. This particular tract can be seen as a thick fiber bundle projecting from the AL in a posterior direction, relatively close to the brain midline (Figs. 5B, 6C, 8G, 9A.). After bypassing the edge of the central body, the m-ALT turns laterally. Before terminating in the LH, it innervates the calyces from the anterior side, as shown in figures 6D and 7A.

The mediolateral antennal-lobe tract: The ml-ALT shares a common root with the prominent m-ALT, halfway to the calyces it bends off laterally, by the anterior edge of the central body (Fig. 6B) projecting directly towards the LH (Figs. 5B, 8C, 8D, 8E). Before terminating in the LH, the ml-ALT diverges into several fiber bundles that innervate distinct areas (Fig. 7A). The ml-ALT appears considerably thinner than the two other classical tracts.

The lateral antennal-lobe tract: The l-ALT exits the antennal lobe in a more ventro-lateral region than the m-ALT. Similarly to the ml-ALT, the lateral tract projects laterally targeting the LH directly (Figs. 6C-6D, 7B-7C). The thickness of this tract varied slightly in the stained preparations; in figure 6, for example, it appears especially thick. Generally, the l-ALT is thicker than the ml-ALT, but thinner than the prominent m-ALT. Notably, the presence of a characteristic sub-branch splitting off from the common path of the l-ALT and projecting in a dorsal direction was observed in one of the frontally oriented preparations (Fig. 8C-8D).

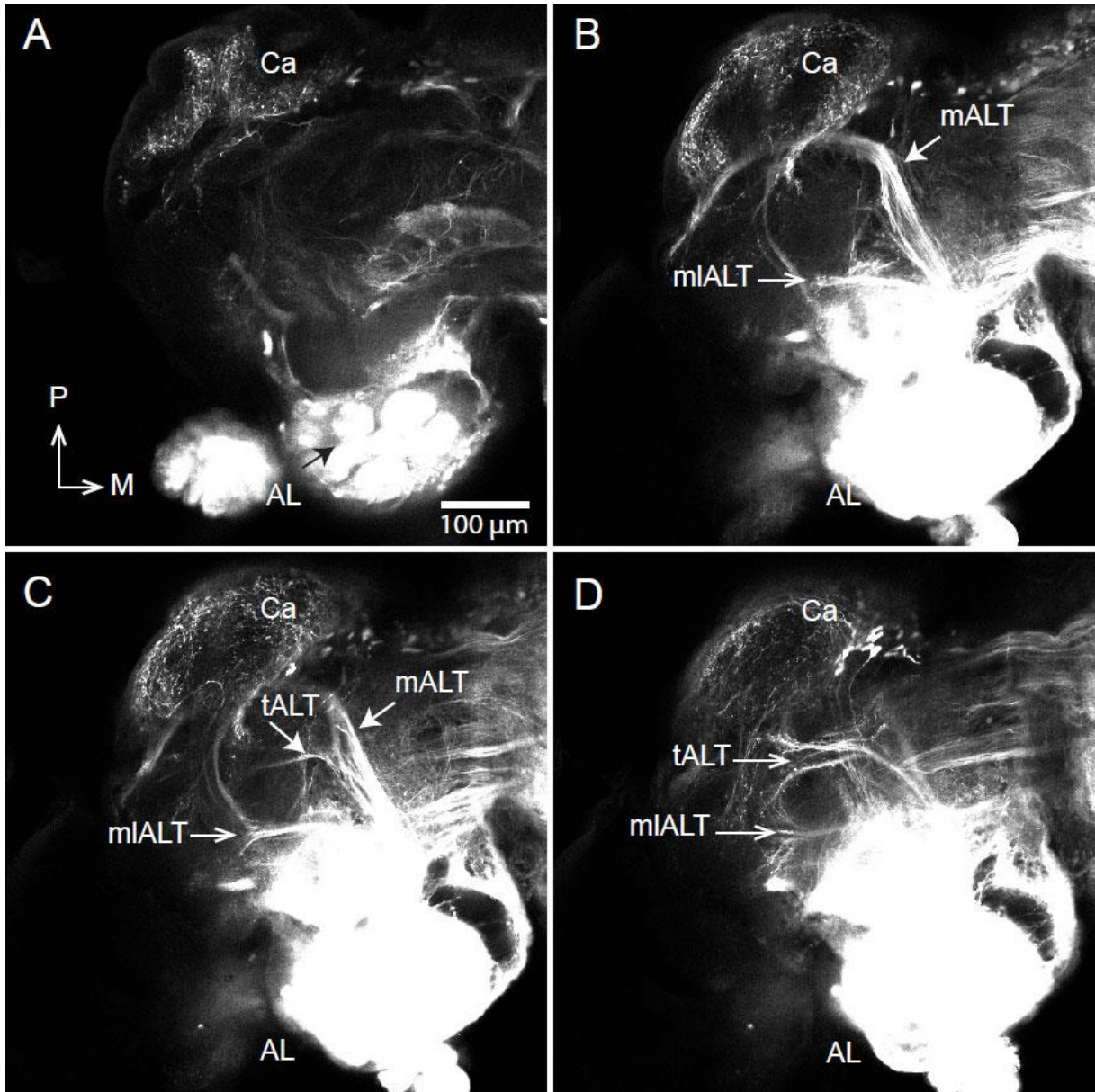


Figure 5: Confocal images of the antennal lobe tracts in the right brain hemisphere, presented from dorsal to ventral position. **A:** One slice showing the stained antennal lobe (AL), and parts of the calyces (Ca). **B:** The prominent medial antennal-lobe tract (m-ALT), projecting posteriorly, and the laterally projecting mediolateral antennal-lobe tract (ml-ALT) can be seen. As shown, the m-ALT innervates the calyces. **C:** In addition to the m-ALT and the ml-ALT, the transverse antennal-lobe tract (t-ALT) can be seen. As demonstrated, it splits off from the m-ALT posteriorly of the ml-ALT. **D:** Most ventral slice showing the t-ALT dividing in two sub-branches, one projecting more posteriorly than the other. Scale bar 100 μm . P posterior, M medial.

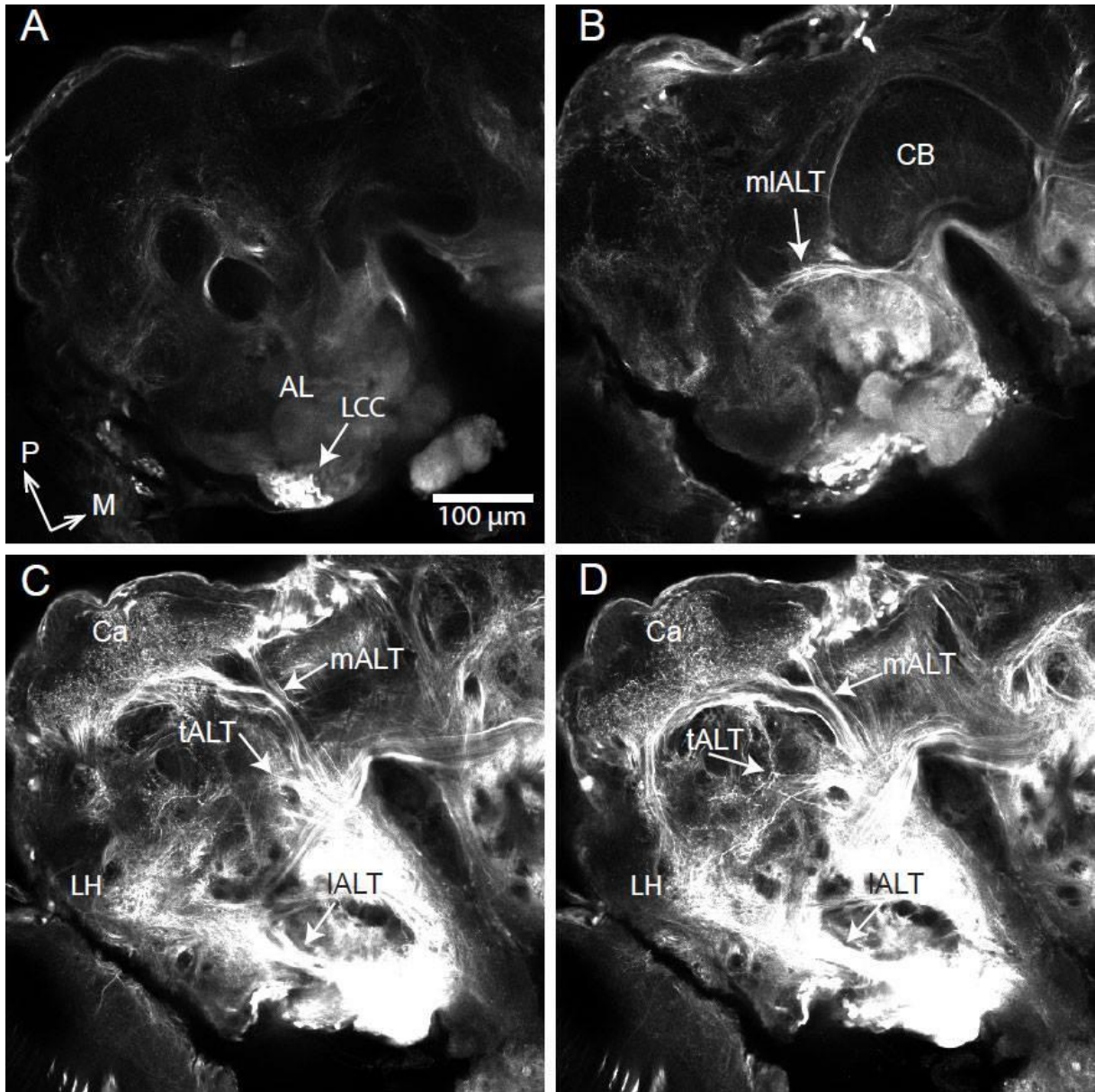


Figure 6: Confocal images showing the left brain hemisphere, represented in a dorsal to ventral position. **A:** One slice showing the lateral cell cluster (LCC), located laterally in the antennal lobe (AL). **B:** Confocal image showing the mediolateral antennal-lobe tract (ml-ALT) leaving the AL. At the edge of the central body (CB) it bends laterally and projects directly towards the lateral horn (LH). **C:** The prominent medial antennal-lobe tract (mALT) is seen leaving the AL. It projects posteriorly, and innervates the calyces (Ca) after turning laterally. The transverse antennal-lobe tract (t-ALT) is seen as it splits off from the m-ALT and projects laterally, though more posteriorly in comparison with the ml-ALT. The lateral antenna-lobe tract (l-ALT) appears as a quite thick bundle in this preparation. As indicated, it leaves the AL and projects laterally, innervating the LH neuropil. **D:** The m-ALT can be seen as it turns anteriorly after innervating the Ca and terminates in the LH. Scale bar 100 μ m. P posterior, M medial.

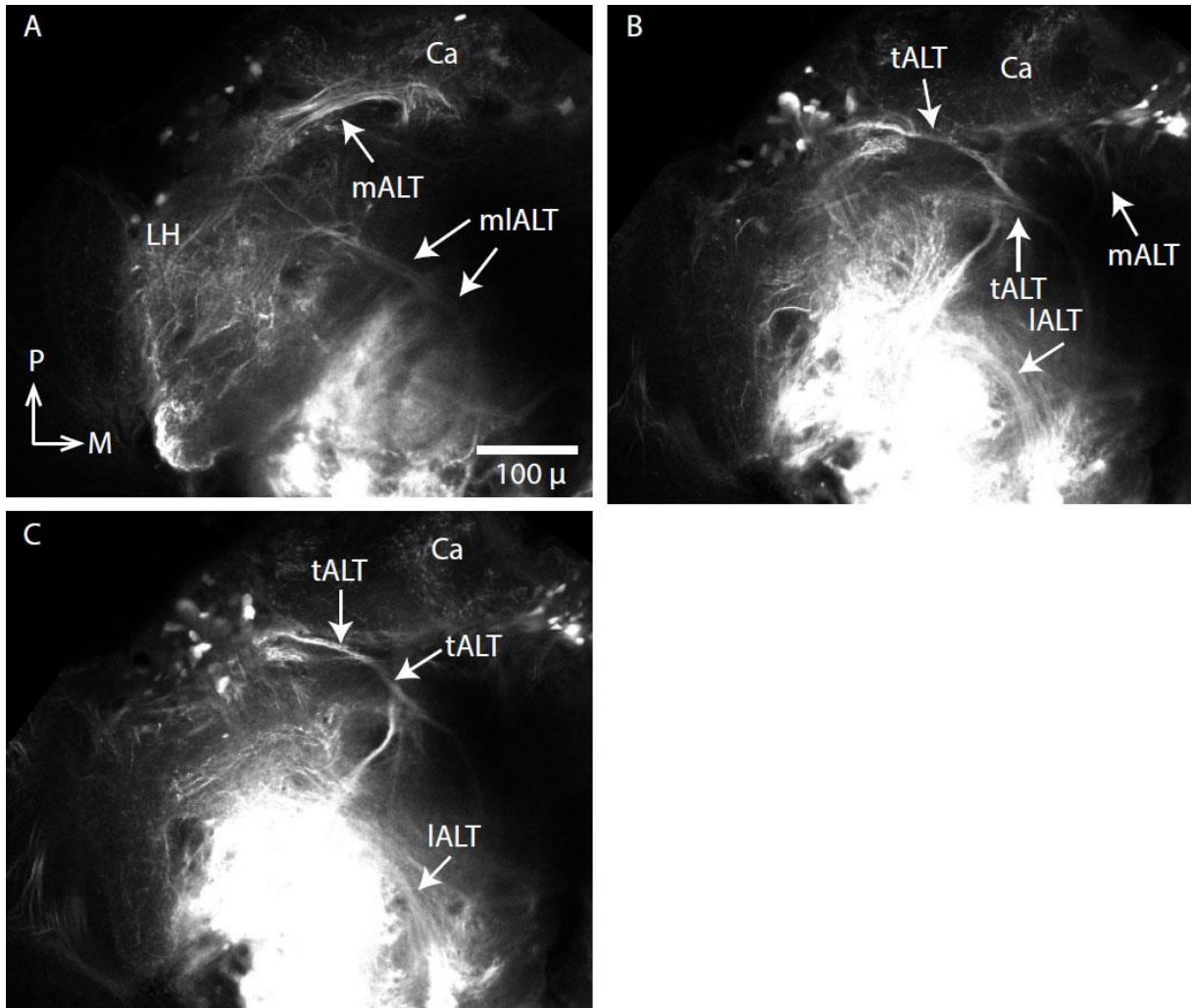
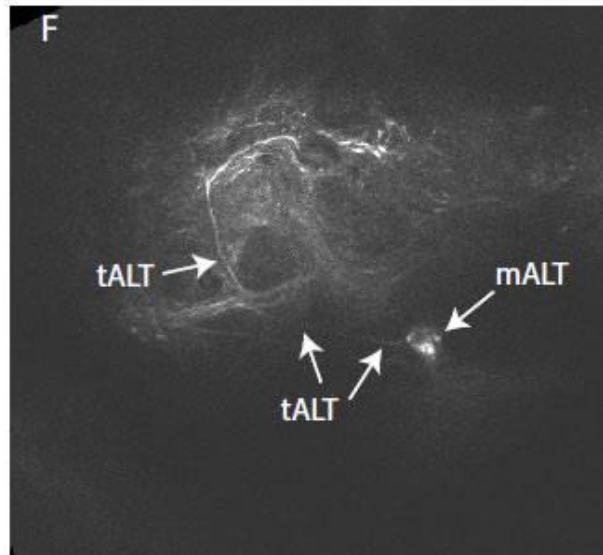
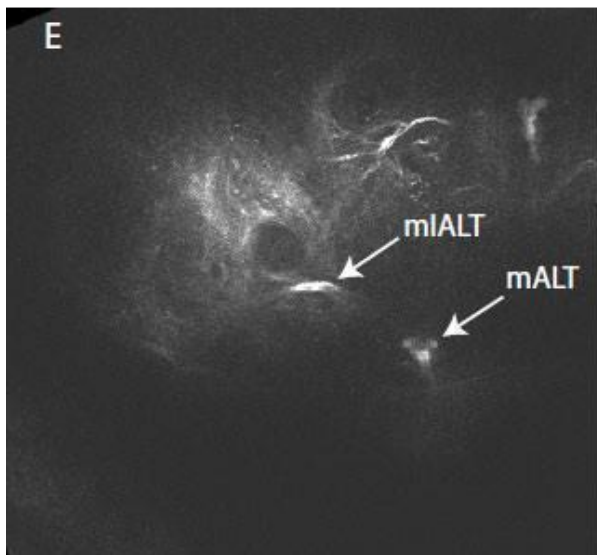
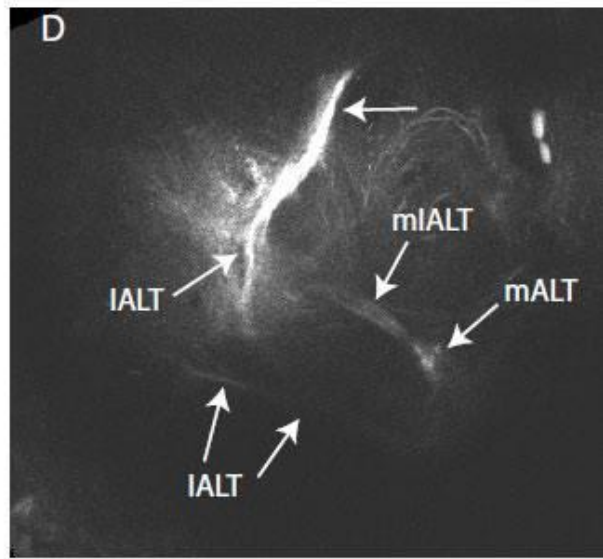
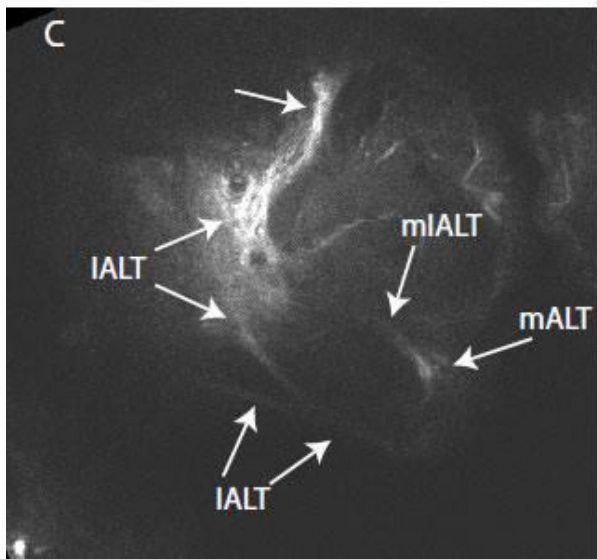
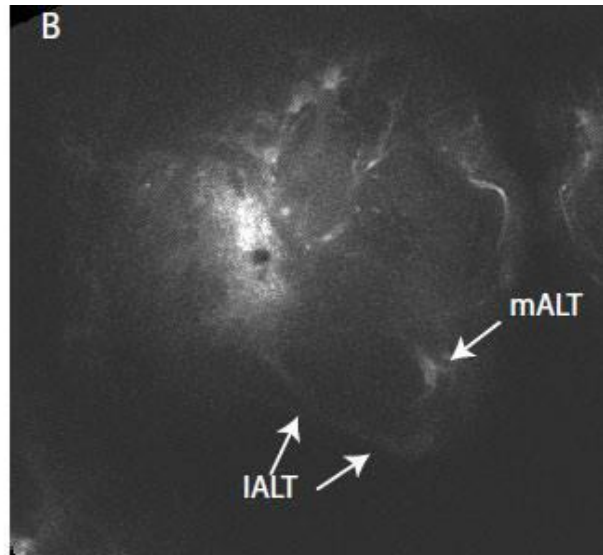
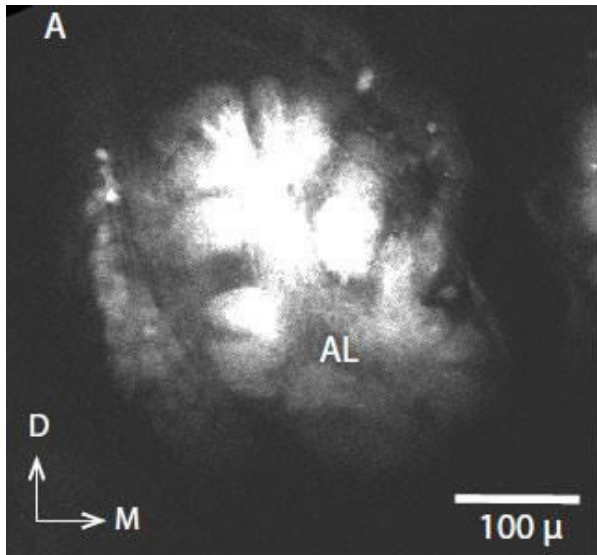


Figure 7: Confocal images illustrating the right brain hemisphere, presented in a dorsal to ventral position. **A:** The final parts of the medial antennal-lobe tract (m-ALT) can be seen as it leaves the calyces (Ca) and terminates in the lateral horn (LH). The mediolateral antennal-lobe tract (ml-ALT) projecting laterally and targeting the LH directly is also visible. **B, C:** The transverse antennal-lobe tract (t-ALT) projects in parallel to the ml-ALT, however, more posteriorly. The t-ALT divides into two sub-branches, one passing more posteriorly and the other more anteriorly. The lateral antennal-lobe tract (l-ALT) is visible, leaving the antennal lobe (AL) before projecting laterally towards the LH. Scale bar 100 μm. P posterior, M medial.



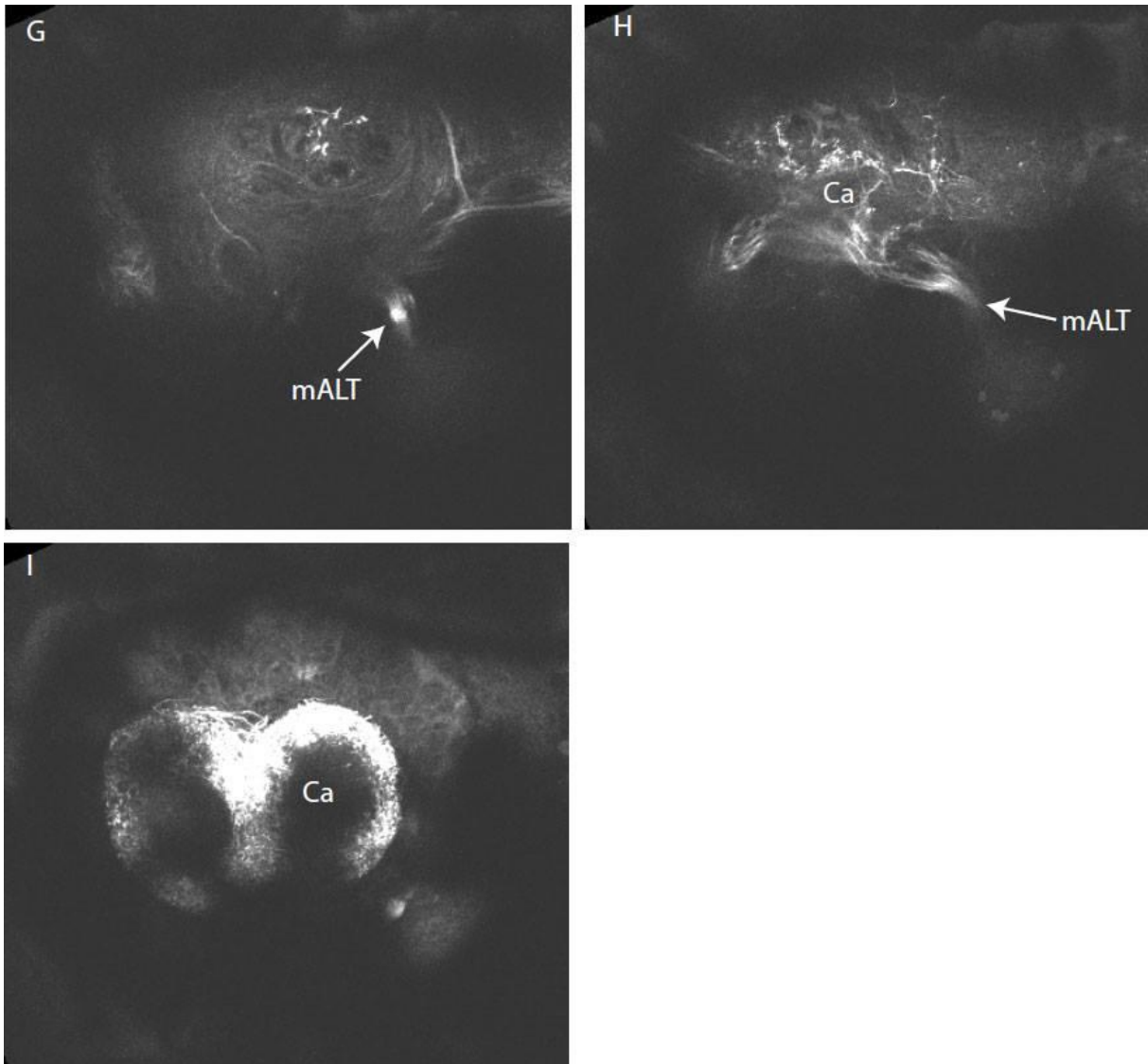


Figure 8: Confocal images of the left brain hemisphere, oriented in a frontal position. The images are presented from an anterior to posterior position. **A:** One slice showing the antennal lobe (AL) in which the dye was applied. **B:** The initial part of the medial antennal lobe-tract (m-ALT) can be observed. In addition, the lateral antennal-lobe tract (l-ALT) is also visible. **C, D:** Two slices showing the mediolateral antennal-lobe (ml-ALT) bending off from the m-ALT. In addition, one strongly stained fiber bundle splitting off from the l-ALT and projecting dorsally can be seen (arrow). **E:** The ml-ALT projecting ventrally can be seen, plus a cross-section of the m-ALT. **F:** One slice showing the transverse antennal-lobe tract (t-ALT), branching off from the m-ALT. One dorsally projecting sub-branch of the t-ALT is visible. **G, H:** The m-ALT is seen as a cross-section before the laterally projecting fiber bundle innervating the calyces (Ca) is visible. **I:** The Ca as occurring in a frontally oriented brain can be seen. Scale bar 100 μ m. D dorsal, M medial.

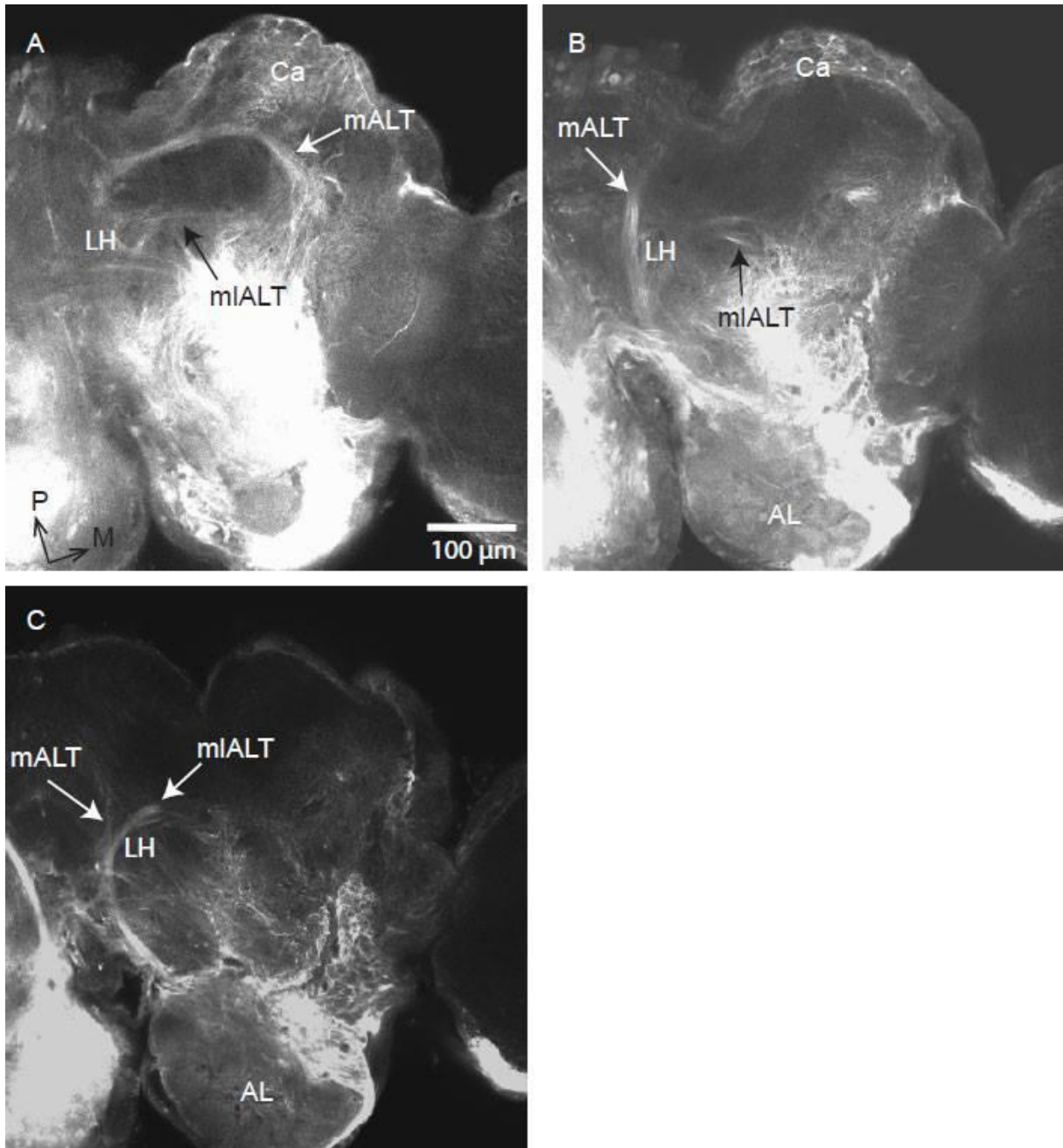


Figure 9: Confocal images of the left brain hemisphere, presented from a dorsal to ventral position. **A:** One slice showing the prominent medial antennal-lobe tract (m-ALT) innervating the calyces (Ca). Though barely visible, the mediolateral antennal-lobe tract (ml-ALT) can be seen as it projects laterally, towards the lateral horn (LH). **B, C:** Two slices showing the m-ALT projecting posteriorly from the antennal lobe (AL). In addition, the ml-ALT can be seen as it splits off from the m-ALT and projects directly to the LH. Outlines of glomeruli are visible within the antennal lobe. Scale bar 100 µm. P posterior, M medial.

Discussion

The successfully stained preparations in this study visualized all the three classical antennal lobe tracts, i.e. the m-ALT, the ml-ALT, and the l-ALT. Additionally, the recently discovered t-ALT was stained in several preparations. All four tracts projected to higher brain regions in the ipsilateral hemisphere. In the consecutive discussion, selected topics including 1) specific properties typifying the t-ALT, 2) general characteristic of the three classical tracts, 3) one distinct observation concerning the l-ALT, plus 4) methodological considerations are commented on.

The transverse antennal-lobe tract

As compared to the classical ALTs, consisting of relatively tight fiber bundles, the t-ALT appeared as more loosely assembled, including at least two sub-bundles. The fact that the t-ALT appeared occasionally, suggests that it is connected to a distinct sub-set of glomeruli in the antennal lobe. This may also be the main reason for its absence in most previous reports. The t-ALT, which was first identified by a former master student in the lab (Lillevoll, 2013), shares some resembling features with the ml-ALT. First and foremost, the t-ALT has a relative thin appearance in stained preparations, similarly to the ml-ALT - albeit appearing even thinner. Secondly, the laterally projecting t-ALT splits off from the m-ALT in a similar pattern as the ml-ALT. Whereas the ml-ALT splits off from the m-ALT halfway to the calyces, however, the t-ALT splits off more posteriorly. In fact, the t-ALT seems to have been stained in a previous study dealing with *H. virescens*, but here it was interpreted as the ml-ALT (Løfaldli, Kvello, Kirkerud & Mustaparta, 2012). Also, a few projection neurons found in *M. sexta*, showing a similar trajectory, seem to have been confined to the m-ALT (Homberg et al, 1988). These neurons named PIC in *M. sexta*, display a similar projection pattern as the t-ALT.

The transverse tract was originally discovered in the fruit fly *D. melanogaster*, by Tanaka et al, in 2012. In this species, the t-ALT is reported to consist of four sub-tracts. Like in the moth, the t-ALT shares a common root with the m-ALT before splitting off from the main path posteriorly of the central body. Each of the four sub-tracts forming the t-ALTs in the fruit fly is reported to consist of a small number of morphological similar neurons, presumable accounting for the somewhat splayed appearance of this path. In the staining experiments performed here, the fluorescent dye was applied into the ventral antennal lobe in all four brains displaying the t-ALT. In the successfully labeled brain not visualizing the t-ALT, on

the other hand, the dye was applied more dorsally in the antennal lobe. These data correspond with the findings from *D. melanogaster* where the t-ALT is connected only to ventrally located glomeruli of the antennal lobe (Tanaka et al, 2012). If the general projection pattern of the transverse tract of the moth is similar to the corresponding tract of the fruit fly, it probably means that this newly discovered tract is not linked to the male-specific MGC which is located dorso-laterally in the antennal lobe. Such a projection pattern would differ from that of the three classical ALTs, which are reported to carry information about both pheromones and plant odors (Homberg et al, 1998; Zhao et al, 2014). A more systematic investigation of the male moth is required for concluding on this particular topic, however.

The three classical antennal-lobe tracts

The identification of three main ALTs, as presented in this thesis, coincides with previous findings from the moth brain (Homberg et al, 1998; Rø et al, 2007). In particular, the finding of one prominent tract, projecting posteriorly from the antennal lobe and innervating the calyces before terminating in the LH, corresponds to previous findings not only in moths, but also various other insect groups (reviewed by Galizia & Rössler, 2010). The prominence of this tract is most likely due to the quantity and diameter of its axons. In the moth *M. sexta*, for example, the m-ALT is reported to comprise roughly 380 fibers in males and 320 fibers in females (Homberg et al, 1998). A relatively large number of individual projection neurons confined to the m-ALT has been described. Interestingly, in male moths, projection neurons originating from the MGC are reported to innervate distinct areas, both in the calyces and LH, from plant odor neurons, which are linked to the ordinary glomeruli (Homberg et al, 1998, Zhao et al, 2014). The higher number of projection neurons in m-ALT of the male, as reported from *M. sexta*, probably include the sub-population connected with the MGC.

The finding of a relatively thin tract splitting off from the m-ALT and projecting to the LH, the ml-ALT, also correspond with previous reports (Homberg et al, 1998, Rø et al, 2007). This tract consists of around 120 axons which are reported to be parts of multiglomerular neurons (Homberg et al, 1998; Rø et al, 2007), a substantial amount of which is GABAergic (Berg et al, 2009).

The observation of the l-ALT as a rather thick tract leaving the antennal lobe more ventrally than the others is in agreement with previous reports as well. In *M. sexta*, this tract comprises more than 300 fibers (Homberg et al, 1998). One interesting finding in the study present here is a distinctive sub-branch splitting off from the common projection pathway of the l-ALT

(Fig. 8C, D). This sub-branch, forming a characteristic column-like structure, extends dorsally in a medial region of the protocerebrum, located relatively close to the posterior border of the antennal lobe. The fact that this particular l-ALT branch was observed only in one of the five successfully stained preparations, suggests that its projection neurons originate from a specific sub-set of glomeruli within the antennal lobe – in a similar manner to the occasional occurrence of the transverse tract. The dye was attempted applied in the ventral part of the antennal lobe in this preparation. Thus, the putative sub-set of glomeruli being connected to the column-shaped structure may therefore be located in this area. Interestingly, in a previous master thesis from the lab (Dahl, 2013), a similar column-formed sub-bundle of axons originating from the labial pit organ glomerulus (LPOG) was found.

The LPOG unit is an enlarged glomerulus situated postero-ventrally in the antennal lobe that is described to receive input about CO₂ from the labial pit organ (Zhao et al, 2015, Kent 1986). Lateral-tract fibers of similar morphological characteristics as those observed in the sub-branch described here have previously been reported in both *H. virescens* (Rø et al, 2007) and in *M. sexta* (Homberg et al, 1988). However, the presence of this column-shaped structure extending from the l-ALT seems to have been somewhat overlooked in review reports (Galizia and Rössler 2010; review article from J. Hildebrand).

Methodological considerations

Various staining techniques have been utilized for anatomical investigation of the insect olfactory pathway. In the study presented her, the mass staining technique was used for exploring the general projection pattern of the antennal-lobe projection neurons, which constitute one of the two major categories of second order neurons. By applying dye into the antennal lobe, the axons' own ability of anterograde transport thus carries the fluorescent markers to the terminal regions in the calyces and the lateral horn. One advantage of choosing this approach is the opportunity to map the overall neural circuitry at this particular level. Due to the small size of the moth brain, this kind of experiment requires a high level of precision during application of the dye. Achieving this skill may require a tremendous amount of practice and patience of the researcher. The relatively few successfully stained preparations obtained in this study are a testament to the amount of effort needed.

Whereas the small size of the moth brain is a challenge during the staining procedure it is a great benefit regarding the experimental part involving confocal microscopy; the opportunity of scanning the whole brain preparation without making physical slices is indeed appropriate.

The anterogradely stained preparations analyzed in this study consequently showed all the three main ALTs. Furthermore, all the successful preparations including dye applied in the ventral part of the antennal lobe also included the t-ALT. The method of applying dye in distinct parts of the antennal lobe is therefore promising for further analysis. However, more staining experiments providing a larger amount of data than that obtained in this master project have to be carried out in future studies.

Conclusions

1. Utilization of the anterograde labeling technique including dye applied to the antennal lobe provided successful staining of the antennal-lobe tracts formed by axons of the projection neurons.
2. All successfully stained preparations revealed the three classical tracts including the medial, the medio-lateral, and the lateral ALT.
3. The occurrence of the transverse ALT, in addition the classical ALTs, in all successfully stained brains that were labeled via dye applied to the ventral part of the antennal lobe, suggests that the t-ALT may be selectively linked to a subset of the glomeruli.
4. The occurrence of a characteristic side-branch splitting off from the main course of the l-ALT indicates that projection neurons forming this path originate from distinct antennal-lobe glomeruli.

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