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Sammendrag

Luktesansen er evolusjonsmessig den eldste sansen, og det å kunne detektere og respondere på kjemiske signaler er fortsatt det primære vinduet for å persipere den eksterne verden for de fleste dyrearter. I denne studien ble møllen *Heliothis virescens* (Lepidoptera: Noctuidae) brukt som modellorganisme for å studere luktesansens nevrale system. Hos denne arten sendes informasjon i andre ordens luktnevroner fra antenneloben til høyere ordens hjerneområder via hovedsakelig tre trakter. To av disse traktene er relativt bra beskrevet hos møll; den mest prominente av disse, den mediale, innerverer både calyces og det laterale horn, mens den medio-laterale trakten projiserer direkte til det laterale horn uten å innervere calyces. Den tredje trakten, den laterale, er mindre beskrevet, og andelen aksoner med direkte forbindelse til calyces i denne trakten har til nå ikke blitt systematisk studert hos møll. Hovedmålet med det nåværende prosjektet var derfor å kartlegge andelen av andreordens luktnevroner som er knyttet til calyces i den laterale trakt. Datainnsamlingen ble gjennomført ved å visualisere disse nevronene ved hjelp av massefarging. Både retrograd (fra antenneloben) og anterograd farging (fra calyces) ble gjennomført. Størsteparten av preparatene ble dobbeltfarget (farget samtidig fra både antenneloben og calyces). Maksimalt seks nevroner som projiserte i den laterale trakten til calyces ble visualisert. Resultatene (høyoppløselige konfokalbilder) viste derfor at de aller fleste nevronene i den laterale trakt har sine terminalområder i det laterale horn, noe som viser at kommunikasjonen mellom antenneloben og calyces hovedsakelig foregår i den mediale antennelobetrakten.

Abstract

The sense of smell is the evolutionary oldest sense, and the ability to detect and respond to chemical signals is the primary window for perceiving the external world for most animal species. In this study, the tobacco budworm moth, *Heliothis virescens* (Lepidoptera: Noctuidae), was used as a model organism to study the neural system linked to olfaction. The second order level of the olfactory pathway in this species includes three main tracts conveying information from the antennal lobe to higher brain areas. Two of the three parallel tracts are relatively well described in moths; thus, the most prominent one, the medial antennal-lobe tract, is known to innervate the calyces of the mushroom bodies and the lateral horn whereas the medio-lateral tract projects directly to the lateral horn without making any connection to the calyces. The third tract, the lateral one, on the other hand, is less well described. In particular, the portion of lateral-tract axons forming a direct connection with the calyces has not been systematically studied in any moth species so far. The main aim of the current project was therefore to map the fraction of lateral-tract neurons linked to the calyces. The data were obtained by visualizing the second-order neurons via mass-staining. Both retrograde (from the antennal lobe) and anterograde staining (from the calyces) were used, plus a combination of the two techniques. Most of the preparations were double-labelled (simultaneously stained from the antennal lobe and the calyces). The results, comprising high-resolution confocal images, showed that most of the neurons in the lateral antennal lobe tract terminate in the lateral horn. At most, six neurons connecting this tract to the calyces were found. Thus, in moths, the connection between the antennal lobe and the calyces is primarily made up by projection neurons confined to the medial antennal lobe tract.

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1 Abbreviations

AL	-	Antennal Lobe
Ca	-	Primary Calyces of the Mushroom Body
CB	-	Central Body
H	-	Mushroom Body Heel
l-ALT	-	Lateral Antennal Lobe Tract
LH	-	Lateral Horn
m-ALT	-	Medial Antennal Lobe Tract
ml-ALT	-	Medio-lateral Antennal Lobe Tract
OSN	-	Olfactory Sensory Neuron
P	-	Peduncle
PN	-	Projection Neuron
Y	-	Mushroom Body T-tract

2 Introduction

The sense of smell is the evolutionary oldest sense, and the ability to detect and respond to chemical signals is the primary window for perceiving the external world for most animal species (Ache & Young, 2005). The fact that all organisms have developed a system for detecting chemical stimuli from their environment indicates its general importance for survival. Thus, both vertebrates and invertebrates can sense chemical cues connected with food, possible partners, and danger.

2.1 The insect as a model organism

More than any other sensory arrangement the olfactory system has been conserved trough evolution. Therefore, the investigation and exploration of the olfactory pathway in invertebrates can give insight into fundamental mechanisms of olfaction in mammals, humans included. Insect brains are structurally different from the brain of vertebrates, yet the basic demands of life are quite similar between various species (Menzel, Leboulle & Eisenhardt, 2006; Wolff, Harzsch, Hansson, Brown & Strausfeld, 2012). Both in vertebrates and invertebrates, particular odorants activate neural pathways displaying remarkable resemblances at various synaptic levels (Ache & Young, 2005). Given the similarities in the olfactory system across species, and exploiting ethical advantages, insects have often been used as models for studying general principles underlying processing of chemosensory information. Also, the relatively simple and easily accessible nervous system of insects makes them ideal objects for experimental research. In these organisms, many neurons can be morphologically identified at a single cell level, which makes it possible to understand the working of the network (Menzel et al., 2006). As concerns investigations of the olfactory system, their reduced number of glomeruli as compared to that of mammals is particularly advantageous.

2.2 The human olfactory system

During breathing, airborne molecules enter the nasal cavity and come in contact with chemosensory neurons located in the olfactory epithelium. This is a specialized epithelial tissue situated in the upper part of the nasal cavity (Bear, Connors & Paradiso, 2007), as seen in figure 1. The olfactory epithelium contains numerous olfactory sensory neurons (OSNs), plus supporting cells, basal cells, and microvillar cells (Morrison & Costanzo, 1990). It is assumed that the total number of OSNs in the human olfactory epithelium exceeds six million (Doty, 2001). The OSNs are bipolar neurons extending two branches from the soma, one

pointing towards the external world, and the other projecting to the brain. The externally exposed part of the OSN is a small dendritic knob with cilia (Moran, Rowley, Jafek & Lovell, 1982). The cilia expand into a mucus layer covering the surface of the epithelium. The membrane of the cilia possesses numerous receptor proteins capable of binding various odorants (Bjering, Deilboll & Mæhlen, 2008; Purves et al., 2012). It has been shown that mammals have approximately 1000 different genes that code for odor receptors (Buck & Axel, 1991).

When a particular ligand binds to a receptor protein, a transduction process is initiated. During this process, the chemical energy is transduced to electrical energy, i. e. action potentials being transferred via the axon membrane (Doty, 2001). The sensory axon projects through the cribriform plate directly into the olfactory bulb where it terminates. This area of the mammalian brain, which is also called the primary olfactory center, is located ventrally of the frontal lobe. In the olfactory bulb, the axons bundle together terminating in about 2000 nest-formed structures called glomeruli. Neurons that express the same type of receptor protein send their axons into the same glomerulus (Fishilevich & Vosshall, 2007; Gao, Yuan & Chess, 2000). About 25 000 OSNs axon terminals are reported to form synapses with dendrites of about 100 second order neurons within each glomerulus (Bear et al., 2007; Connors & Paradiso, 2007). The great difference between the number of first and second order neurons shows that there is a strong convergence at the current synaptic level. In the olfactory bulb, there are different cell types including projection neurons passing to cortical brain regions and local interneurons being restricted to the olfactory bulb.

The projection neurons (PNs) comprise mitral and tufted cells carrying the olfactory information via the olfactory tract to higher brain areas, as shown in figure 1. The target regions of the PNs are cortical areas located in phylogenetically old regions of the temporal lobe. The areas receiving direct input from the olfactory bulb is termed the primary olfactory cortex. These include the piriform and the entorhinal cortex. The olfactory cortex is connected to hippocampus and amygdala (Bear et al., 2007; Purves et al., 2012). The hippocampus is an area important for memory whereas the amygdala, receiving information both directly from the olfactory bulb and from the olfactory cortex, plays a key role in regulating emotional conditions (Purves et al., 2012). In addition, olfactory information is sent to the hypothalamus, an area involved in control of the autonomic nervous system and the endocrine arrangement (Bear et al., 2007). Parts of the odor signals are forwarded from the allocortex to neocortical areas in the frontal lobe, either directly or relayed through the thalamus (Brodal,

2007; Purves et al., 2012). The fact that olfactory information is in contact with so many different areas of the brain demonstrates the complexity of the system, and its importance for the survival of the various organisms through evolutionary history.

The olfactory pathway is different from the other sensory arrangements in the mammalian brain by having a direct connection to the cortical region. All other sensory pathways, that of taste included, are relayed through the thalamus before entering the cortical areas (Bear et al., 2007). Generally, the anatomical organization of the olfactory pathway indicates that smell is less connected to our consciousness than the information about other sensory modalities.

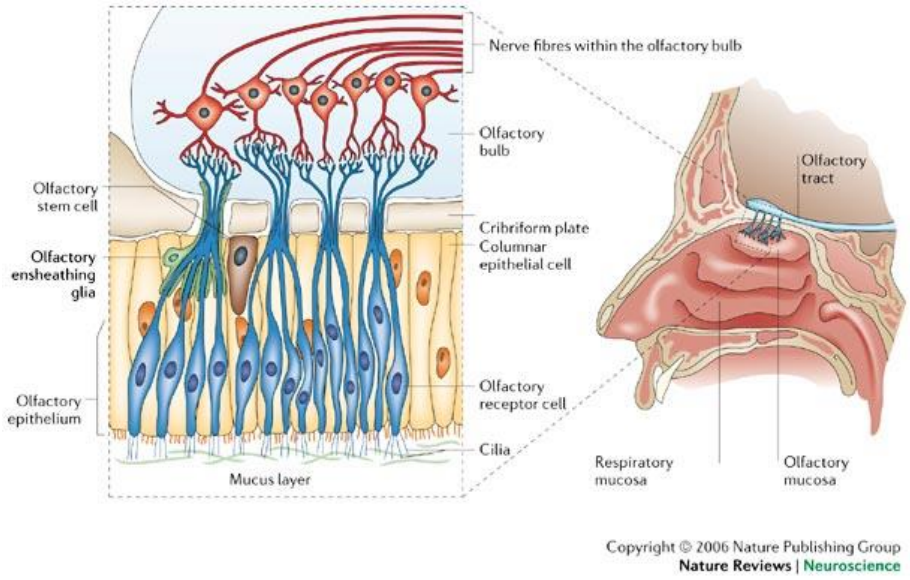


Figure 1: Schematic drawing showing the first order level of the olfaction system in humans. The chemical stimulus is taken up in the mucus layer situated in the olfactory epithelium of the nasal cavity. Here, the odorants bind to numerous olfactory sensory neurons having axons crossing the cribriform plate, and terminating in the olfactory bulb. The sensory neuron terminals form connections with projection neurons in olfactory-bulb glomeruli (not shown on the figure). The projection neurons send axons to higher brain areas through the olfactory tract (Thuret, Moon & Gage, 2006).

2.3 The insect olfactory system

2.3.1 The peripheral olfactory system

The insect's main sensory organ for detecting airborne odorants is the flagellum, a segment of the antenna covered by a large number of sensory hairs called sensilla (Dacks et al., 2009). Each olfactory sensillum contains one to several OSNs which are first order neurons capable of detecting odors. As in mammals, the OSNs are bipolar cells, having a dendrite that extends toward the external world – here, into the lumen of the sensillum, and an

axon that projects through the antennal nerve directly into the primary olfactory center of the brain. In order to perceive the odor information the signals being carried by distinct types of OSNs have to be processed within the relevant neural circuits of the brain. In insects, the primary olfactory processing center, corresponding to the mammalian olfactory bulb, is the so-called antennal lobe (AL) (Boeckh & Tolbert, 1993; Dacks et al., 2009; Haupt, Sakurai, Namiki, Kazawa & Kanzaki, 2010; Homberg, Christensen & Hildebrand, 1989; Kaupp, 2010; Keil, 1999).

2.3.2 The primary olfactory center, the antennal lobe

As in other insects, the ALs of moths contains three main types of neurons, the already mentioned OSNs, local inter-neurons (LNs) and the projection neurons (PNs) (Fukushima & Kanzaki, 2009; Homberg et al., 1989). As in mammals, the OSNs terminate in glomeruli within the ALs where they connect with the two main categories of AL neurons; LNs and PNs (Homberg et al., 1989). The spheroidal glomerular structures are neuropil regions made up of densely packed neural processes. In the lepidopteran model species *Heliothis virescens* each AL contains 65 glomeruli (Berg, Galizia, Brandt & Mustaparta, 2002; Løfaldli, Kvello & Mustaparta, 2010). The LNs are confined into the AL where they have arborizations in many glomeruli (Reisenman, Dacks & Hildebrand, 2011). The PNs, on the other hand, connect the AL with higher order brain centers, mainly the ipsilateral lateral horn (LH), an area defined as the part of the lateral protocerebrum being innervated by the AL PNs (Ito et al., 2014), and the mushroom body calyces (Homberg, Montague & Hildebrand, 1988).

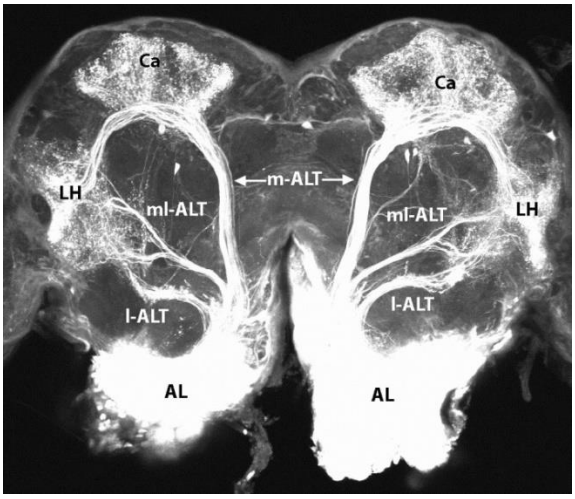


Figure 2: Confocal image showing the three main tracts connecting the antennal lobe (AL) with the lateral horn (LH) and the calyces (Ca). As shown in the figure, neural branches project into the calyces from the prominent medial antennal lobe tract (m-ALT) before terminating in the LH. The two other tracts, the medio-lateral antennal lobe tract (ml-ALT) and the lateral antennal lobe tract (l-ALT) project directly to the LH. Dorsal view. Shown with permission from Xin-Cheng Zhao (unpublished data).

2.3.3 The antennal lobe tracts

In the thoroughly studied sphinx moth, *Manduca sexta*, approximately 800 PNs connect each AL to higher order brain areas involved in olfaction via three main tracts (Homberg et al., 1988). Corresponding tracts have been found in several other moth species, including *H. virescens* (Iwano & Kanzaki, 2005; Rø et al., 2007). According to the new nomenclature suggested by Ito et al. (2014), these tracts are named the medial antennal lobe tract (m-ALT), the medio-lateral antennal lobe tract (ml-ALT), and the lateral antennal lobe tract (l-ALT). There are mainly two higher order areas involved in odor information processing, the calyces of the mushroom body and the LH. For an overview of the second order level of the moth's olfactory pathway, see figure 2 and 3. Not much is known about the functional significance of the parallel antennal lobe tracts (ALTs), and how olfactory information is processed within these pathways.

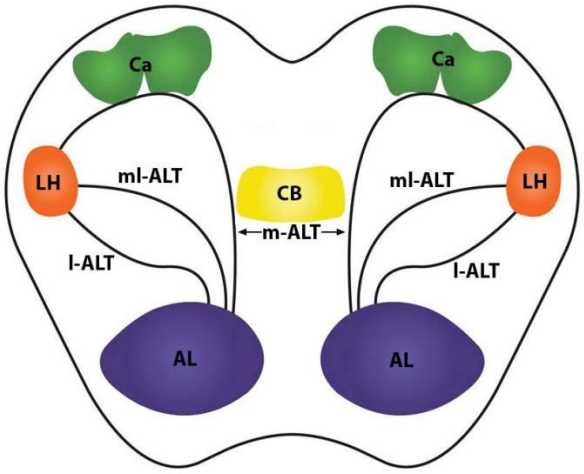


Figure 3: Schematic drawing showing the second-order olfactory pathways in the moth brain. From the antennal lobe (AL), the three main antennal lobe tracts (ALTs) project to the higher brain regions. Ca calyces, CB central body, LH lateral horn, m-ALT medial ALT, ml-ALT medio-lateral ALT, l-ALT lateral ALT.

The medial antennal lobe tract: Two roots within the AL, the dorsal and the ventral, leave the AL fusing at the posterior-lateral edge of the central body, forming the prominent m-ALT. The projection of this tract is shown in figure 2 and 3. In *M. sexta,* the m-ALT contains approximately 320 fibers in male, and 280 in female (Homberg et al. 1988). The m-ALT projects posteriorly, innervating a part of the ipsilateral mushroom bodies known as the calyces before turning antero-laterally terminating in the LH (Homberg et al., 1988; Ito et al., 2014; Rø et al., 2007; Zhao & Berg, 2010), as shown in figure 2. The m-ALT contains mainly uniglomerular PNs, which means that these neurons have dendritic arborizations in one

glomerulus in the AL. It is assumed that most of the PNs in this tract have cell bodies located in the medial cell cluster (Homberg et al., 1989).

The m-ALT is found in many insect species, always displaying a similar projection pattern to that described above (for review see Galizia & Rössler, 2010). This demonstrates the general importance of this tract for odor processing. Most of the individual PNs that have been morphologically and physiologically identified in moths, *H. virescens* included, are uniglomerular PNs passing in the m-ALT. (Christensen, Harrow, Cazzocrea, Randolph & Hildebrand, 1995; Berg et al., 1998; Vickers, Christiansen & Hildebrand, 1998; Zhao et al., submitted article).

The medio-lateral antennal lobe tract: The ml-ALT leaves the AL together with the m-ALT before it turns laterally at the edge of the central body, as shown in figure 2 and 3. The ml-ALT is significantly smaller than the m-ALT by containing approximately 120 neurons (Homberg et al., 1988). This tract projects directly to the LH, bypassing the calyces (Berg, Schachtner & Homberg; 2009; Rø et al., 2007). Further, the neurons in the ml-ALT are mainly multi-glomerular, which means that the PNs have arborizations in several glomeruli (Homberg et al., 1988). The cell bodies of the neurons in this tract are located in the lateral cell cluster. Also, the somata are smaller than those of neurons in the m-ALT. (Homberg et al., 1988; Homberg et al., 1989). A considerable number of these neurons are shown to be GABAergic, also in *H. virescens* (Berg et al., 2009; Iwano & Kanzaki, 2005).

The lateral antennal lobe tract: The l-ALT leaves the AL medio-ventrally. It runs laterally, directly to the LH, however, in a more antero-ventral position than the ml-ALT (Fig. 2) (Homberg et al., 1988; Iwano & Kanzaki, 2005; Rø et al., 2007). Many of its fibers continue in different directions and therefore it soon loses its tract-like appearance. The number of axons leaving the AL along the l-ALT in *M. sexta,* is estimated to 286 to 390 in females and 335 to 390 fibers in males (Homberg et al., 1998). At least 75 of these never target the LH (Homberg et al., 1988). Whether this is the same in *H. virescens* remains to be known. However, a substantial number of the axons confined to the current tract in *H. virescens* have been shown to target other brain regions than the lateral protocerebrum (Lillevoll, 2013, master thesis). The PNs projecting in the l-ALT is assumed to be primarily multi-glomerular, however, uniglomerular neurons have been identified as well (Rø et al., 2007). The cell bodies of the PNs in this tract are situated in the lateral cell cluster ((Homberg et al., 1989; Iwano & Kanzaki, 2005). Also, a characteristic feature of the current axons is their thin fibers, usually being less than 1 µm (Homberg et al., 1988). Some of the neurons

that project from the AL in the l-APT of *H. virescens* are reported to be GABAergic (Berg, Schachtner & Homberg, 2009). Iwano and Kanzaki, however, did not find such neurons in the silk moth, *Bombyx mori* (2005).

2.3.4 Higher order areas involved in processing olfactory information

Like in other insect species, the olfactory information is carried to two main intergration centres in the protocerebrum, the calyces of the mushroom bodies and the lateral horn (reviewed by Galazia and Rössler, 2009). The mushroom body calyces (Fig. 2) is the first structure innervated by PNs in the m-ALT. This is a paired structure located posterior-dorsally in the moth brain, formed by two cups, one medial and one lateral, with the adjacent walls completely fused (Rø et al., 2007; Sjöholm, Sinakevitch, Ignell, Strausfeld & Hansson, 2005). At the base of the calyces, the two cups merge and form the pedunculus which runs anteriorly and branches into three main and several smaller lobes (Sjöholm et al., 2005). A second tract, called the Y tract, also leaves the calyces at its base and follows a route towards the mushroom body lobes. (For a more detailed overview of the different lobes, see Rø et al., 2007; Fukushima & Kanzaki, 2009; Sjöholm et al., 2005).

The calyces are built up by third order neurons that receive input from the AL PNs. These cells are known as Kenyon cells. The exact number of these cells is not known, but in the moth species, *Spodoptera littoralis*, the calyces contain around 4000 Kenyon cells (Sjöholm et al., 2005). Moths possess at least four different types of Kenyon cells having different dendritic arborization patterns in the calyces and distinct projections to different subdivisions in the lobes (Fukushima & Kanzaki, 2009; Sjöholm et al., 2005; Strausfeld, Sinakevitch, Brown & Farris, 2009).

As the calyces receives information from the AL, and are found in the brain of most insects that have olfactory glomeruli (Sjöholm et al., 2005; Strausfeld et al., 2009), it supports the theory that this area is heavily involved in processing olfactory information. Generally, the mushroom bodies are known to play an important role in mechanisms underlying learning and olfactory associative memory (Heisenberg, 1998; Menzel et al., 2006; Sjöholm, 2005; Tanaka, Awasaki, Shimada & Ito, 2004). Based on findings from the silk moth, *Bombyx mori*, demonstrating only a few pheromone collaterals targeting the calyces, it has been hypothesized that this area is mainly involved in processing non-pheromonal information (Kanzaki, Soo, Seki & Wada, 2003). Different, from *B. mori* however, pheromone neurons of most other moths species, *H. virescens* included are reported to innervate the calyces significantly (Vickers et al. 1998; Berg, Almaas, Bjaarlie & Mustaparta, 1998; Zhao et al.,

submitted article). Furthermore, Sjöholm et al. (2005) hypothesized that since there are such fundamental similarities in morphology of the mushroom bodies across different taxa, the distinct subdivisions may be of essential significance for terrestrial insects (Sjöholm et al., 2005).

The LH has been assumed to be a premotor area (Rø et al., 2005), However, as recently reported,there is no direct connection between the LH and the ventral cord in *H. virescens* (Pramod KC, master thesis, 2014). The LH has also been presumed to be sufficient for mediating direct non-associative responses to odors (Tanaka et al., 2004). Its putative role in evaluating odor concentration and quality has also been mentioned (Homberg et al., 1988), as well as its importance for processing pheromonal information (Kanzaki et al., 2003; Seki et al., 2005). Based on previous studies showing individual l-ALT PNs continuing into the calyces after projecting to the LH both in *H. virescens* and other moth species (Homberg et al., 1988; Rø et al., 2007; Sun, Tolbert & Hildebrand, 1997), it has been assumed that a substantial part of the current PNs send branches into the calyces. However, the recent master project of A. Berg (2013), including retrograde labeling of PNs in *H. virescens,* indicated that only a few axons connect the antennal lobe to the calyces via this pathway. The fact that the current part of the l-ALT is not yet systematically explored is the main motivation for the investigation carried out in the current project.

2.4 Goal of the present study

Principal goal: By using the double-fluorescence labelling technique on the moth brain, the detailed projection pattern of the antennal lobe tracts will be mapped, including the connection between the primary olfactory center and the calyces via neurons confined to the lateral tract.

Specific goal:

- To perform staining of second order olfactory neurons by applying dye in the antennal lobe and the calyces, respectively.
- To establish a technique for double-labeling including application of two different fluorescent dyes in the same individual, one in the antennal lobe and the other in the calyces.
- To study the projection pattern of the l-ALT specifically for determining the extent of axons connecting the antennal lobe to the calyces via this route.

3 Method

The method used in this study included fluorescent staining of brain tissue in living moths. The dye which was placed in specific parts of the brain, was carried by means of the neurons' own transport system. By using two different dyes, one being carried anterogradely from the AL, and another being carried retrogradely from the calyces, it was possible to explore which of the AL projection neurons that are connected to the calyces.

3.1 Insects

In this study, the tobacco budworm *H. virescens* (Lepidoptera: Noctuidae: Heliothis), was used. Most of the moths were reared in the laboratory. A few arrived as pupae, delivered by Bayer Crop Science, Germany. Before emerging the pupae were sorted by gender. After hatching, the insects were kept in cylinders of plexiglas in two heating cabinets at 22-23 °C, one for each sex (Refitherem 6E incubator, Struers), with a day-night cycle of 14 hours with light followed by 10 hours dark. The humidity in the cabinets was 70 %. In these cabinets the insects were fed with a sucrose solution (50 grams sucrose for 1 liter water), or a mixture of honey and water. Experiments were performed on moths one to 14 days after emergence; the mean age was five days.

3.2 Staining and dissection

88 moths were used in this study; 46 males, 41 females, and one where the sex was unknown. The moth was mounted into a plastic tube with the exposed head immobilized with the help of dental wax (Kerr Corporation, Romulus, MI, USA). Preparation, staining and dissection were carried out using a stereomicroscope (Leica, MZ 12.5). Of the prepared moths, 21 were stained by applying fluorescent dye into the AL only, 10 by applying dye into the calyces only, whereas 57 preparations were double-labelled by applying one dye into the AL and another into the calyces.

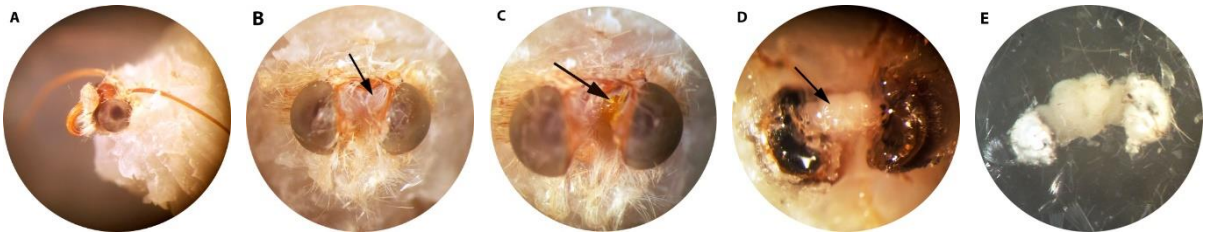


Figure 4: Images showing the procedure including dissection and staining of a preparation. **A: The** moth mounted in a plastic tube, immobilized in dental wax, scales removed. **B:** The cuticula between the eyes has been removed, as well as the antennas, the proboscis, and the mouth parts. The arrow indicates where the dye is going to be injected. **C:** Preparation where the right antennal lobe has been stained by Micro-Emerald, a yellow dye. This is shown by the arrow. **D:** Preparation where the moth's head has been cut from the thorax. The preparation is mounted into melted wax exposing the posterior-caudal parts of the moth brain. The arrow indicates the position of the calyces. **E:** A dissected brain.

Dye applied to the antennal lobe: In order to stain the AL, scales on the moth's head were removed, and a rectangular hole was cut in the cuticle, dorsally, with a razorblade knife. To expose the brain, the muscle tissue and trachea were removed, and a small cut was made in one of the ALs. Crystals of the dye were applied with a micro-needle, or the tip of a glass pipette. Three different types of dye were tested; Alexa 488 (Hydrazide, 10000MW, excitation/emission maxima ~495/519 nm, Life Technologies), Micro-Emerald (Dextran, Fluorescein and Biotin, 3000 MW, excitation/emission maxima ~494/518 nm, Life Technologies), and Micro-Ruby (Dextran tetramethylrhodamine, 3000MW, excitation/emission maxima ~555/580 nm, Life Technologies). Micro-Emerald was preferred over Alexa 488 if both dyes were accessible, due to better staining of the neurons. Micro-Emerald is hence used in all preparations showed in the results. After dye application, the stained brain was stored two hours at room temperature, or overnight at 4°C soaked in Ringer-solution (50ml: NaCl: 0.4383, KCC: 0.0112, TES: 0.1145, Sucrose: 0.4275, CaCl2: 0.0167).

Dye applied to the calyces: Different methods were tried to get access to the calyces. The one that appeared to be the most successful was to remove the head from the thorax, mounting it into melted wax exposing the posterior caudal parts of the brain. Cuticula and trachea were then removed in order to uncover the calyces. Dye was inserted using a micro-needle, or the tip of a glass pipette into the calyces. After the dye was injected into the calyces the brain was kept for one or two hours at room temperature.

Double-labelling experiments: During double-labeling experiments, dye application to the calyces was performed directly after the dye was injected into the AL, using the methods described above. In these experiments, the dye in the AL was Micro-Emerald, and the dye injected into the calyces was Micro-Ruby. In most of the preparations, dye was inserted in the

calyces of the ipsilateral hemisphere. In some of the brains, the dye was inserted into the contralateral hemisphere.

3.3 Fixation and dehydration.

After the brain was dissected out of the head capsule it was fixated overnight in 4 % paraformaldehyde (Roti Histofix, Carl Roth GmbH + Co. KG, Germany) in a refrigerator at 4 °C. After fixation, the brain was dehydrated by a series of alcohol (10 min each: 50 %, 70 %, 90 %, 96 %, 100 %, 100 %). Finally, the brain was mounted on a metal plate in methylsalicylat (methyl 2-hydroxybenzoate), covered on each side with a glass-plate. The brain was then kept dark in a refrigerator at 4 °C.

3.4 Confocal microscopy and analyses of the data.

The brains were scanned using a confocal laser microscope (LSM 510 META Zeiss, Jena, Germany). Both a 10x air objective and a 20x air objective were used. The resolution in X-Y direction was normally set to 1024x1024 pixels. A Helium-Neon laser was used to visualize Micro-Ruby and the 488-nm line of an Argon laser was used to visualize Alexa 488 and Micro-Emerald. The brains were scanned frontally with 2-4 µm intervals.

More than 50 preparations were scanned. The scanned brains were viewed on the computer program LSM 510 Image browser (Carl Zeiss Microscopy, Jena, Germany, Version 4.2). Photoshop CC (Adobe systems, San Jose, CA) was used for editing and final adjustment of the confocal images.

3.5 Ethics

When working with insects there are no laws concerning ethics. Therefore, no restrains regarding the use of *H. virescens* in research exist. This means that no application for ethical approval was needed. Nevertheless, the insects were treated carefully in the lab. They were regularly fed, and kept in a clean environment.

4 Results

In this study, 88 moths were attempted stained in total. Among these, 43 preparations were successfully labelled, including ten preparations that were stained from the AL only, seven from the calyces only, and 26 being stained from both regions simultaneously.

Some of the confocal images have been laterally inversed to make it easier for the reader to understand the data, in particular for comparing the stained axons in the different brain preparations. In the figures, a small schematic drawing of the moth brain indicating which ALTs that were stained is added. An overview of the moth brain is shown in figure 5, including the m-ALT as appearing when dye is selectively applied to the calyces (red labeling in the left hemisphere) and into the AL (green labeling in the right hemisphere).

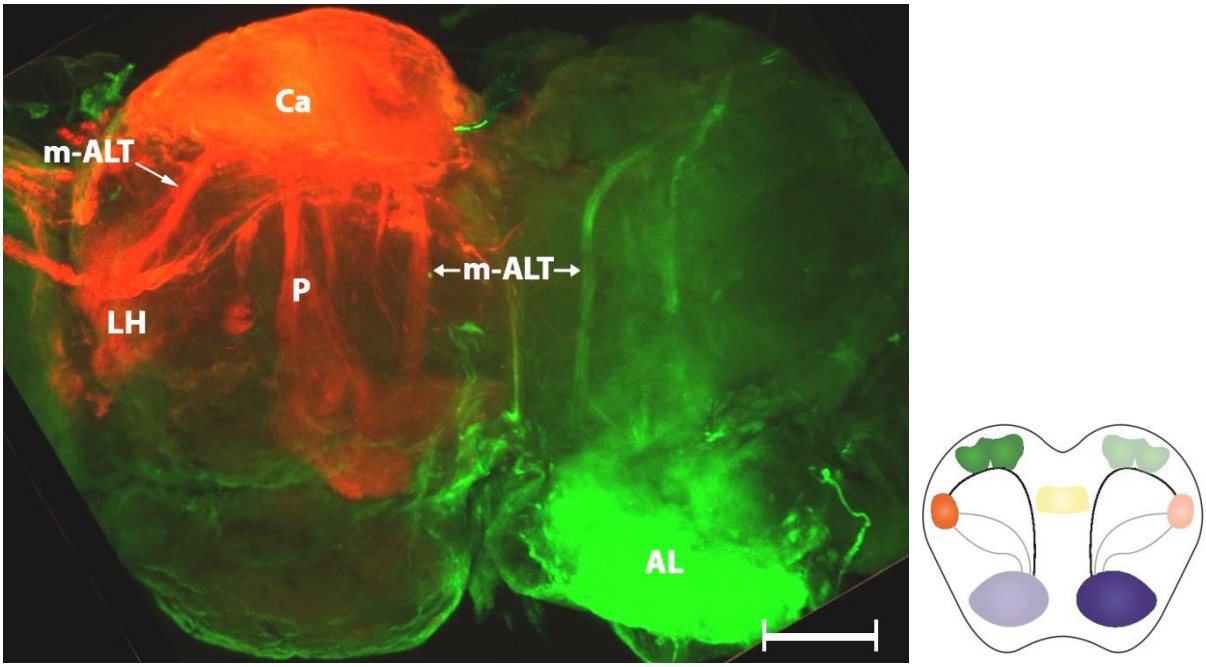


Figure 5: Confocal image showing the brain of preparation 69 stained from the antennal lobe (AL) (green) and the calyces (red), respectively. Due to auto-fluorescence from the neural tissue, parts of the brain where no dye was applied also appear green. As the dorsally oriented image shows, the medial antennal lobe tract (m-ALT) is stained from both regions. Ca calyces, LH lateral horn, P peduncle. 10x objective. Scale bar 100 µm.

4.1 Projection pattern in preparations stained from the antennal lobe exclusively

In preparations stained with fluorescent dye from the AL only, the three main ALTs identified as the m-ALT, the ml-ALT, and the l-ALT were visualized (Table 1; Fig. 6 and 7). The m-ALT, which was visible in all the successfully stained brains, leaves the medial part of the AL and runs posteriorly towards the calyces. After bypassing the central body, the m-ALT turns laterally and runs along the anterior edge of the calyces. Here, it innervates the calyces before turning anterior-laterally, terminating in the LH (this can be seen, though weakly, in figure 7).

The considerably thinner ml-ALT follows the m-ALT for a short distance before turning laterally at the anterior edge of the central body, terminating in the LH without making any connection with the calyces (Fig. 6 and 7). The current tract seems to split into several small bundles on its route; hence it projects into different parts of the LH.

The third tract, the l-ALT leaves the AL more ventro-laterally than the two other ALTs, projecting directly to the LH (Fig. 6 and 7). As shown in figure 7, this tract is also much thicker than the ml-ALT. In addition, it is more loosely formed than both the m-ALT and ml-ALT (Fig. 6 and 7).

In addition to the three main tracts, one other tract, identified as the second medio-lateral ALT (Lillevoll, 2013, master thesis) was stained in some of the preparations (Fig. 8). This tract, which is even thinner than the ml-ALT, leaves the AL together with the m-ALT before it bends off running laterally, however, in a more posterior position than the ml-ALT. The second medio-lateral ALT targets the LH and a region adjacent to the calyces.

Table 1: Stained neurons appearing in the different antennal lobe tracts when inserting dye into the antennal lobe only.

Preparation	m-ALT	ml-ALT	l-ALT
7	**X**	**X**	**X**
12	**X**	**X**	**X**
20	**X**	**X**	**X**
23	**X**	**X**	**X**
42	**X**	**X**	**X**
56	**X**	**X**	**X**
60	**X**	**X**	**X**
62	**X**	**X**	**X**
74	**X**	**X**	**X**
75	**X**	**X**	**X**

*Note: **X** indicates that the tract was heavily stained. m-ALT, medial antennal lobe tract, ml-ALT medio-lateral antennal lobe tract, l-ALT lateral antennal lobe tract.*

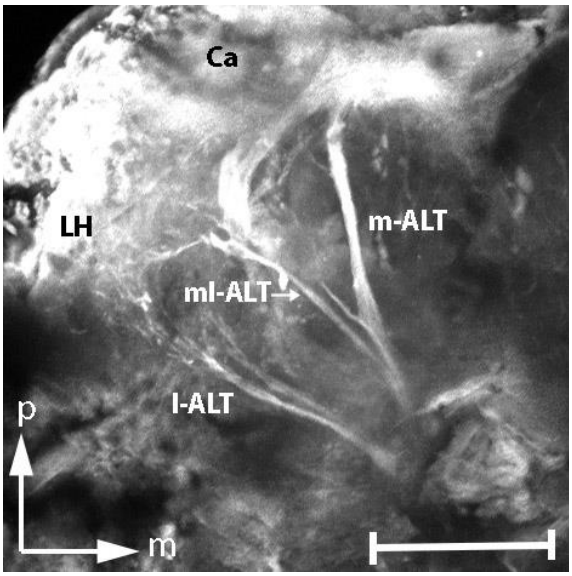
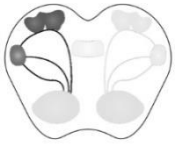


Figure 6: Confocal image of preparation 60 showing stained projection neurons in all the three main tracts passing from the antennal lobe (AL) to the lateral horn (LH). These are the medial antennal lobe tract (m-ALT), the medio-lateral antennal lobe tract (ml-ALT), and the lateral antennal lobe tract (l-ALT). As shown in the image, the m-ALT is thicker than the ml-ALT. Also, the l-ALT is more loosely formed. The current staining pattern was obtained by applying dye into the AL. Ca calyces, m medial p posterior. 10x objective. Dorsal view. Scale bar 100 µm.



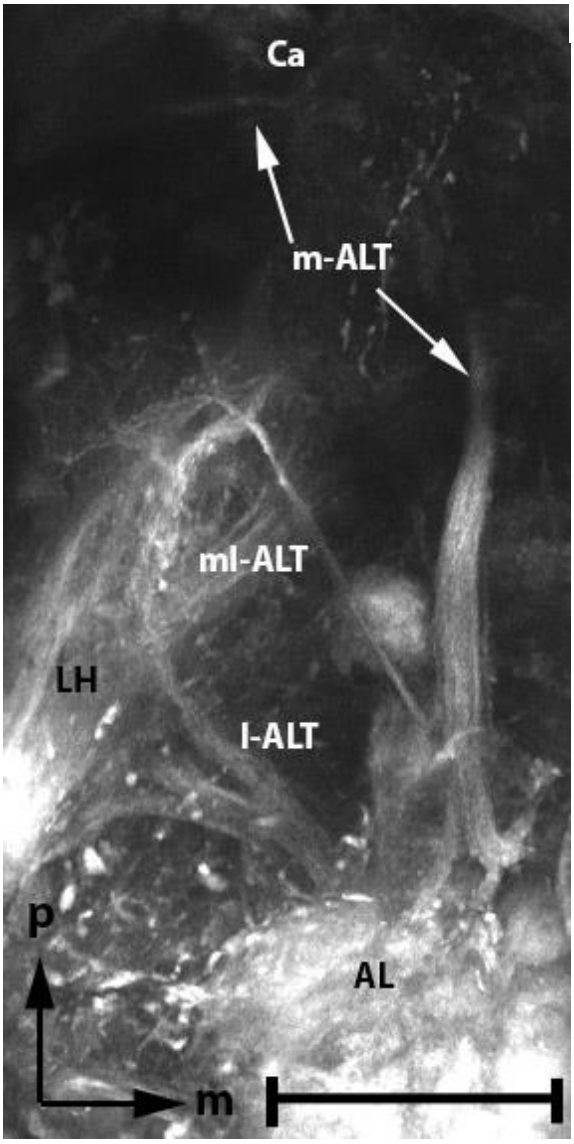
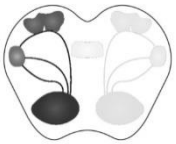


Figure 7: Confocal image of preparation 74 showing the three main antennal lobe tracts including the medial antennal lobe tract (m-ALT), the medio-lateral antennal lobe tract (ml-ALT), and the lateral antennal lobe tract (l-ALT). The l-ALT forms a loose fibre bundle which terminates in different parts of the lateral horn (LH). The current staining pattern was obtained by applying dye into the antennal lobe (AL). Ca calyces, m medial, p posterior. 10x objective. Scale bar 100 µm.



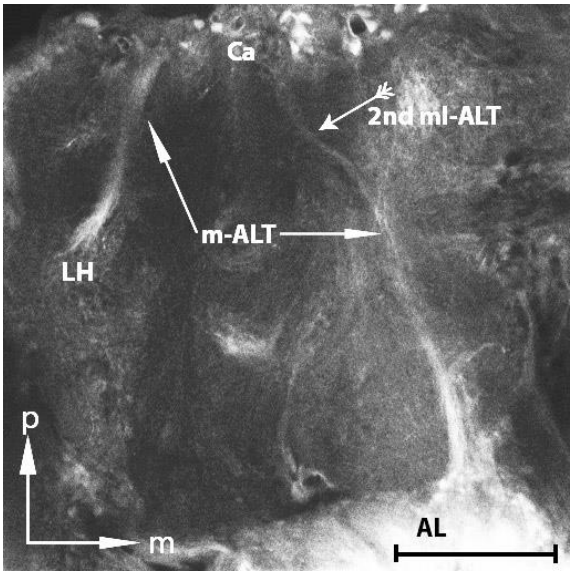
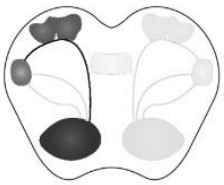


Figure 8: Confocal image of preparation 88 showing, in addition to the prominent medial antennal lobe tract (m-ALT), the so called second medio-lateral antennal lobe tract (2nd ml-ALT, double arrow). The current staining pattern was obtained by applying dye into the antennal lobe (AL). Ca Calyces, LH lateral horn, m medial, p posterior. 10x objective. Scale bare 100 µm.



4.2 Projection pattern in preparations stained from the calyces exclusively

All preparations successfully stained from the calyces showed the prominent m-ALT (Table 2; Fig. 9). Similarly to the brains stained from the AL the current preparations included staining of the m-ALT projecting in a posterior direction from the AL, following a route close to the central body. As shown by the current staining technique, the m-ALT has arborizations in several AL glomeruli (Fig. 10A), and terminates in different parts of the LH (Fig. 10B). In some of the preparations stained from the calyces, the two AL roots of the current tract, i.e. the dorsal and the ventral root, were visible. As shown in figure 9, the ventral root is connected to glomeruli located more laterally in the AL. A few stained axons projecting outside these two roots were also observed in the AL (data not shown). As expected, the ml-ALT was not stained when inserting dye into the calyces (Fig. 9).

The third main tract, the l-ALT, was not visualized as a prominent fiber bundle such as when applying dye into the AL. Instead, only three of the current preparations (seven in total) contained a few stained fibers passing along the l-ALT (Table 2; Fig. 11 and A1 in the Appendix). The second medio-lateral ALT was visualized in a few preparations being stained from the calyces (Fig. 11).

Table 2: Stained neurons appearing in the different antennal lobe tracts when inserting dye into the calyces only.

Preparation	m-ALT	ml-ALT	l-ALT
21	**X**		
64	**X**		x (2)
66	**X**		
68	**X**		x (1)
73	**X**		x (2)
82	**X**		
87	**X**		

X *indicates that the tract is heavily stained, and x that it is weakly stained. Number of visible neurons is stated in parenthesis. m-ALT medial antennal lobe tract, ml-ALT medio-lateral antennal lobe tract, l-ALT lateral antennal lobe tract.*

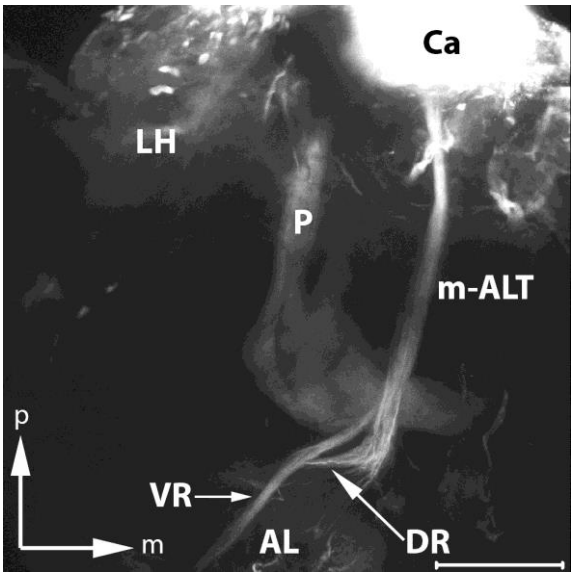
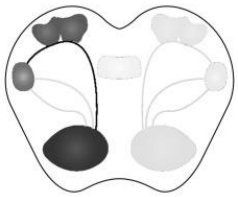


Figure 9: Confocal image of brain preparation 64 demonstrating the typical staining pattern obtained when applying dye in the calyces. Only one main tract is visualized in the current image, the medial antennal lobe tract (m-ALT). In addition, the image shows the two roots of the m-ALT, the dorsal root (DR) and the ventral root (VR), projecting within the antennal lobe (AL). Ca calyces, LH lateral horn, m medial, p posterior, P peduncle. 20x objective. Scale bar 100 µm.



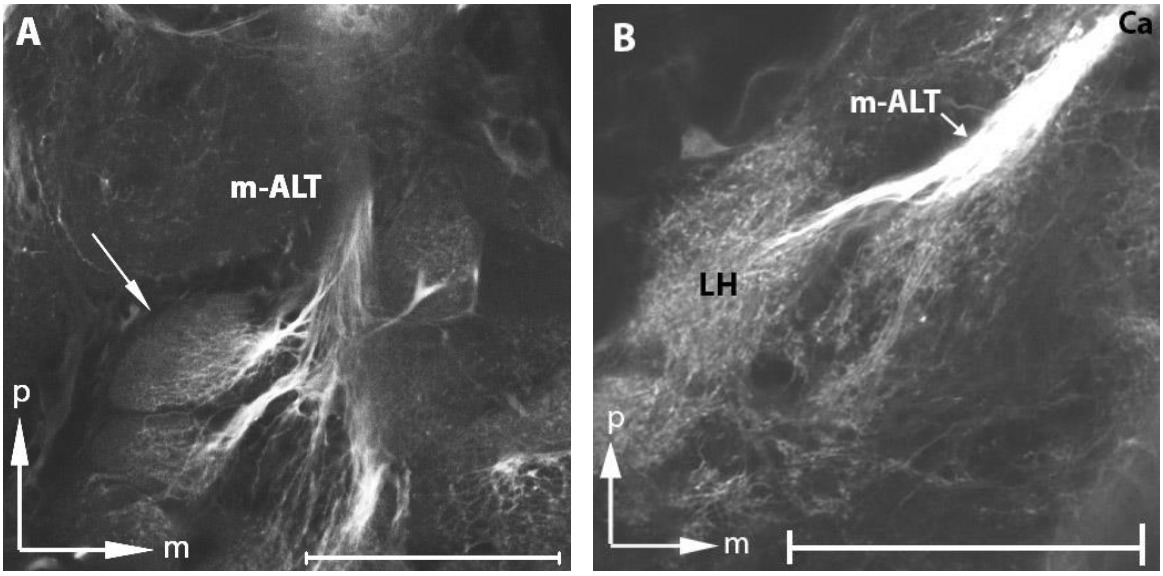


Figure 10: Confocal images of two brain preparations labelled from the calyces (Ca), one showing dendritic arborisations of neurons in the medial antennal lobe tract (m-ALT) within distinct glomeruli of the antennal lobe (AL) and the other image showing terminal regions of the same neuron category projecting into the lateral horn (LH). **A:** Image of preparation 68, showing glomerular innervations of neurons confined to the m-ALT. The arrow points at one of the innervated glomeruli in the AL. **B:** Image of preparation 86 showing the terminal area of the m-ALT neurons in the LH. This image shows that neurons connecting the calyces and the LH project into different parts of the LH. m medial, p posterior. 20x objective. Dorsal view. Scale bar 100 µm.

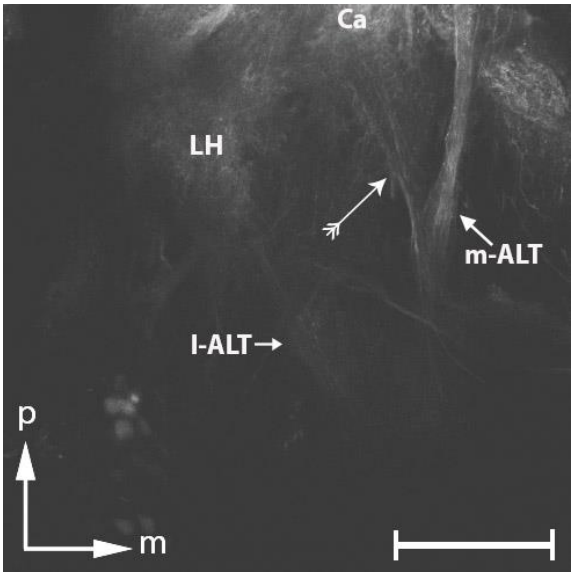
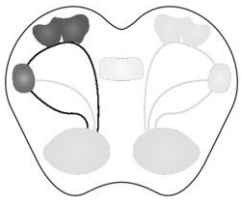


Figure 11: Confocal image of preparation 73 being stained from the calyces (Ca). As demonstrated, numerous fibres of the medial antennal lobe tract (m-ALT) are clearly stained, whereas only a few axons passing along the route of the lateral antennal lobe tract (l-ALT) are labelled. In addition, the second medio-lateral antennal lobe tract can be seen (double-arrow). LH lateral horn, m medial, p posterior. 20x objective. Scale bar 100 µm.



4.3 Projection pattern in double-labelled preparations

In the double-labelled preparations, obtained by applying two different dyes into the same brain, i.e. one dye inserted into the AL and the other into the calyces, a combination of the staining patterns described above was seen. Hence, the m-ALT was stained from both the AL and the calyces (Fig. 12), whereas the ml-ALT was labelled from the AL only (Fig. 13). The m-ALT appeared as a prominent fiber bundle passing in an anterior-posterior direction in the moth brain. Also, the m-ALT appeared as the thickest tract in the double labelled preparations.

The l-ALT was stained mainly via the dye inserted into the AL. Only ten of the 26 successfully double-labelled preparations possessed l-ALT PNs that contained dye applied to the calyces (Table 3). From the confocal images of this study it is therefore clear that most of the neurons confined to the l-ALT terminate in the LH. The few visible neurons in the l-ALT connecting the AL to the calyces were counted in the relevant preparations, and the numbers included one to six neurons in each preparation (Table 3). Three of these preparations are shown in figure 12, 13 and 14 (for another double-labelled preparation, see figure A2 in the Appendix). From figure 13, it is clear that the l-ALT neurons target the LH along slightly different routes, some of the axons being positioned more laterally than the others. No differences between the sexes were seen.

The two roots of the m-ALT, the dorsal and the ventral, were also observed in some of the double-labelled preparations. As shown in the confocal image in figure 15, it is obvious that these two roots, here being stained by the dye inserted into the calyces, originate[1] in different parts of the AL.

[1]Provided that the dendritic region forms the starting point of the neuron.

Table 3: Staining pattern of the antennal lobe tracts as appearing when two different dyes were applied to the same brain, one in the antennal lobe (AL) (green), and the other in the calyces (Ca) (red).

Preparation	**m-ALT**		**ml-ALT**		**l-ALT**	
(Stained from)	*(AL)*	*(Ca)*	*(AL)*	*(Ca)*	*(AL)*	*(Ca)*
15	X	X	X		X	x (2)
24	X	X	X		X	x (3)
29	X	X	X		X	x (3)
40	X	X	X		X	
46	X	X	X		X	
49	X	X	X		X	
55	X	X	X		X	
56	X	X	X		X	
59	X	X	X		X	
61	X	X	X		X	x (2)
63	X	X	X		X	
67	X	X	X		X	
68	X	X	X		X	
69	X	X	X		X	
73	X	X	X		X	
74	X	X	X		X	x (3)
75	X	X	X		X	
79	X	X	X		X	x (3)
80	X	X	X		X	
81	X	X	X		X	
83	X	X	X		X	
86	X	X	X		X	x (2)
88	X	X	X		X	x (6)
89	X	X	X		X	x (1)
90	X	X	X		X	
91	X	X	X		X	x (2)

X *indicates that the tract is heavily stained; x indicates that the tract is weakly stained. Number of visible neurons is stated in parenthesis when known. m-ALT medial antennal lobe tract, ml-ALT medio-lateral antennal lobe tract, l-ALT lateral antennal lobe tract.*

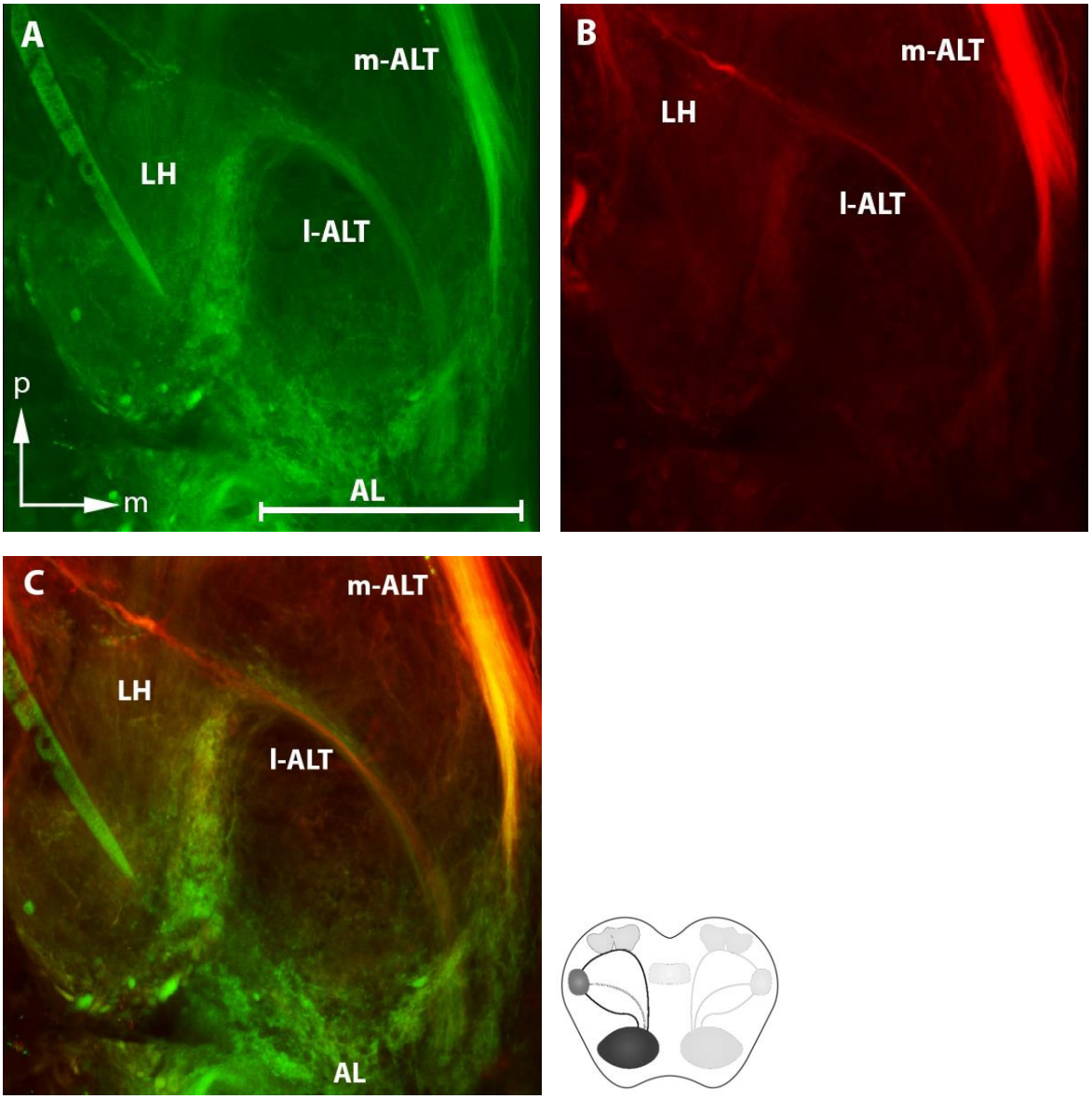


Figure 12: Confocal images of the double-labelled preparation 89 showing projection pattern of the antennal lobe (AL) output neurons. **A:** Dye applied to the AL (Micro-Emerald) shows the medial antennal lobe tract (m-ALT) and the lateral antennal lobe tract (l-ALT). **B:** The same two ALTs labelled from the calyces (Micro-Ruby). **C:** Overlay of the confocal images in A and B. As demonstrated, the number of l-ALT neurons being labelled from the calyces (red) is considerably lower than that being labelled from the AL (green). The ml-ALT was not included in the scanned region. LH lateral horn, m medial, p posterior. 20x objective. Scale bar 100 µm.

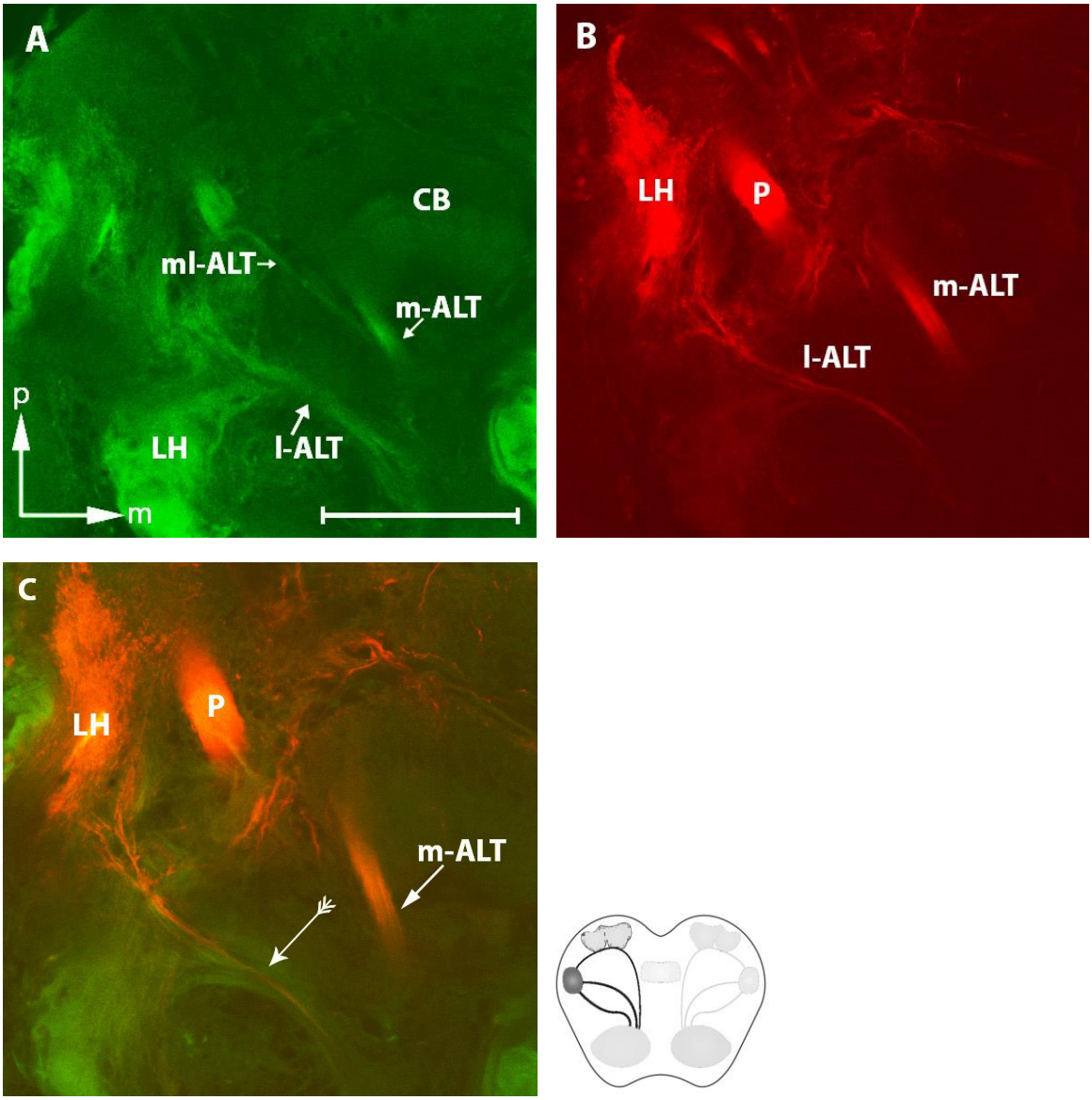


Figure 13: Confocal images of the double-labelled preparation 79 showing the antennal lobe tracts (ALTs). **A:** The three main ALTs, the medial ALT (m-ALT), the medio-lateral ALT (ml-ALT), and the lateral ALT (l-ALT), as appearing when stained by Micro-Emerald from the antennal lobe (AL). **B:** The ALTs as appearing when stained by Micro-Ruby from the calyces. As demonstrated, only the m-ALT and the l-ALT are visible. **C:** Overlay of the confocal images in A and B. The double-arrow is pointing at the l-ALT. As demonstrated, the number of l-ALT neurons being labelled from the calyces (red) is considerably lower than that being labelled from the AL (green). CB central body, LH lateral horn, m medial, P, peduncle, p posterior. 20x objective. Scale bar 100 µm.

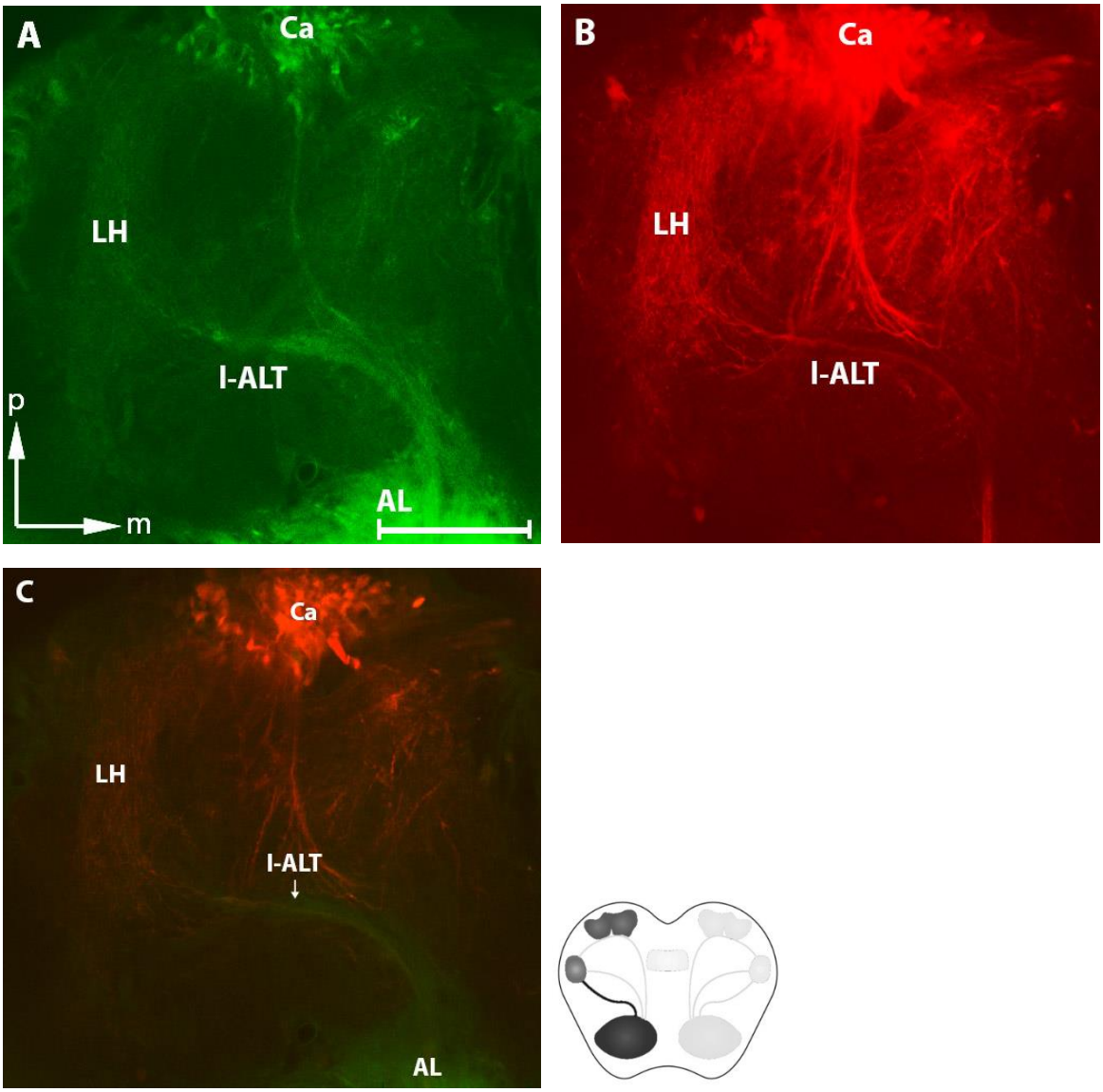


Figure 14: Confocal images of the double-labelled preparation 88 showing projection pattern of antennal lobe (AL) output neurons. **A:** Dye applied to the antennal lobe (Micro-Emerald) shows the lateral antennal lobe tract (l-ALT). **B:** The l-ALT stained by dye (Micro-Ruby) applied to the calyces (Ca). **C:** Overlay of the confocal images in A and B. As demonstrated, the number of l-ALT neurons being labelled from the calyces (red) is considerably lower than being labelled from the AL (green). The m-ALT is not visible due to the layer chosen. LH lateral horn, m medial, p posterior. 20x objective. Scale bar 100 µm.

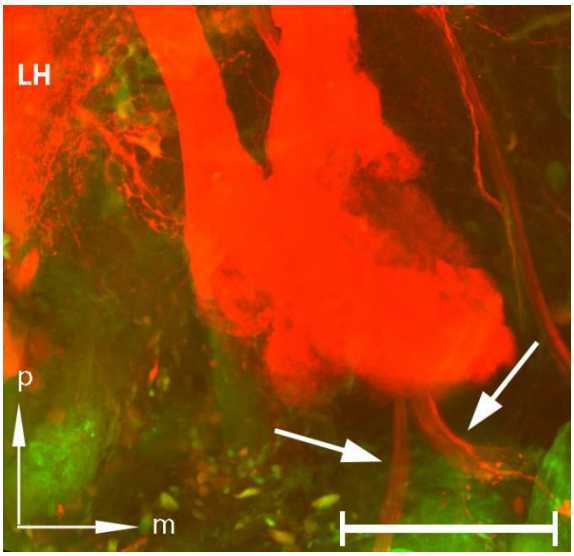


Figure 15: Confocal image of the double-labelled preparation 79 showing the two antennal lobe roots of the medial antennal lobe tract (arrows). The red colour represents neurons stained with Micro-Ruby from the calyces. The antennal lobe was stained with Micro-Emerald (green).The lateral horn (LH) can be seen in this image, as well as parts of the mushroom bodies (not named). The image is ventrally oriented. m medial, p posterior. 20x objective. Scale bar 100 µm.

4.4 Additional observations

In addition to the AL PNs, some other neuron categories connected to the calyces were visualized in the preparations stained from the current region. One major category includes the numerous Kenyon Cells forming the mushroom body lobes. The mushroom body lobes is located posteriorly of the AL, mainly dorsally of the m-ALT. The particularly strong straining of this structure, including the peduncle and the Y-lobe which connect the calyces to the mushroom body lobes, can be seen in figure 16 and figure A3 in the Appendix. Cell bodies of the Kenyon cells, situated at the surface of the calycal cups, can be seen in figure A4 in the Appendix).

Other clusters of cell bodies being connected with the calyces were also visualized. One of these was a strongly labelled cell cluster located in the LH, adjacent to the optic lobe (Fig. 16). Furthermore, the data showed a previously described bundle of neurons connecting the calyces to the ipsilateral optic lobe (Sjöholm et al., 2005) (Fig. 17A, Fig. A4 in the Appendix).

Finally, a bundle of neurons projecting from the calyces to the central body was stained (Fig. 17B). These neurons follow a path from the calyces close to the m-ALT before turning medial-anteriorly and entering the central body. The neurons connected to the calyces form a characteristic spherical region in the central body (Fig. A5 in the Appendix).

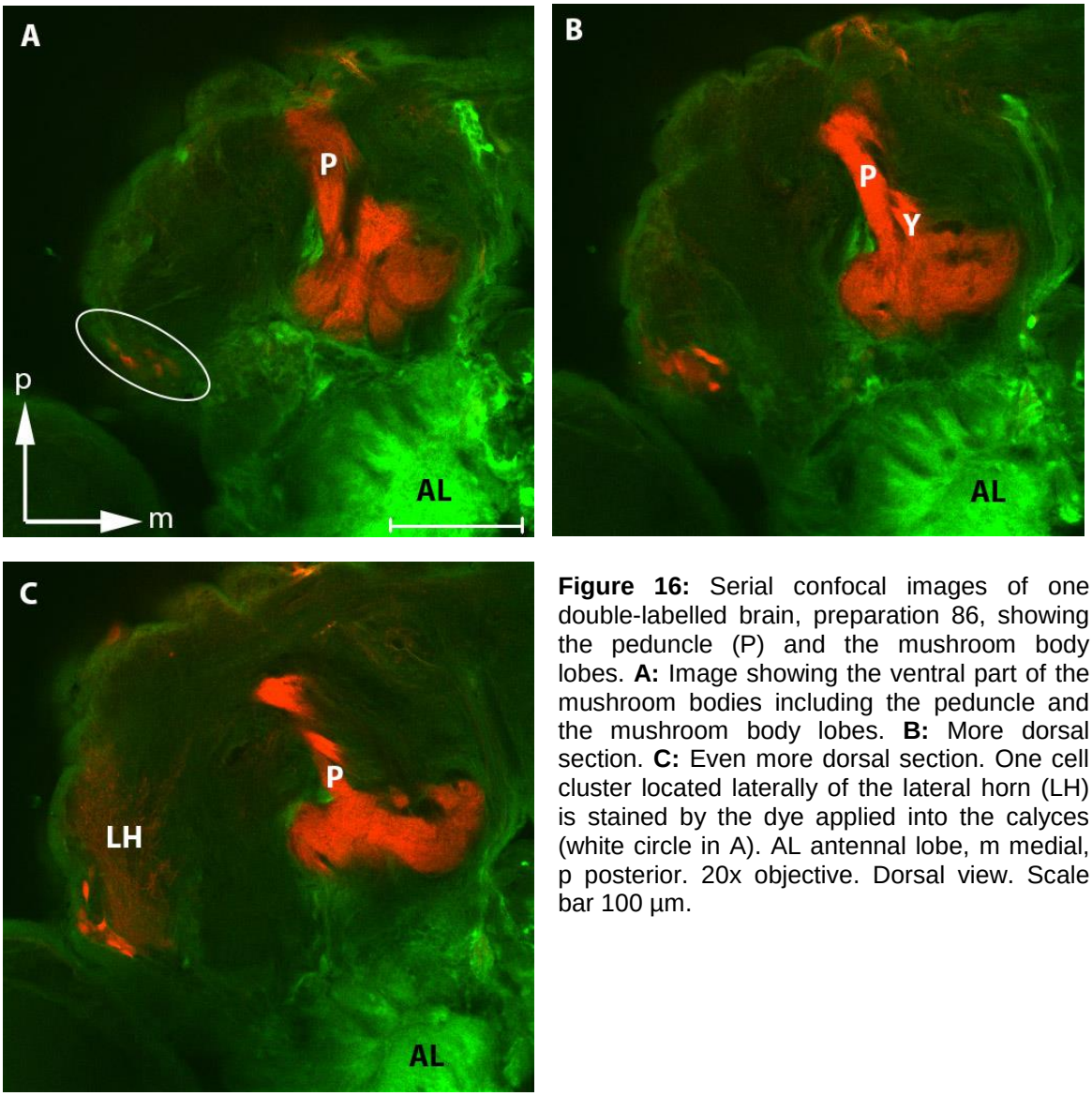


Figure 16: Serial confocal images of one double-labelled brain, preparation 86, showing the peduncle (P) and the mushroom body lobes. **A:** Image showing the ventral part of the mushroom bodies including the peduncle and the mushroom body lobes. **B:** More dorsal section. **C:** Even more dorsal section. One cell cluster located laterally of the lateral horn (LH) is stained by the dye applied into the calyces (white circle in A). AL antennal lobe, m medial, p posterior. 20x objective. Dorsal view. Scale bar 100 µm.

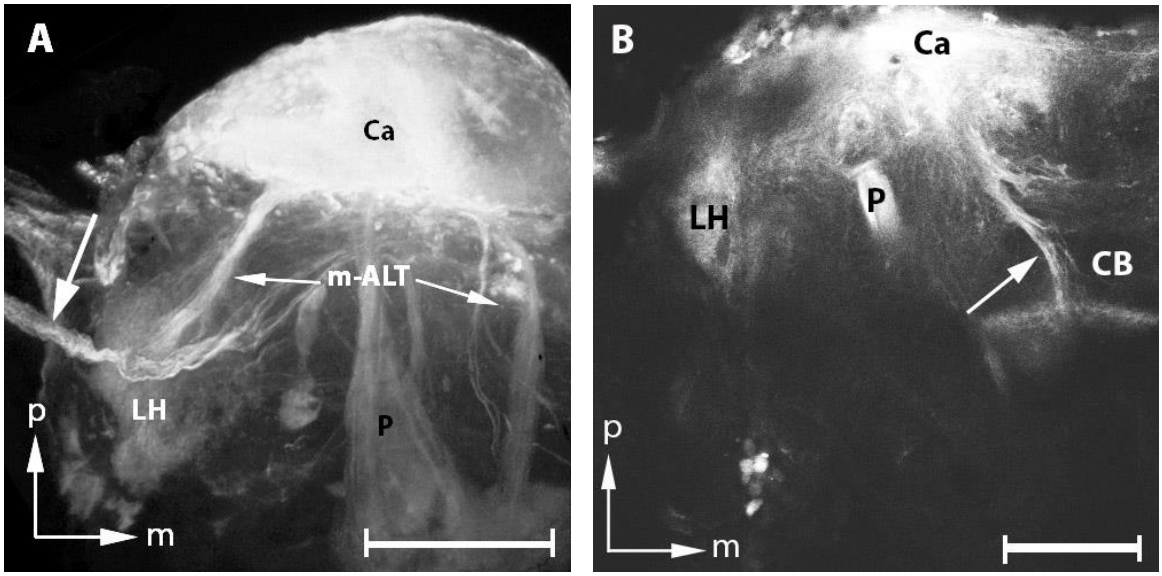


Figure 17: Confocal images showing non-odorant neurons projecting from/to the calyces. **A:** Confocal image of preparation 69 showing a bundle of neurons connecting the optic lobe with the calyces (Ca), indicated by one large arrow. The calyces consist of two cups both visible in the image. Confocal image, 10x objective. **B:** Image of preparation 74 showing neurons connecting the calyces with the central body (CB) (arrow). The bundles of neurons send branches towards different parts of the CB. LH Lateral Horn, m medial, P Peduncle, p posterior. 20x objective. Scale bars 100 µm.

5 Discussion

The specific aim of the current project was to explore to which extent the l-ALT constitutes a pathway connecting the primary olfactory center of the moth brain, the AL, with the calyces of the mushroom body. The data obtained in this study provide detailed knowledge about morphological characteristics of the l-ALT in the moth, *H. virescens*. Particularly, the findings demonstrate that relatively few PNs confined to the l-ALT send an axon into the calyces. Hence, most of the l-ALT PNs terminate in the LH. Also, the data obtained, describe the two other ALTs, the m-ALT and the ml-ALT, as well as different brain areas that are connected to the calyces, including the optic lobes and the central body.

5.1 Projection pattern of the lateral antennal lobe tract

In this study, all the three main ALTs, the m-ALT, the ml-ALT and the l-ALT, were stained when dye was applied into the AL (anterograde staining). This is in consistency with previous findings in different moth species (for review see Galizia & Rössler, 2009). The finding of the m-ALT as the most prominent tract, the ml-ALT as the thinnest, and the l-ALT as a thick but relatively loosely formed fiber bundle terminating in different parts of the lateral protocerebrum, is also in agreement with previous reports (Homberg et al., 1988; Rø et al., 2007). Retrograde labeling of AL PNs, on the other hand, implying application of dye into the calyces, has not been performed in any moth species previously – except for one master project labeling the m-ALT of *H. virescens* by placing dye into the calyces (Berg, 2013). Interestingly, this former investigation also resulted in only one prominently labeled tract, i.e. the m-ALT. However, as the previous master project did not include double-labeling experiments, the detailed projection pattern of the l-ALT was not completely determined. The double-labelling experiments hence made it possible to identify which of the lateral-tract PNs projected into the calyces and which did not. Therefore, this staining technique was particularly appropriate for the current investigation since the complete axon bundle forming the l-ALT was stained. The data from the investigation presented here demonstrate that only a minor proportion of the numerous lateral-tract projections leaving the AL send an axon into the calyces. Actually, a maximal number of six stained l-ALT axons being connected to the calyces were counted in this study. The repeated finding of either none or a few axons within the l-ALT being stained by the dye applied to the calyces shows that most of the information carried between the AL and the calyces follows the m-ALT in the moth brain.

Previous studies have found morphologically different neurons projecting in the l-ALT in several different moth species. In *H. virescens,* for example, Rø et al. (2007), performing

intercellular recordings from the AL, found totally eight PNs confined to the l-ALT, three of which projected into the calyces. The last mentioned category were uni-glomerular neurons sending two branches towards the calyces, one targeting the medial calycal cup and one the lateral calycal cup. The remaining lateral-tract neurons were reported to be multi-glomerular (Rø et al. 2007). In *M. sexta,* one class of multiglomerular neurons projecting via the l-ALT into the calyces has been reported (Homberg et al. 1988). These neurons innervated the same areas in the calyces as neurons projecting in the m-ALT. In addition to this type of PN, three other categories of lateral-tract neurons were identified in *M. sexta,* none of which innervated the calyces (Homber et al. 1988). In the moth species *Spodoptera littoralis,* one class of lateral-tract neurons innervating almost the entire calycal structure has been found (Sjöholm et al., 2005). As the current study included mass staining experiments, however, the individual staining pattern of the neurons remain unexplored. Even though the majority of lateral-tract neurons previously identified in these moth species seemed not to project into the calyces, a relatively considerable amount did. The reason for the apparent discrepancy between the previous findings including a rather significant proportion of lateral-tract neurons projecting to the calyces, and the present findings encompassing a minimal percentage, may be that the relatively few PNs of the current type are larger than those terminating in the LH and therefore being more often obtained during intracellular recording experiments. An alternative explanation might be that the current neuron population innervates only distinct parts of the calyces and is thus more rarely being stained by the current technique – however, so far, no such lateral-tract PNs has been reported. Therefore this explanation seems unlikely. Generally, mass-staining experiments can obviously never ensure that all neurons in a specific tract have been stained.

The finding of lateral-tract PNs terminating mainly in the LH and not in the calyces differs essentially from the arrangement described in honey bees and ants. In these hymenopteran species, a non-overlapping dual pathway including uni-glomerular PNs passing along the m-ALT and the l-ALT, both terminating in the calyces, is reported (Kirschner et al. 2006). In the honeybee, *Apis mellifera*, the PNs projecting in the m-ALT and the l-ALT terminate in different parts of the calyces and the LH, and the terminals of the PNs remain largely segregated (Gronenberg & López-Riquelme, 2004; Kirschner et al., 2006). That these two tracts project to largely non-overlapping areas in the calyces and the LH may mean that the PNs are connected to different third order neurons. Hence, the PNs in the m-ALT and the

l-ALT have been suggested to play different functional roles in this species (Kirschner et al. 2006).

That the majority of neurons in the l-ALT terminates in the LH, as found here, remains consistent with former findings in the silk moth, *Bombyx mori*; thus, in this species most l-ALT neurons are in fact reported to terminate in the LH (Examples of such articles: Iwano & Kanzaki, 2005; Seki, Aonuma & Kanzaki, 2005). As previously mentioned, since the neurons in l-ALT are thinner than those in the two other tracts, i.e. the medial and the medio-lateral, they are not that easy to stain individually (Homberg et al. 1988). This might explain why many articles showing single PNs stained from the AL reports of no stained neurons in the l-ALT at all (For instance: Kanzaki, Arbas & Hildebrand, 1991; Kanzaki, Arbas, Strausfeld & Hildebrand, 1989). Mass-staining experiments, as performed here, may visualize thin neurons, however, and is thus a suitable approach to general morphological characterization of projection neurons passing in the l-ALT.

The general assumption that the majority of PNs in the l-ALT of moths projects into the calyces, should be re-considered.

5.2. Projection patterns of the medial and the medio-lateral antennal lobe tract

The finding of the m-ALT as the most prominent tract both when applying dye into the AL and the calyces is in correspondence with previous studies performed on the same species (Rø et al., 2007; Berg, master thesis, 2013). Particularly, the m-ALT being similarly labeled by the two staining techniques used here, confirms that the absolute majority of projection neurons passing in this tract innervates the calyces. The fact that the m-ALT was equally stained by both dyes was also used to consider the quality of the staining, and to ensure that the dye had been inserted into the relevant parts of the brain.

The data obtained in this study shows that neurons confined to the m-ALT leave the AL through two bundles known as the dorsal and the ventral root. Previous studies have shown that the axons in the lateral root are connected to somata located in the lateral cell cluster whereas the axons running in the dorsal root are connected to somata positioned in the medial cell cluster (Homberg et al., 1988; Berg, master thesis, 2013). Also, the appearance of medial-tract axons forming characteristic glomerular structures within the AL, as shown in figure 10A, corresponds with previous investigations reporting that the neurons projecting in the m-ALT are mainly uniglomerular (Homberg et al., 1989). A relatively large amount of uniglomerular medial-tract neurons have been morphologically and physiologically

characterized in different moth species, *H. virescens* included (Christensen et al. 1995; Vickers et al. 1998; Berg et al. 1998; Zhao et al., submitted article).

The appearance of the considerably thinner ml-ALT when staining from the AL and its absence when staining from the calyces, as demonstrated here indicates the total lack of connection between the current tract and the calyces. Again, the data confirms previous findings obtained in several moth species, *H. virescens* included (Homberg et al., 1988; Rø et al, 2007). This tract consists of mainly multiglomerular PNs (Homberg et al., 1988; Rø et al., 2007), many of them being GABAergic (Berg et al., 2009).

Some of the confocal images of the moth brain showed the second medio-lateral ALT, which was always thinner than the three main ALTs, hence seemed to contain significantly fewer neurons. Lillevoll (master thesis, 2013) found that this tract targets several regions, including an area nearby the calyces, plus the LH. In this study, only the bundle projecting towards the calyces was found when labelling the neurons from the AL, and from the confocal images it seems as this bundle innervates the calyces. Also, the appearance of the second ml-ALT in some of the preparations being stained from the calyces, as found here, indicates its innervation of the current neuropil. The axons forming this tract are suggested to originate only from a few glomeruli (Lillevoll, master thesis, 2013) explaining why this tract is not always stained when applying dye in the AL.

5.3 Parallel antennal lobe tracts

It is not yet understood why the PNs follow different routes towards the higher brain centres in the insect brain. Based on the previous and present results dealing with the ALTs of the heliothine moth, we can conclude that the calyces get input primarily from one tract, i.e. the m-ALT. The other prominent integration centre, the LH, receives input from all three main ALTs. Whether the axon terminals from the various tracts overlap in the LH, has not yet been systematically investigated. However, it is commonly accepted that there is at least a certain extent of overlaying branches from the three tracts in the current brain region. As mentioned, the l-ALT PNs have generally thinner axons than the PNs confined to the other tracts. Since information travels slower in thinner neurons, it is likely that information following the l-ALT is conveyed at a lower speed and thus arrives at its destination later than signals being carried in the other tracts, a theory previously mentioned by Homberg et al. (1988) and Galizia & Rössler (2010), among others. This may be one of the explanations of why different tract terminates in the LH.

Interestingly, it has been shown that the two main categories of odor cues, i.e. pheromone and plant odor information, is conveyed along all three main ALTs in moths (Homberg et al. 1988; Lillevoll, master thesis, 2013; Zhao et al., submitted article). This indicates that the various tracts seem to be associated not with distinct function or behavior, but rather with particular properties characterizing the stimulus. In a recent master thesis, Dahl (2013) found that neurons conveying information about CO_2 follow the l-ALT and ml-ALT, and not the m-ALT, showing that neurons containing qualitative distinct odor information may actually travel in particular tracts.

The main neurotransmitter of the AL PNs is not yet identified in moths. However, neurotransmitters utilized by distinct sub-populations of neurons have been identified. Both in *M. sexta* and *H. virescens*, for example, it is assumed that as many as 70 GABAergic neurons follow the ml-ALT (Kent, Hoskins & Hildebrand, 1987; Berg et al., 2009). Some of the neurons in the l-ALT of *H. virescens* are found to be GABAergic as well (Berg et al., 2007). Also, a few lateral-tract axons are reported to contain FMRFamide (Berg et al., 2007). Whereas no GABA or FMRFamide has been found in the m-ALT of the silk moth, *B. mori*, some serotonergic projection neurons have been reported in this species (Iwano & Kanzaki, 2005). Altogether, these examples enlighten that in addition to olfactory information passing through different ALTs, the information flow within each tract may also be differentiated by the presence of various neurotransmitters.

5.4 Additional observations

The calyces are connected to the rest of the mushroom bodies via numerous Kenyon Cells (Heisenberg, 1998), hence these cells were strongly labelled when dye was applied to the calyces in this study. As the confocal images show, it is possible to distinguish several of the lobes named by Rø et al. (2007). Due to time constrains, however, no attempt was made to identify the different lobes in the current project. The various kinds of Kenyon cells have their somata in different places around the calyces. The data showed for instance numerous Kenyon cell bodies that are situated at the surface of the calycal cups, which is in correspondence with previous findings (Fukushima & Kanzaki, 2009; Sjöholm et al. 2005). Furthermore, cell bodies located posterior-dorsally in the calyces, as shown in figure A4 in the appendix, may belong to the Kenyon cells as well. In the moth species *S. littoralis,* the cell bodies of the neurons in the Y-tract are situated on the lateral side of the calyces (Sjöholm et al., 2005). This is the same location as the cell bodies surrounded by a small circle in figure A6 in the Appendix.

Also, the experiments including dye application into the calyces resulted in prominent staining of a cell cluster located most laterally in the lateral protocerebrum, adjacent to the optic lobe (Fig 16). This cell cluster seems to be connected to the so-called protocerebral calycal tract (PCT), being identified as a GABAergic network in a previous master thesis (Jacobsen; 2012).

Furthermore, when labelling the calyces, a bundle of neurons connecting the calyces and the optic lobe were visible in several of the preparations. Sjöholm et al. (2005) have previously reported about a bundle following the same pathway. As the calyces are presumed to be one of the areas responsible for memory in the moth brain it is not surprising that these two brain areas are connected; similarly to olfactory information, visual information needs to be memorized. In *Drosophila,* only the LH receives direct visual information (Tanaka et al., 2004). This is different from ants and in bees where the calyces receive visual input from the optic lobes (Gronenberg & López-Riquelme, 2004). The areas in the calyces that receive optical information are reduced and less complex in the ant brain, compared to the brain of bees (Gronenberg & López-Riquelme, 2004). This is logical, as flying insects depend more on visual information for movement. As the moth is a flying insect as well, the calyces may hence be one of the places in the moth brain where olfactory and visual information are integrated.

The preparations stained from the calyces also included neural processes connecting to the CB. As the confocal images demonstrate, this is a relatively large structure located centrally between the two brain hemispheres (Strauss, 2002). Also, the images show a bundle of neurons connecting the calyces and the CB. This bundle projected close to the m-ALT before turning medially and projecting into the CB where the neurons spread out as a fan into large areas of the current structure. A characteristic spherical configuration was also visualized in one of the current preparations. It could be speculated that the current neurons project from the central body and into the calyces, not the other way around. Further it may be assumed that there is one similar structure connected to the other hemisphere. In *Drosophila*, the CB has namely been shown to play an essential role in locomotion control in interplay with the mushroom bodies (Strauss, 2002). Little is known about the CB in moths, but it may serve a similar function as in the fruit fly. As the central body is a structure being present in the brain of all insects (Strauss, 2002), is it presumed to be essential for the survival of these organisms.

5.5 Similarities and differences between the olfactory system in mammals and insects

Due to the many similarities between the olfactory system in mammals and insects, it is beneficial to do research on the relatively simple and easy accessible arrangement in insects to obtain a greater knowledge on basic principles characterizing the olfactory system.

Both in mammals and in insects, second order neurons having comparable morphologies, in particular mammalian Mitral cells and insects' uniglomerular projection neurons, carry olfactory information from the primary processing center to higher brain centers. Also the higher order centers, including the calyces in insects and the olfactory cortex in mammals, share striking similarities by having a folded structure (Kaupp, 2010). Not only that; these higher order areas are essentially involved in memory processes. In particular, the hippocampus, a subcortical area in the mammalian forebrain involved in memory formation, has often been compared to the calyces in insects (Sjöholm et al., 2005; Strausfeld, Hansen, Li, Gomez & Ito, 1998). Also, the calyces, plus the LH in the insect brain, have been compared with secondary centers in the mammalian brain, as the piriform cortex and the olfactory tubercle. Furthermore, the connection of these higher sites to regions like the thalamus and frontal cortex in the mammalian brain has been compared with the calyces' connection to the mushroom body lobes in the insect brain (Tanaka et al., 2004). As there are so many striking similarities between the olfactory pathways among different taxa, it may imply that there is more or less one optimal solution on how to detect and discriminate between different odors (Ache & Young, 2005).

5.6 Methodological considerations

The main challenge during the experimental work was to apply dye into the calyces. The method that gave successful results, was first to put dye into the AL, before removing the head from the thorax. The head was then positioned in melted wax so that its posterior part faced upwards allowing easy access to the calyces where the dye crystals were inserted.

Different dyes were used; mainly Micro-Ruby and Micro-Emerald, both being successful in staining detailed patterns of the brain structures, for instance the mushroom body lobes. Alexa 488, emitting light at the same wavelength as Micro-Emerald, was also tested but showed relatively poor staining quality and was therefore not used. Micro-Ruby stained the neurons in even greater detail than Micro-Emerald and as Micro-Ruby was used in the calyces it was easier to see single neurons projecting from the calyces than PNs stained from the AL being marked with Micro-Emerald. As parts of the same neurons are stained

both from the AL and the calyces, the dyes used here works both retrogradely and anterogradely. Hence, it is characteristics of the dye itself that is the reason for the different quality of the stained neurons, not internal properties of the neurons. When scanning the preparations stained with Micro-Emerald in the confocal microscope it became clear that even those parts of the brain that were unstained emit light, i.e. so-called auto-fluorescence. This relatively strong background staining made it harder to see the structures stained by Micro-Emarald.

One disadvantage of the mass-staining technique is the uncontrolled amount of dye entering the tissue. In many of the preparations shown here, the calyces, the peduncle, and the lobes were successfully stained, indicating that a sufficient amount of dye was applied into the relevant brain region. Still, mass staining experiments may sometimes mark irrelevant neurons. In this study, for example, the ml-ALT was stained when inserting dye in the calyces in one of the preparations. As the ml-ALT is not connected to the calyces (Rø et al., 2007) this shows that areas outside the calyces must have been stained. This preparation was excluded from the study.

It is still not completely clarified whether dye can be transmitted from one neuron to another. Such a mechanism would imply the possibility that the neurons stained here were actually several neurons linked together and not individual neurons. This seems unlikely, and has therefore not been considered.

6 Conclusion

Whereas the medial and the medio-lateral ALTs of moths have been mapped previously, the detailed projection pattern of the lateral ALT has not been fully explored until now. From this study, the following conclusions can be drawn:

- The major portion of axons passing in the l-ALT terminates in the LH. Only a few axons of the numerous fibers passing in the l-ALT project into the calyces.
- The projection pattern of the l-ALT shows that the direct neural connection between the antennal lobe and the calyces in the heliothine moth brain is formed essentially by antennal-lobe projection neurons confined to the m-ALT.
- The medial and the lateral ALTs of the moth are therefore not equivalent to the similarly named tracts of the honey-bee

Additional observations:

- The calyces of the heliothine moth are directly connected to the central body through a bundle of neurons.
- Also, the calyces are directly connected to the optic lobe via a neural bundle.

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Appendix

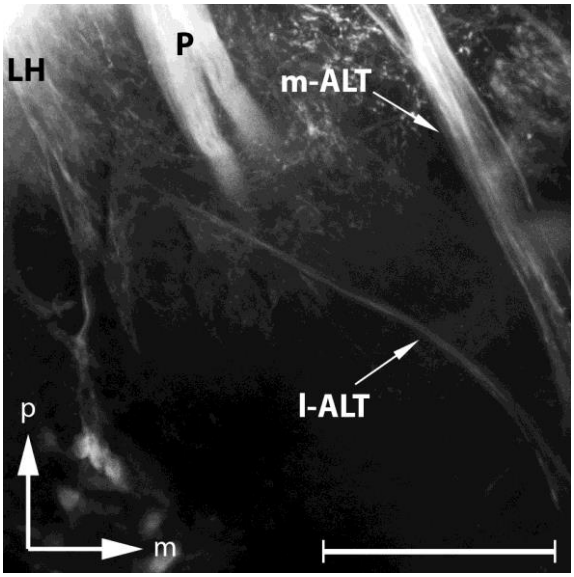
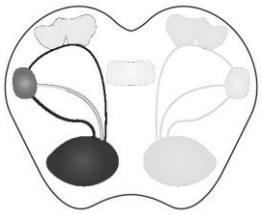


Figure A1: Image of preparation 61 being stained from the calyces. In addition to the heavily stained antennal lobe tract (m-ALT) a weak staining of the lateral antennal lobe tract (l-ALT) appears. LH lateral horn, m medial, p posterior, P peduncle. Confocal image, 20x objective. Scale bar 100 µm.



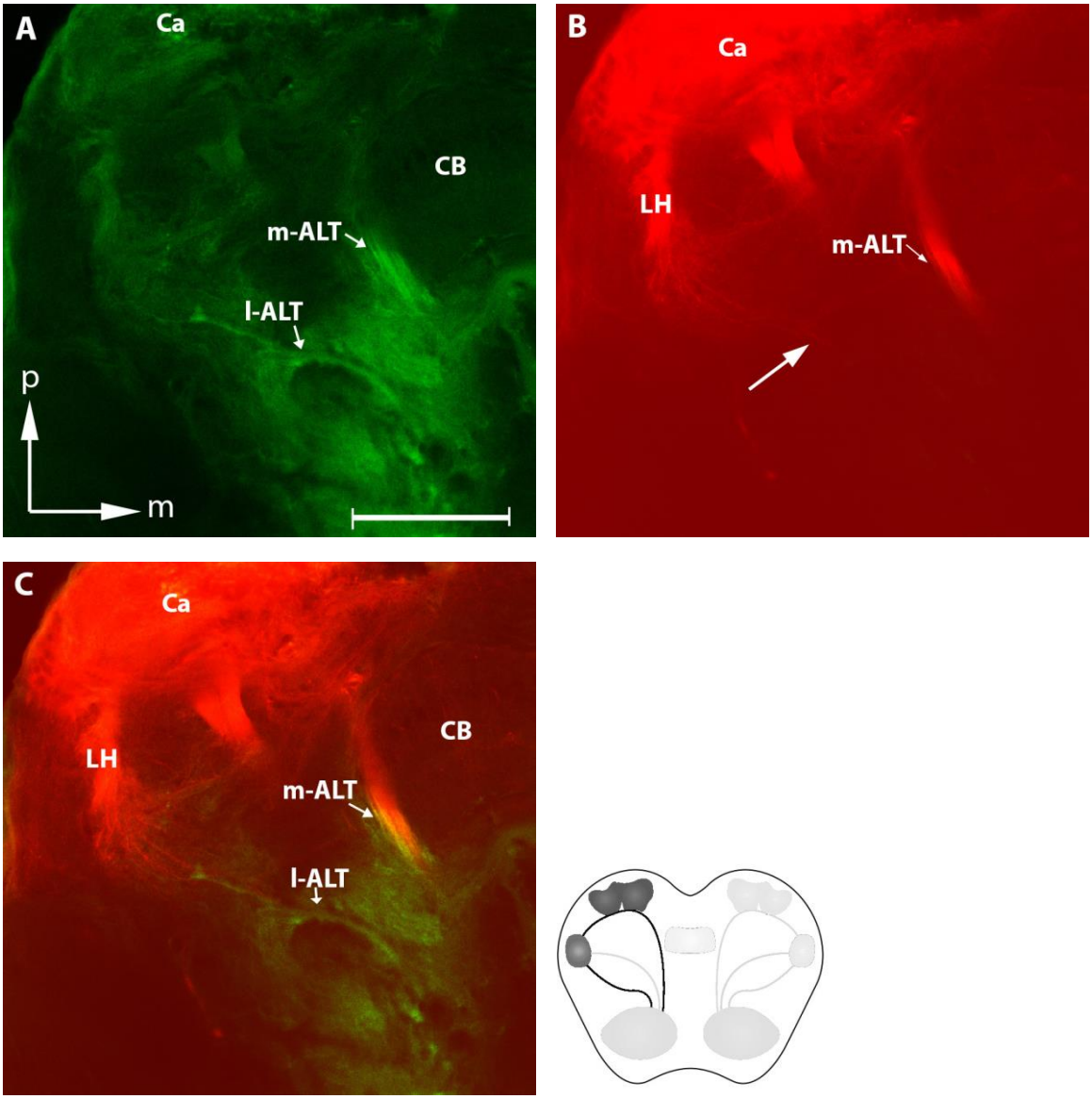
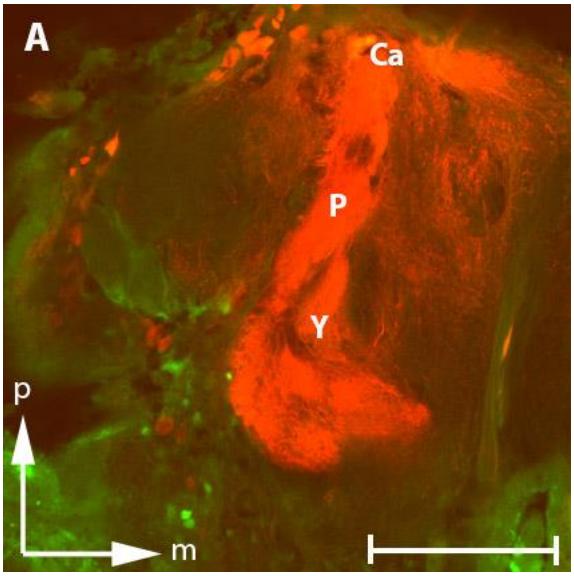
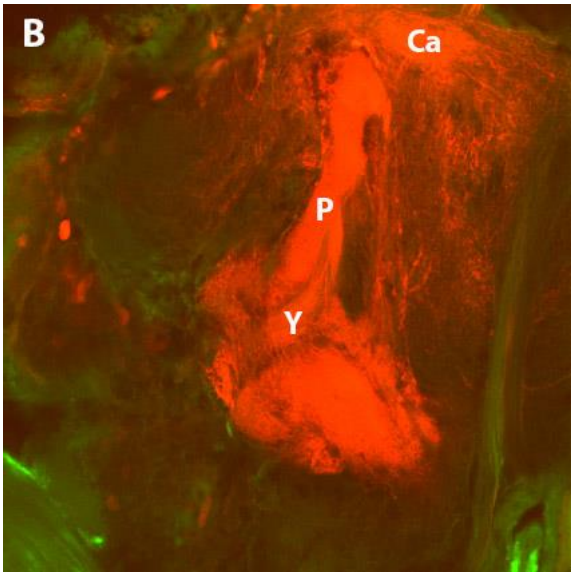


Figure A2: Confocal images of the double-labelled preparation 86 showing the antennal lobe tracts (ALTs). **A:** Image of the preparation stained from the antennal lobe (AL) showing the medial ALT (m-ALT) and the lateral ALT (l-ALT). **B:** The same preparation showing the neurons stained from the calyces (Ca). **C:** Overlay of the images shown in A and B. Due to weak staining it is not easy to see the neurons stained from the calyces connecting the calyces and the AL in the l-ALT. As the AL is not visible in these confocal images it cannot be stated that these neurons are connected to the AL but from their location and pathway in all likelihood they are. CB central body, LH lateral horn, m medial, p posterior. 20x objective. Scale bar 100 µm.





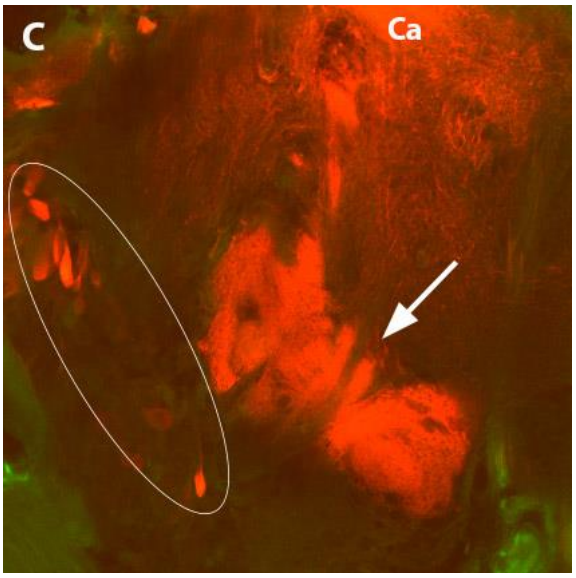


Figure A3: Serial slices from dorsal to ventral of the double-labelled preparation 73 showing the mushroom bodies. **A:** Image of the mushroom bodies in a dorsal view. **B:** The same preparation in a more ventral view. **C:** Image of even more ventral section. The circle shows some of the cell bodies belonging to cells connected to the calyces (Ca). m medial, P peduncle, p posterior, Y Y-tract. 20x objective. Dorsal view. Scale bar 100 µm.

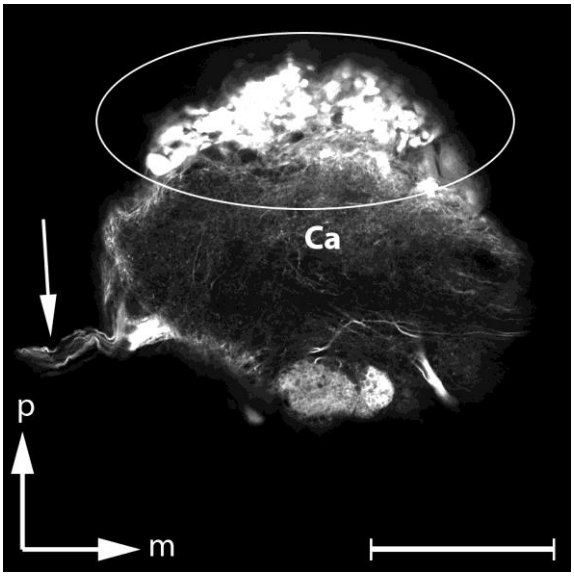


Figure A4: Confocal image of preparation 68 showing cell bodies of neurons connected to the calyces (Ca) located posteriorly in the moth brain. This image also shows a bundle of neurons branching from calyces towards the optic lobe (arrow). 20x objective. Scale bar 100 µm.

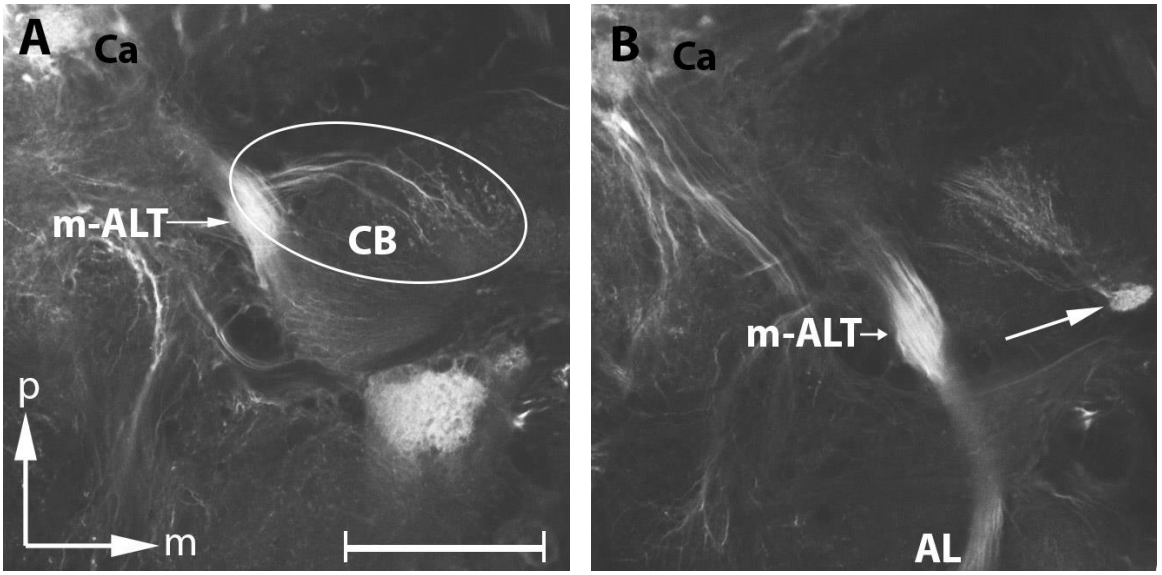
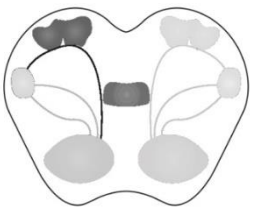


Figure A5: Confocal images showing neurons branching from the calyces (Ca) into the central body (CB), in preparation 68. The preparation is stained in the calyces with Micro-Ruby. **A:** Image showing neurons projecting into the CB. **B:** Image showing the projection pattern of the current neurons within the CB, including a small spherical region that was strongly stained (arrow). AL antennal lobe, m-ALT medial antennal lobe tract, m medial, p posterior. 20x objective. Scale bar 100 µm.



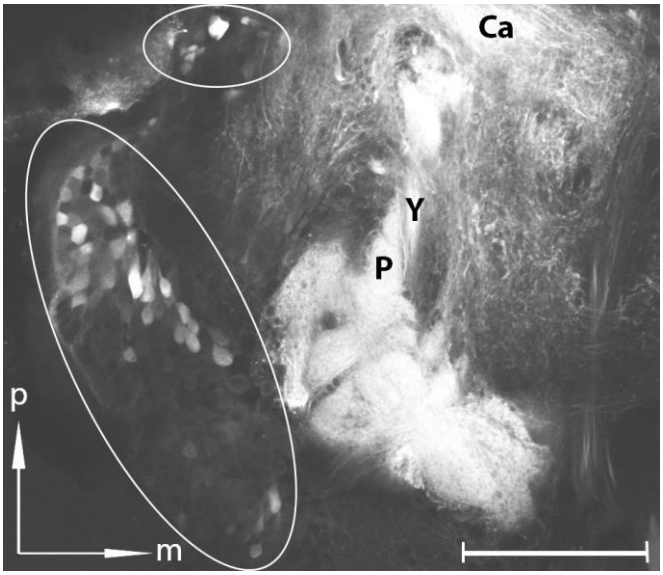


Figure A6: Image showing cell bodies of preparation 68 belonging to neurons connected to the calyces (Ca). These neurons are situated over a large area laterally to the peduncle (P) and the calyces (circles). m medial, p posterior, Y mushroom bodies Y-tract. 20x objective. Scale bar 100 µm.