Anatomical organization of second order neurons in the primary olfactory brain center of the model organism *Heliothis virescens*

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Aleksander Berg

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Sammendrag

Luktesansen er den eldste av sansene. Grunnet dens mindre håndgripelige natur sammenliknet med de andre sansene er den ofte blitt mystifisert. Selv om flere hundre millioner år skiller virveldyr og insekters felles stamfar evolusjonsmessig, er luktesansens underliggende nevrale organisasjon sammenliknbar på tvers av arter. I dette studiet ble hjernen til nattsvermerarten *Heliothis virescens* (Lepidoptera: Noctuidae) brukt som modell for å studere nevrale prinsipper knyttet til luktesansen. Dataene gav detaljert informasjon om anatomisk organisering av det første synaptiske nivå i luktebanen hos denne arten. Det primære luktsenter, som hos insektet betegnes antenneloben, har slående likheter med menneskets luktelapp og utgjør derfor et system av generell interesse knyttet til å studere blant annet luktesansen. Ved å benytte teknikken med retrograd farging av andre ordens luktenevroner knyttet til ett av hjernens høyere integrasjonsområder (calyces), ble anatomisk organisering av det primære luktsenter, samt de nevrale traktene som frakter luktinformasjon fra antenneloben til calyces visualisert. En fremtredende bunt med axoner (den mediale antennoprotocerebrale trakt: M-APT), som projiserer til calyces, ble beskrevet i detalj med sine spredte røtter i antenneloben. Grunnet beskjeden farging av en annen antennoprotocerebral trakt (den laterale antennoprotocerebrale trakt: L-APT), ser det ut til at få fibre terminerer i calyces gjennom denne trakten. Et fjerde cellekluster i antenneloben, som tidligere ikke er beskrevet, ble funnet og navngitt parvus (Latin: lite) anterior cellekluster på grunn av dets moderate størrelse. Mens den retrograde fargingen av andre ordens projeksjonsnevroner viste at dendritter fra disse nevronene forgreiner seg fra ett punkt og sprer seg innvendig i hvert glomerulus, viste anterograd farging av sensoriske axoner fra antennenerven at disse projiseringene innerverer hovedsakelig periferien av antennelobens glomeruli.

Abstract

The puzzling and often mystified sense of smell is the oldest of the senses. Though several hundred million years separates the common ancestor of vertebrata and invertebrata in evolution, the general underlying neural organization of olfaction is preserved and highly similar across species today. In the present study the tobacco budworm *Heliothis virescens* (Lepidoptera: Noctuidae) was used as a model organism for studying the neural system linked to olfaction. This provided a detailed anatomical account of the first synaptic level of the olfactory pathway in this particular species. The anatomical organization of the primary olfactory center (antennal lobe), and the neural pathways conveying olfactory information from here to a higher level brain area (calyces of the mushroom body), were visualized by retrograde staining of second-order neurons targeting the calyces. A prominent axonal bundle (the medial antennoprotocerebral tract: M-APT), conveying olfactory information to the calyces, was described in detail along with its sub-divisional roots in the antennal lobe. Due to sparse staining of another antennoprotocerebral tract (the lateral antennoprotocerebral tract: L-APT), it is suggested that only a few fibers terminate in the calyces through this tract. A fourth antennal-lobe cell cluster, previously not described, was found. It was named the parvus (Latin: small) anterior cell cluster due to its modest size. Whereas the retrograde staining of second order projection neurons demonstrated dendrites entering each glomerulus at its base and branching within the core, anterograde staining of sensory axons from the antennae showed that these projections target the periphery of each glomerulus.

Introduction

Throughout evolution the sense of smell has been fine tuned to meet the needs of various organisms adapting to specific habitats. Be it mating behavior, danger avoidance or feeding behavior, the sense of smell has probably assisted different species through their environments as a vital mechanism facilitating survival. In modern human existence, olfactory perception continuously represents the external world in our brains in a way not graspable by the other senses. The exception may be gustation, which seems to be closely interconnected with olfaction (Shepherd, 2006). This connection is indeed prominent in a pleasant way when exhaling after having swallowed a particularly odorous piece of food like chicken curry paste or after you have tasted a rich red wine. Conversely, malodorous volatiles like the smell of feces, rotten food or toxic gasses are very likely to cause avoidance behavior and maybe emesis in most people. Despite the obvious presence of olfactory impressions in our daily lives and the assumed importance of this sense in an evolutionary perspective, its significance has often been sublimed and mystified. This is even somewhat observable today in that for instance the American Medical Association's (AMA) "Guides to the evaluation of permanent impairment" (1993) rates anosmia (inability to smell) as being equivalent to 3% impairment of the whole person, in contrast to a 35% impairment for complete loss of hearing and 85% impairment for complete loss of vision. Though the importance of the different senses depends on age, occupation, and general life situation there is no doubt we are mainly utilizing visual information in our everyday life. However, might this disproportionate ranking of importance partially be a result of insufficient knowledge about the primordial, mystified sense of olfaction some only tend to appreciate once deprived of during a flue? Though modern science has unearthed many different and previously unknown features concerning olfactory perception in different species (e.g. Shepherd, 2006; Zhou & Chen, 2009; Dahlin, Bergman, Jansson, Bjork & Brittebo, 2000; Ramaswamy, Ma & Baker, 1987), it might still be the sense holding the most fertile grounds for new discoveries in terms of morphology and physiology in both vertebrates and invertebrates. In addition to a demystification of the sense in itself, further studying the olfactory system might also reveal important fractions of information useful in the process of understanding complex human and animal behavior.

The human olfactory system

When odor molecules from an external source hit the human nose they interact with olfactory-receptor proteins expressed by sensory neurons in the olfactory epithelium located dorsally in the nasal cavity (Figure 1A). The olfactory epithelium covers an area of about 5 cm^2 and contains several million sensory neurons. Each neuron extends one dendrite ending up in numerous hair-like structures, cilia, which are surrounded by a layer of mucus providing an appropriate molecular and ionic environment for odor detection. The olfactory sensory neurons are bipolar and unmyelinated, and from its basal pole it projects an axon directly into the primary olfactory center of the brain, the olfactory bulb, located ventrally of the prefrontal cortex (Figure 1B). On its way to the olfactory bulb, it passes up through the cribriform plate (Kandel, Schwartz & Jessel, 2000). In the olfactory bulb each sensory axon converges into one glomerulus, i.e. a tiny spherical module that further codes the odor signal. The organization of receptor zones evenly distributed throughout the olfactory epithelium is mirrored at the next level of information coding by glomeruli also being clustered into different spatial zones in the olfactory bulb. Within the glomeruli the sensory neuron axons terminate on relay neurons called mitral and tufted cells. Encircling the individual glomeruli are periglomerular interneurons making connections between glomeruli within the olfactory bulb. From the olfactory bulb the mitral and tufted relay neurons project in the olfactory tract terminating on the dendrites of pyramidal neurons in phylogenetically old regions in the temporal lobe, which have been named the olfactory cortex (Kandel et al. 2000; Kandel, Schwartz, Jessel, Siegelbaum & Hudspeth, 2013). These regions, including the piriform and entorhinal cortex, plus parts of the amygdala, overlap with portions of the limbic system (Zald & Pardo, 2000). The olfactory information is further mediated to hippocampus and hypothalamus. After processing in the olfactory cortex, parts of the signals also reach through the thalamus to the orbitofrontal cortex in the frontal lobe which is thought to be responsible for the identification and discrimination of odor qualities (Kandel et al. 2000). The limbic system is a set of brain structures commonly described as a coherent entity involved in processing sensory signals as well as information about emotion and memory. Thus, these areas are important for the organism in order to benefit from the olfactory signal. The overlap between the olfactory pathway and the limbic system is evident in that extensive lesions in the limbic system are very likely to cause anosmia (Tranel & Welsh-Bohmer, 2012). An arrangement that makes the olfactory pathway unique as compared to those of the other senses is that the signals do not pass through thalamus before projecting to the cerebral cortex.

This combined with the remaining path of the olfactory signal, targeting regions of the limbic system, as mentioned above, connect brain structures important for emotion, motivation, and certain kinds of memory particularly closely connected to the external world (Bear, Connors & Paradiso, 2007).

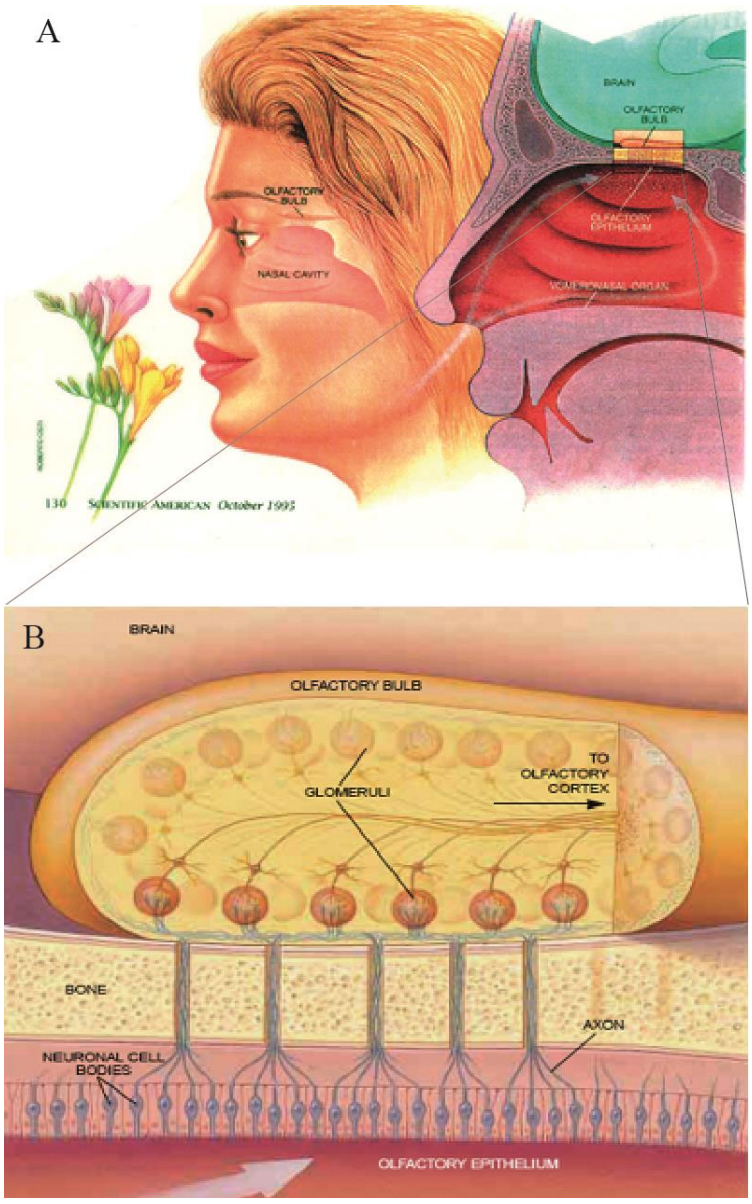


Figure 1: (A) Schematic drawing of a human head showing the olfactory epithelium located just under the cribriform plate. On top of the cribriform plate and under the prefrontal cortex lies the olfactory bulb. **(B)** Enlarged view of the square from figure A, showing the olfactory bulb with sensory input neurons synapsing with second order projection neurons making up the olfactory tract (Axel, 1995).

Studying olfaction using a model system

Studying the neural underpinnings of olfaction, though depending on how we study it, is not done without its challenges. Due to ethical considerations, the human brain is relatively inaccessible in terms of investigating neural networks, especially when it comes to data obtained in vivo. Tracing individual neurons and their pathways cannot be performed using in vivo experiments on human brains with today's technology. Therefore, model brains from various organisms have been, and are being used to study principles underlying processing of sensory information in general. Due to their exquisitely developed sense of smell and easily accessible nervous system, insects are often used for studying olfaction in various experimental settings. Moths, honeybees, locusts, cockroaches, and the fruit fly have successfully been investigated and so provided useful information about general and specific coding mechanisms of olfactory information in the brain. Because the neural principles underlying olfaction is very similar across different organisms, these experimentally favorable animals can provide broadly representative insights regarding chemosensory perception and its neural encoding mechanisms.

Except from the design of the external sensory organ, i.e. the nose of vertebrates (humans included) and the antennae of insects, a number of striking similarities characterizes the organization of the olfactory system across these groups of organisms. Firstly, the olfactory epithelium in humans, described above, though not at all seemingly similar, is in fact highly comparable to the olfactory organ of insects at a microscopic level. Thus, the tiny hair-like structures on the insect antennae also contain bipolar receptor neurons mediating the olfactory information directly into glomeruli of the brain olfactory center, here called the antennal lobe (Hansson, 2010). As the different spatial zones of the vertebrate olfactory epithelium contain stochastic olfactory receptors within each zone, the antenna of *D. melanogaster* has functionally identical sensilla randomly distributed in different zones (reviewed by Kaupp, 2010). In addition to functional similarities in the olfactory system, new research has reported deep homology in the arthropod (i.e. moths included) central complex and vertebrate basal ganglia. These are complexes of deeper brain structures that in both phyla are involved in the initiation of a behavioral outcome following for instance an olfactory input (Strausfeld & Hirth, 2013). In addition, homology of higher processing brain centers, i.e. the cortex and the mushroom bodies in respectively vertebrates and annelids are suggested (Tomer, Denes, Tessmar-Raible & Arendt, 2010). Together, this research point to a substantial general

similarity in the system that receives and process sensory information across phyla. Because the mushroom body shares a certain molecular arrangement and gene patterning with the developing cortex, Tomer et al. (2010) suggest with an evolutionary point of view that these brain units evolved from the same structure in a common ancient ancestor of vertebrates and invertebrates before diversifying. With these homologous tendencies taken into account, the scientific advantage of using the brain of a model organism that possesses a mushroom-body structure becomes evident.

The olfactory system of the moth

The antennal lobes

The antennal lobe is the primary olfactory center in moths, corresponding to the vertebrate olfactory bulb. It receives input from olfactory receptor neurons located on the antenna and consists of spheroidal neuropilar sub compartments termed olfactory glomeruli. The glomeruli are situated as a layer in the periphery of the antennal lobe, making a central hollow compartment consisting of interneuron processes that also reaches through the glomeruli. The majority of the glomeruli are so-called ordinary glomeruli involved in processing information about general odorants. Dorsally in the antennal lobe the male-specific macroglomerular complex (MGC) is located. This distinctive complex of glomeruli is well known for its role in guiding males towards the location of females. The largest glomerulus in the MGC, the so-called cumulus, is positioned closest to the antennal nerve (Hansson, Almaas & Anton, 1995). This unit is known to process information about the major pheromone component *Z*-11-hexadecenal (Z11-16:AL), excreted by female moths for mating purposes. In addition, the MGC has a dorso-medial, an antero-medial, and a ventro-medial glomerulus. The dorso-medial glomerulus receives input from neurons that selectively respond to the second pheromone component *Z*9-tetradecenal (Z9-14:AL), whereas the antero-medial and ventro-medial units are involved in recognizing sympatric species and thereby play a role in inhibiting mating behavior (Vickers, Christensen & Hildebrand, 1998; Berg, Almaas, Bjaalie & Mustaparta, 1998). Among the many heliothis species examined in this kind of research, the two enlarged glomeruli, out of three, in the MGC of *Helicoverpa assulta* have similarly shown to process information about the primary pheromone component and the behavioral antagonists, respectively, while the smallest receives information about the secondary pheromone component (Berg, Almaas, Bjaalie & Mustaparta, 2005; Zhao & Berg, 2010). During the metamorphic transformation from larva to adult, the MGC is the first structure to form in the male antennal lobe, i.e. before the ordinary glomeruli. A study on the sphinx

moth, *Manduca sexta*, showed this in that its cumulus and two toroidal MGC glomeruli separated early from the ordinary glomeruli and that the basic organization of the MGC was established within 3-4 days after ingrowth of the first antennal olfactory receptor cells (Rössler, Tolbert & Hildebrand, 1998). Thus, despite minor or extensive species specific variations in morphology and physiology, we see that the neural underpinnings of olfaction are similarly and reliably observable across different species. Exploring the microstructures of the olfactory system and studying old findings up against new data is therefore very valuable when mapping variations in function and structure to provide a continuum of thorough theoretical framework for future studies in this field.

Antennoprotocerebral tracts

From the insect antennal lobes, second order projection neurons organized in three cell clusters relay olfactory information to higher-order brain centers via several parallel pathways called the antennoprotocerebral tracts (reviewed by Galizia & Rössler, 2010). The most prominent one travels along the brain midline (i.e. medially). This medial antennoprotocerebral tract (M-APT; former term: inner antennocerebral tract, I-ACT) targets two main higher-order centers, the calyces of the mushroom bodies and the lateral protocerebrum (Homberg, Montague & Hildebrand, 1988; Rø, Müller & Mustaparta, 2007). In addition to the M-APT, a lateral and a mediolateral antennoprotocerebral tract (L-APT and ML-APT) are identified in several insect species, moths included (reviewed by Galizia & Rossler, 2010). The M-APT initially projects from the antennal lobe to the calyces before it projects further to the lateral protocerebrum. The axons in the L-APT first project to the lateral protocerebrum and then sends axonal fibers to the calyces, whereas the ML-APT follows the M-APT for a short distance before bending laterally at the level of the central body and projecting to the lateral protocerebrum only (Homberg et al. 1998; Rø et al. 2007). In *M. sexta* the M-APT is described as projecting from the antennal lobe by the means of two main sub-unit branches, or so-called roots, and in-between them a smaller bundle. These roots are the axons of (mostly uniglomerular) projection neurons from the lateral, the medial and the anterior cell cluster of the antennal lobe which fuse and make up the M-APT (Homberg et al. 1988). The cell clusters are given their names due to their position in the antennal lobe whereby the lateral is the biggest and the anterior the smallest. All clusters are positioned in the peripheral part of the antennal lobe, together enveloping the glomeruli.

Calyces of the mushroom bodies

The mushroom bodies cover the brain area behind the antennal lobes that the APTs, described above, extends through. In addition to the two cup-shaped structures located posteriorly, the so-called calyces, the mushroom bodies consist of several lobes. Numerous intrinsic Kenyon cells make up these sub-compartments. Thus, the dendrites of the Kenyon cells form the calyces, whereas the axons and terminals make up the lobes. In general, the calyces are described as a distinctive multimodal sensory integration neuropil (Hansson, 2010). Further on, the calyces are thought to play a key role in learning and memory (reviewed by Heisenberg, 2003). Of the APTs, the prominent M-APT projects directly to the calyces (Homberg et al. 1988). In some species of neoptera and coleoptera, the absence of an antennal lobe and glomeruli can be correlated with a prominent reduction or total loss of the calyces (Strausfeld, Sinakevitch, Brown & Farris, 2009). As formerly mentioned, there is a long tradition, actually dating back to the latter half of the 1800s, of seeking knowledge about human neuroanatomy by mnemonic usage of model organisms. When it was discovered that the calyces of species depending on multisensory integration was particularly big, the functional analogy of mushroom-body structures to human cognitive processes were established (Strausfeld, 2012). Consequently, there are some strong indications that olfactory information is somehow processed in these areas in order for previously processed stimuli to be retrieved, compared, and integrated with current stimuli. These observed functions make the calyces a valid analogy of the human olfactory cortex.

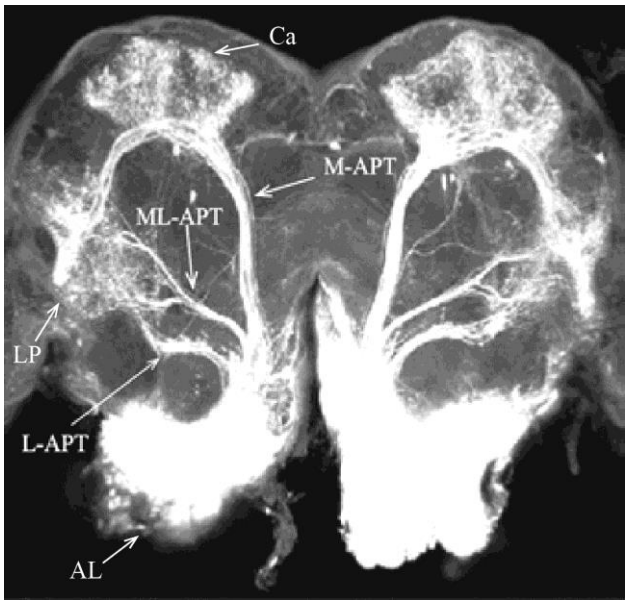


Figure 2: Dorsal view of the moth brain showing the three main antennoprotocerebral tracts in each hemisphere, the calyces (Ca), the lateral protocerebrum (LP), and the antennal lobe (AL). Used with permission from Xin-Cheng Zhao, unpublished data.

Model organism: Heliothis virescens

Increasingly evident when reviewing literature on insect biology is the nearly infinite multitude of species and experimental varieties used when researchers in this field are obtaining data. Despite numerous inter-species specific variations, certain fundamental principles in anatomy and function are readily observable. The species available in our lab at MTFS/Dragvoll is the tobacco budworm *H. virescens* (Lepidoptera: Noctuidae). This species' anatomical and physiological appearance is very similar to the fundamental principles introduced above. There is an array of existing detailed knowledge about anatomy and physiology in *H. virescens* (e.g. Rø et al. 2007; Kvello, Løfaldli, Rybak, Menzel & Mustaparta, 2009; Løfaldli, Kvello & Mustaparta, 2010; Vickers et al. 1998; Zhao, Pfuhl, Surlykke, Tro & Berg, 2012). However, a complete account of the morphological organization concerning the various antennoprotocerebral tracts is currently still in progress.

Given the striking similarity in synaptic organization of the primary olfactory center between mammals and insects, and that the calyces can be said to be corresponding in function to areas of the mammalian olfactory cortex, the use of the moth brain as a model for investigating higher order processing of olfactory information in general is very valuable, as is underlined above. As noted by Hildebrand (1995), a comparative approach exploiting experimentally favorable model systems is among the foundations of this type of research and is likely to reveal valuable principles of organization and function of the sensory systems in vertebrates and invertebrates alike if studied with a neuroethological approach.

Objectives

Main objective:

- Gain further knowledge about chemosensory encoding principles by visualizing the population of second order olfactory neurons connecting the calyces to the primary olfactory center in the moth brain.

Sub-goals:

- Mass stain all projection neurons linked to the calyces in order to explore their glomerular innervation pattern and to gain further knowledge about the antennoprotocerebral tracts linked to this particular neuropil.
- Stain antennal sensory neurons in order to visualize the connectivity pattern between the sensory and the second order neurons in the antennal lobe.

Materials and methods

Insects

Male moths of the species *H. virescens* (Lepidoptera: Noctuidae) were used during the experiments. The insects were obtained from Zyngenta (Switzerland) and received as pupae via mail. When the pupae arrived they were sorted by gender and placed in separate heating cabinets (Refritherm, 6E) with a light-dark cycle of 14-10 hours and humidity of 70%. After hatching, the insects were placed in a plexi glass container and given a solution of water and sucrose for food. Experiments were conducted within 2-7 days after hatching.

Ethics

Since the moth is an invertebrate it is not formally regulated for experimental purposes in Norwegian law. However, the insects were handled with care and kept in proper conditions of light, temperature, and food supplies.

Equipment

All equipment necessary for making the incisions was available at the Neuroscience unit (gruppe for nevrofag) at NTNU. The dissection tools, delivered by Fine Science Tools (Dumont, Switzerland), included a fine micro-scissor, forceps, and a fine hollow needle. In addition, a razor-blade knife and a syringe were used. A soldering iron was used to melt the wax where the insect head was placed. A Leica microscope (Wild 355110, Heerbrugg, Switzerland) was used to visualize fine details in the preparations while working.

General procedure

Firstly the moth was placed in a plastic tube, about 3cm long and 7mm wide, made from a plastic pipette with the narrow end cut off. An easily conformable type of wax was used to fasten the specimen in the plastic tube, exposing the head with the antennae sticking out, leaving the thorax and the wings inside the tube (See figure 4).

Figure 3: Posterior view of the cut off moth head mounted in wax. The orange substance covering the right hemisphere is melted crystals of a fluorescent dye (in this case Alexa 488) used to visualize second order neurons.

Staining procedure

Visualization of the antennal-lobe projection neurons linked to the calyces was performed by retrograde labeling. This labeling technique utilizes the internal axonal transportation system of the neuron so that dye taken up by the terminal processes is carried back to the cell body and the dendrites. In order to access the calyces and apply the dye, the head was removed from the thorax. After separating the head from the body, the head was melted into a piece of wax with the frontal parts pointing down, exposing the posterior/caudal part of the head. The posterior parts of the head cuticula and some underlying trachea was removed to expose the right calyces. The preparation was kept moist with Ringer's solution (50 ml: NaCl: 0.4383, KCC: 0.0112, TES: 0.1145, Sucrose: 0.4275, CaCl2: 0.0167) during the procedure to keep the brain from drying out, and thereby getting spoiled. The area of the calyces was then drained, using a thin kind of lens paper. Once cleared of trachea, muscle tissue, and moisture, minor cuts were made on the brain surface in the area of the right calyces. Crystals of fluorescent dye were placed on top of the cuts. Two different dyes were used, Micro-ruby (Dextran tetramethylrhodamine, 3000MW, excitation/emission maxima ~555/580 nm) or Alexa 488 (hydrazide, 10000MW, excitation/emission maxima ~495/519 nm). Once in contact with liquid, the dye crystals melted and were absorbed into the brain (See figure 3). The preparation was covered in pieces of paper moistened with Ringer's solution to keep from

drying, and put in room temperature for ca. 2 hours. To maintain moisture, drops of Ringer's solution were also added to the lens paper approximately every 30 minutes during the 2 hours.

Experiments involving dye applied to the antenna for anterograde labeling of the sensory neurons were also performed. This staining procedure started by firstly cutting off the right antenna just above the pediculus, a ball-and-socket-joint near the antennal base. Then, crystals of the fluorescent dye (Alexa 488 or Micro-ruby) were applied to the cut antennal stump, using a fine hollow needle (see figure 4). For the dye to be absorbed and transported by the axons, the preparation was either placed in a refrigerator overnight or in room temperature for 3 hours. In an amount of preparations, anterograde staining of sensory neurons and retrograde staining of projection neurons were performed simultaneously in the same individual by injecting one fluorescent dye in the antenna and the other in the calyces. Since dye application to the calyces was always performed on a decapitated preparation, these double-labeling experiments always started by staining the sensory neurons. In order to obtain consistency in the procedure, only the right side of the brain (seen from the moth's point of view) was stained.

Figure 4: Image showing the preparation with the thorax inside a small plastic pipe. The right cut off antenna is exposed, showing melted red crystals of a fluorescent dye (in this case Micro-ruby) applied to visualize sensory neurons. The white material surrounding the head is an easily conformable type of wax keeping the head in place.

Dissection, fixation, and dehydration

After transportation of the dye, the brain was carefully dissected out of the head capsule, leaving the brain intact without any muscles or surrounding tissue. After dissection, the brain was fixated in paraformaldehyde (4%) in room temperature for 1 hour, or overnight in a refrigerator. After this it was washed in PBS for 10 x 2 minutes followed by dehydration in an ethanol series (5min: 50%, 70%, 90%, 96%, 10 min x 2: 100%). Finally, the brain was mounted on a metal plate in methylsalicylate, covered with one glass-plate on each side.

Confocal microscopy

The brains were scanned with a confocal laser microscope (LSM 510 META Zeiss, Jena, Germany) with a 40x and 20x objective (C-Achroplan 40x/0,8 W and Plan-Neofluar 20x/0,5. The X-Y resolution was set to 1024 x 1024 pixels and the z-stack resolution to 4µm. An argon laser was used for visualization of Alexa 488 and a Helium-Neon laser (HeNe1) for visualization of Micro-ruby.

Data analysis and depiction

The confocal images were viewed and interpreted using the LSM image browser. This allowed scanning through the entire sample and also to make three-dimensional reconstructions that could be rotated for detail interpretation. The data were edited using Adobe Photoshop CS6, and Adobe Illustrator CS6 was used for final adjustments and completion of the illustrations.

Results

In addition to the mass staining experiments that labeled the antennal-lobe projection neurons by applying dye into the calyces, the present study included visualization of olfactory sensory neurons by applying dye into the antenna, as well as a combination of the two techniques by applying two different dyes into the same preparation. Of the 43 preparations made, 14 successfully stained brains were taken to the confocal microscope for imaging. (All illustration images are collectively presented following this results section).

1. Retrograde staining of antennal-lobe projection neurons innervating the calyces

In addition to labeling the antennal-lobe projection neurons and the antennal lobe, dye application into the right calyces resulted in staining of several brain regions in the ipsilateral hemisphere including the lateral protocerebrum, the mushroom body lobes, and the pedunculus (Figure 5). Also, a previously described GABAergic cell cluster connected to the protocerebral calycal tract (Bente Jacobsen, 2012, master's thesis), located near the lobula of the optic lobe was visualized (Figure 5 B-D). Of the main antennoprotocerebral tracts, the M-APT was strongly stained. The L-APT, on the other hand, contained only a few labeled fibers. As expected, no fibers in the ML-APT were stained.

1.1 The M-APT and its origin in the antennal lobe

By applying dye to the calyces, the M-APT projecting as a thick bundle of fibers along the medial part of the brain in an anterior-posterior direction, bypassing the central body dorsally, was visualized. With it, the dorsal and ventral root, which fuse and make up the M-APT when projecting from the antennal lobe, was visualized (Figure 6 and 7A). The staining shows that the fibers of the dorsal root innervate mainly dorsally located glomeruli and that they are connected to somata in the medial cell cluster (Figure 7C). Due to the density of stained somata in this particular cell cluster, it appears that it is selectively linked to the M-APT. The ventral root, which is slightly thinner and stretches ventro-laterally, innervating ventro-laterally located glomeruli, is clearly connected with somata in the lateral cell cluster (Figure 7 B and D). Unlike the dense and strongly stained medial cell cluster, the stained somata in the lateral cell cluster appeared as intermingled with numerous unstained somata. In between the two prominent roots there is a third root (hereafter called the *medial root*), less dense and briefly following the dorsal root before fusing with the ventral root and forming the M-APT.

Seen as entering the antennal lobe[1], it detaches the two other roots and splits into 6 smaller branches (Figure 7 A and C). These branches appear to innervate mainly glomeruli in the ventromedial half of the antennal lobe and seem to be linked to somata in the anterior cell cluster (Figure 7 B and C). Slightly posterio-dorsally of the anterior cell cluster, i.e. between the anterior and the medial cell cluster, the most successfully stained preparation displayed a group of ca. 10 somata (hereafter called the *parvus anterior cell cluster; PACc*), each having a diameter of approximately 5µm, i.e. one third the size of the adjacent anterior cell cluster somata (Figure 8A). These tiny somata connect to a small bundle of thin fibers projecting medially in an anterior-posterior manner and seem to exit the antennal lobe in between the medial and the dorsal root (Figure 8B and C).

1.2 L-APT

As mentioned, only one or a few fibers in the L-APT were stained. As seen in figure 9, the L-APT exits the antennal lobe beneath the ventral root of the M-APT (the small dot in figure 9A). From there the fibers project laterally and dorsally, reaching the lateral protocerebrum (the connection to the calyces is invisible due to the large number of stained fibers in this region).

1.3 Glomerular arborization pattern

The retrograde staining from the calyces visualized a distinctive glomerular arborization pattern formed by the dendrites of the projection neurons. Apparently, all glomeruli contain dendritic branches from projection neurons. Thus, as shown in figure 7C, a thick dendrite, connected to one of the roots of the M-APT, enters the glomerulus at its base, decreasing in thickness as it reaches the periphery. Though, in one glomerulus located ventrally in the antennal lobe, this pattern appears less prominent. This particularly big glomerulus, which is significantly weaker stained than the others, is likely to be the so-called labial pit organ glomerulus (LPOG), known to receive sensory fibers from the labial palps (Figure 10A). Regarding the general glomerular arborization pattern, a difference between the innervation in ordinary glomeruli and the MGC unit seems to be that the MGC is densely innervated by the dorsal root of the M-APT whereas the ordinary glomeruli are innervated by the ventral and the medial root. Most of the MGC-projection neurons seem therefore to have their somata located in the medial cell cluster, as previously mentioned (Figure 10B).

[1] The flow of information actually runs from the antennal-lobe roots to the calyces via the M-APT, however, in this example the direction was reversed in order to explain the appearance of the medial root in the antennal lobe.

2. Anterograde staining of the antennal sensory neurons

Mass staining of the antennal sensory axons also visualized the antennal-lobe glomeruli, however, in a somewhat different manner than the pattern formed by projection neurons. In the successfully stained preparations an array of spherical structures, i.e. the glomeruli, located in the peripheral part of the antennal lobe could be seen. The central region of the antennal lobe was not stained by the sensory processes (Figure 11A).

Following the sensory axons in the antennal nerve at the entrance of the antennal lobe, the four MGC sub-units, positioned next to the more numerous ordinary glomeruli, could be identified. In both the ordinary glomeruli and the MGC, the staining of sensory terminals showed innervation mainly in the periphery of the individual glomeruli (Figure 11). In addition, the sensory neurons innervated the collection of glomeruli from the periphery of the antennal lobe (Figure 11 B-D).

3. Double labeling of sensory terminals and second order projection neurons

Analyzing the preparations labeled by the two dyes, confirmed the previous observation that the sensory neurons innervate the periphery of each glomerulus whereas the projection neurons target the more central part (Figure 12).

4. Additional observations

4.1 Antennal neurons projecting outside the antennal lobe

In one particular preparation, a number of projections from the antenna, bypassing the antennal lobe ventrally, were strongly stained. A few projections terminated in three big somata positioned ventrally of the antennal lobe, close to the antennal mechanosensory and motor center (AMMC; Figure 13, appendix). In addition to a portion of fibers ending up in the AMMC, the remaining terminated further towards the medial part of the subesophageal ganglion (SOG). These projections are assumingly axons of gustatory receptor neurons located on the antenna (Jørgensen, Kvello, Almaas & Mustaparta, 2006; Figure 13, appendix).

4.2 Labelled microstructures surrounding the cell nucleus

In many of the stained cell bodies, particularly those located in the lateral cell cluster, which could be more easily observed due to their appearance among unstained cell bodies; several tiny dark spots surrounding the nucleus could be observed (Figure 14, appendix). Considering the proportions of these structures relative to structures surrounding the vertebrate nucleus, though speculative, the present data might have visualized mitochondria. Mitochondria are known to be involved in the generation of cell energy in vertebrates (Kandel et al. 2013) and invertebrates (Boardman, Terblanche, Hetz, Marais & Chown, 2012).

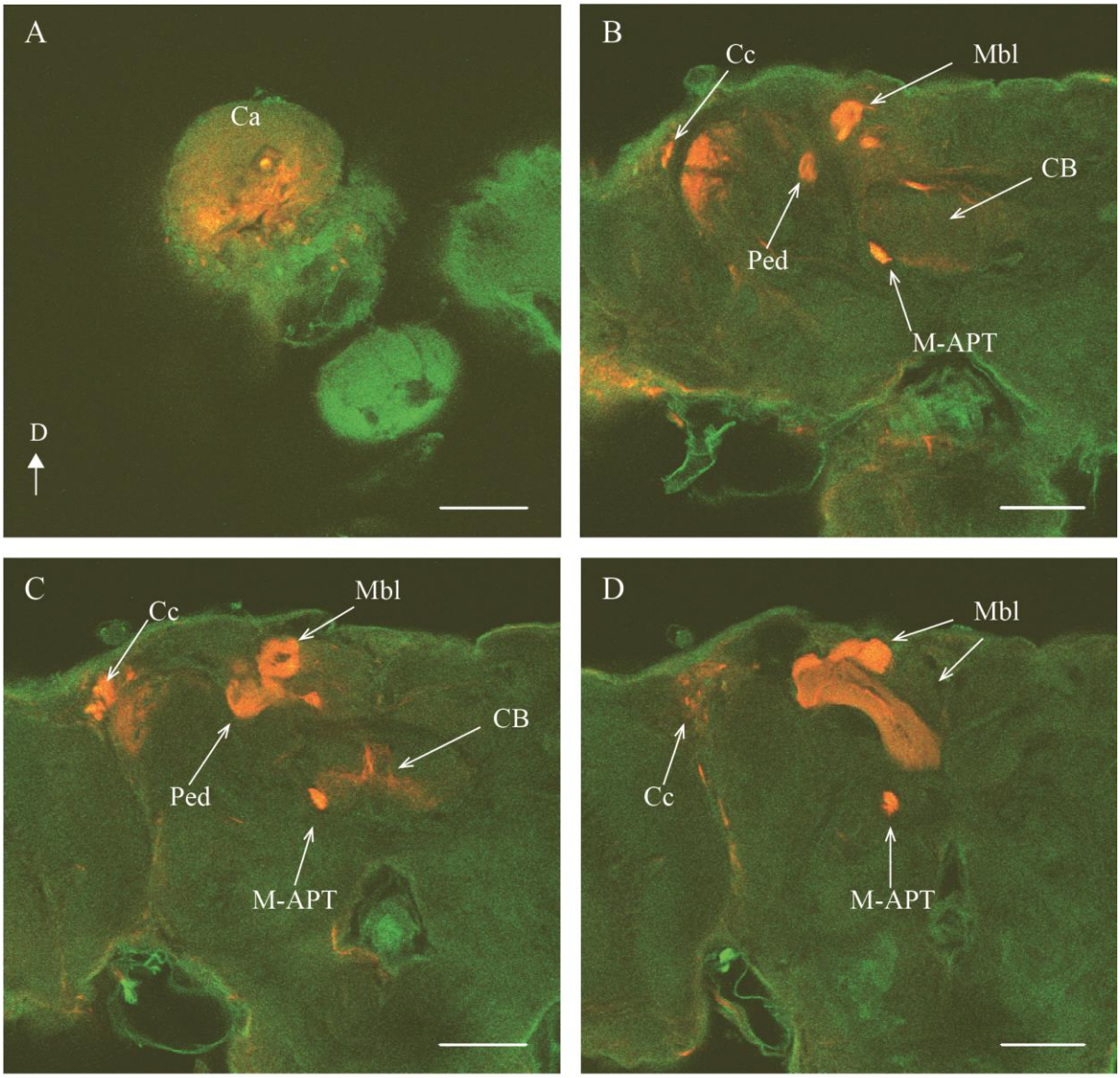


Figure 5: Serial slices of confocal images in a frontal view (posterior-anterior progression from A-D), showing the calyces (Ca), pedunculus (Ped), mushroom-body lobe system (Mbl), a cell cluster of GABAergic neurons belonging to the protocerebral calycal tract (Cc), the central body (CB), and the medial antennoprotocerebral tract (M-APT). Confocal scan, 20x objective. D=dorsal. Scale bar 100 µm.

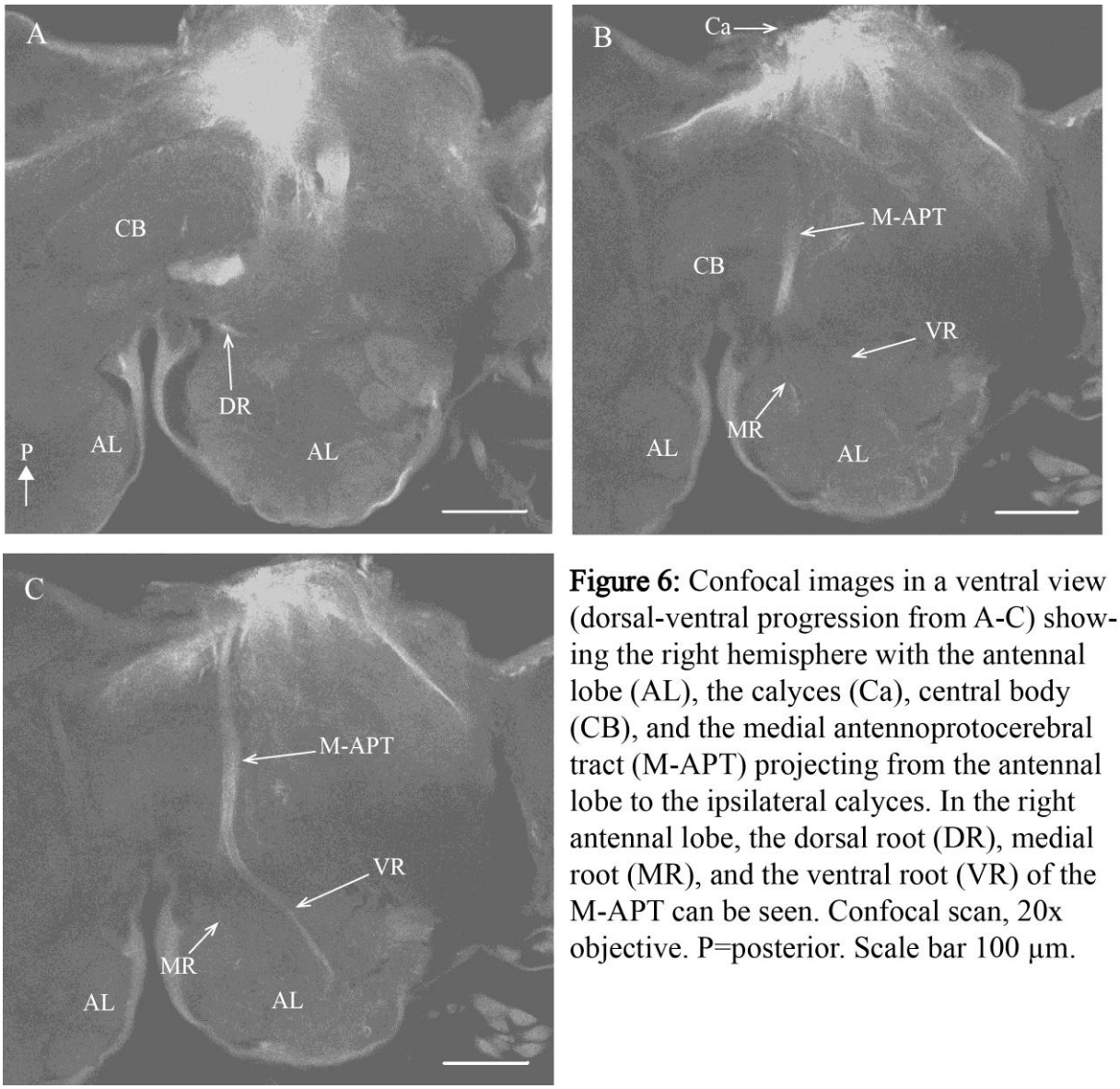


Figure 6: Confocal images in a ventral view (dorsal-ventral progression from A-C) showing the right hemisphere with the antennal lobe (AL), the calyces (Ca), central body (CB), and the medial antennoprotocerebral tract (M-APT) projecting from the antennal lobe to the ipsilateral calyces. In the right antennal lobe, the dorsal root (DR), medial root (MR), and the ventral root (VR) of the M-APT can be seen. Confocal scan, 20x objective. P=posterior. Scale bar 100 µm.

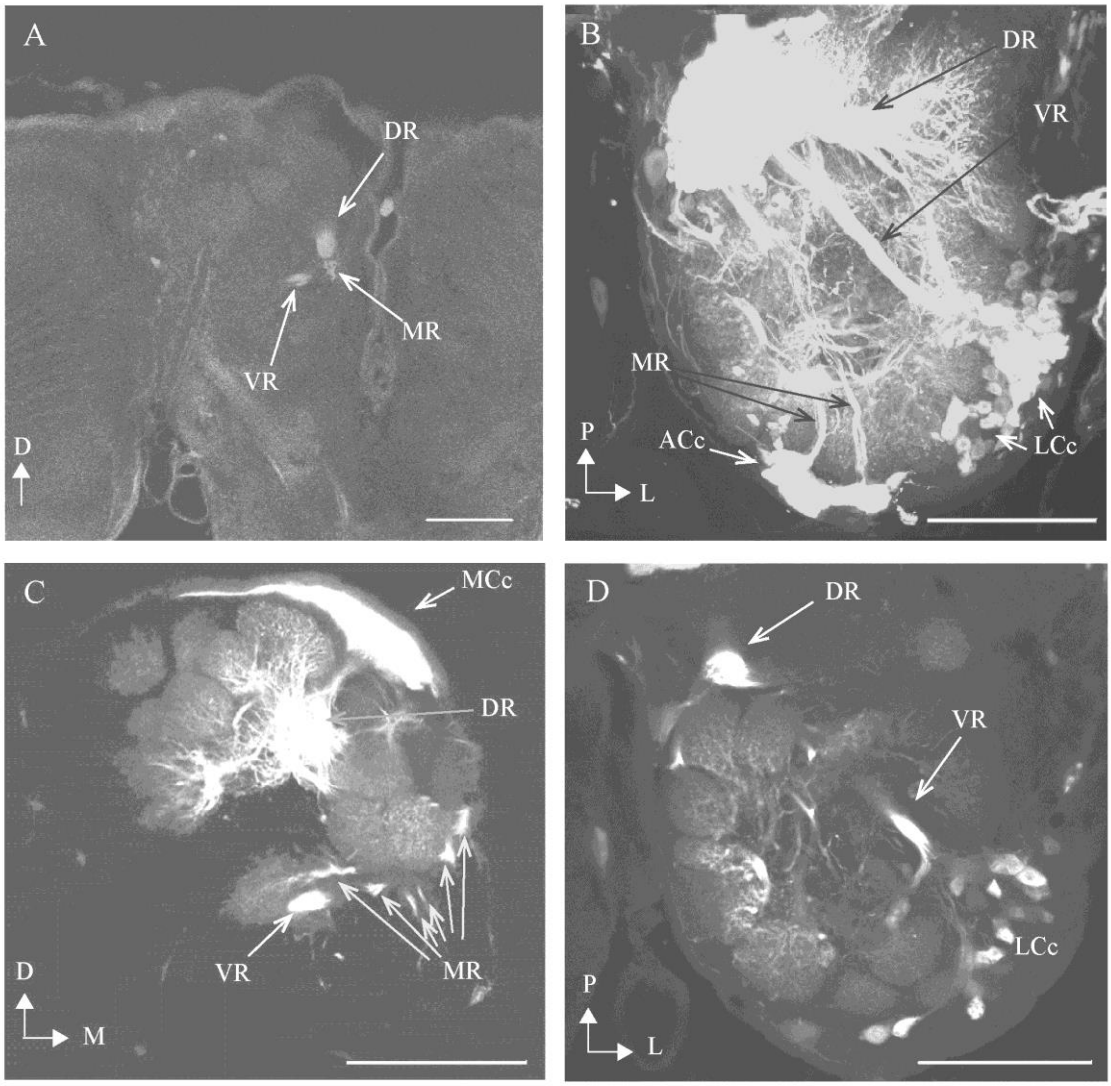
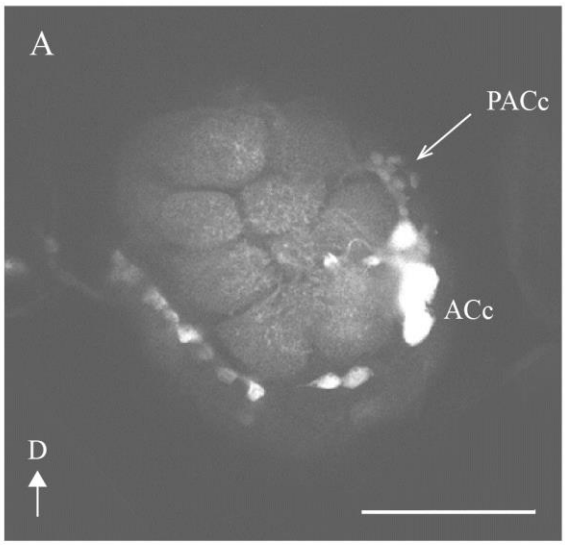
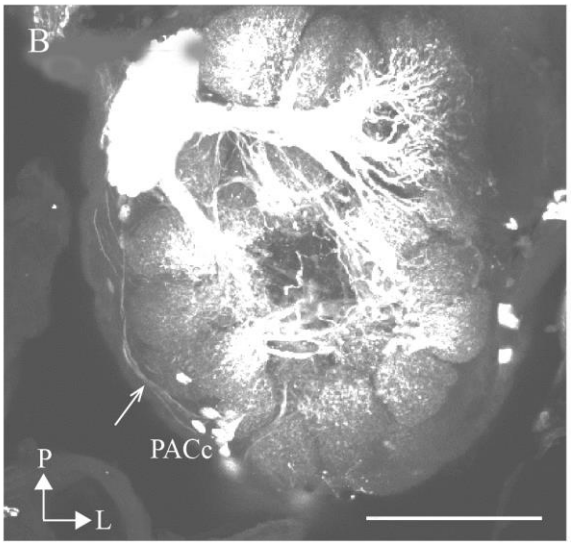


Figure 7: Confocal images of the three roots of the medial antennoprotocerebral tract (M-APT). **(A)** Frontal view of the right hemisphere showing the three roots; ventral root (VR), medial root (MR), and dorsal root (DR) projecting from the antennal lobe before they merge in the M-APT. **(B)** 3D reconstruction: Ventral view of the right antennal lobe showing fibers of the medial root connecting with the anterior cell cluster (ACc). The ventral root connect with the lateral cell cluster (LCc). The dorsal root are also visible (DR). **(C)** Frontal view of the right antennal lobe showing the dorsal root spreading within dorsally located glomeruli and connecting to the medial cell cluster (MCc). The ventral root and fibers of the medial root are also visible. **(D)** Ventral view of the right antennal lobe showing the ventral root connecting with the lateral cell cluster. The dorsal root is also shown. Confocal scan, A: 20x, B: 40x, C: 40x, D: 40x objective. D=dorsal, P=posterior, L=lateral, M=medial. Scale bar 100 µm.





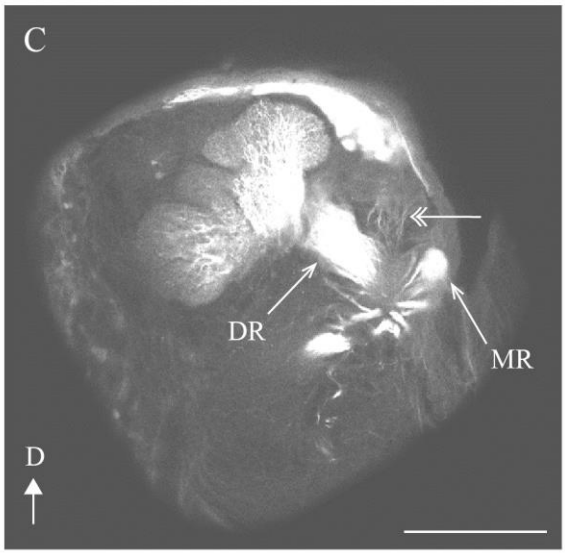


Figure 8: Confocal images of a fourth cell cluster, here referred to as the parvus anterior cell cluster (PACc), and its primary neurites exiting the antennal lobe. **(A)** Frontal view of the right antennal lobe showing the PACc above the anterior cell cluster (ACc). **(B)** Ventral view of the right antennal lobe showing thin fibers (arrow) connecting with the small somata in the PACc. **(C)** Double-pointed arrow showing the thin fibers from the PACc shortly before exiting the antennal lobe inbetween the dorsal most branch of the medial root (MR) and the dorsal root (DR) of the medial antennoprotocerebral tract. Confocal scan, 40x objective. D=dorsal, P=posterior, L=lateral. Scale bar 100 µm.

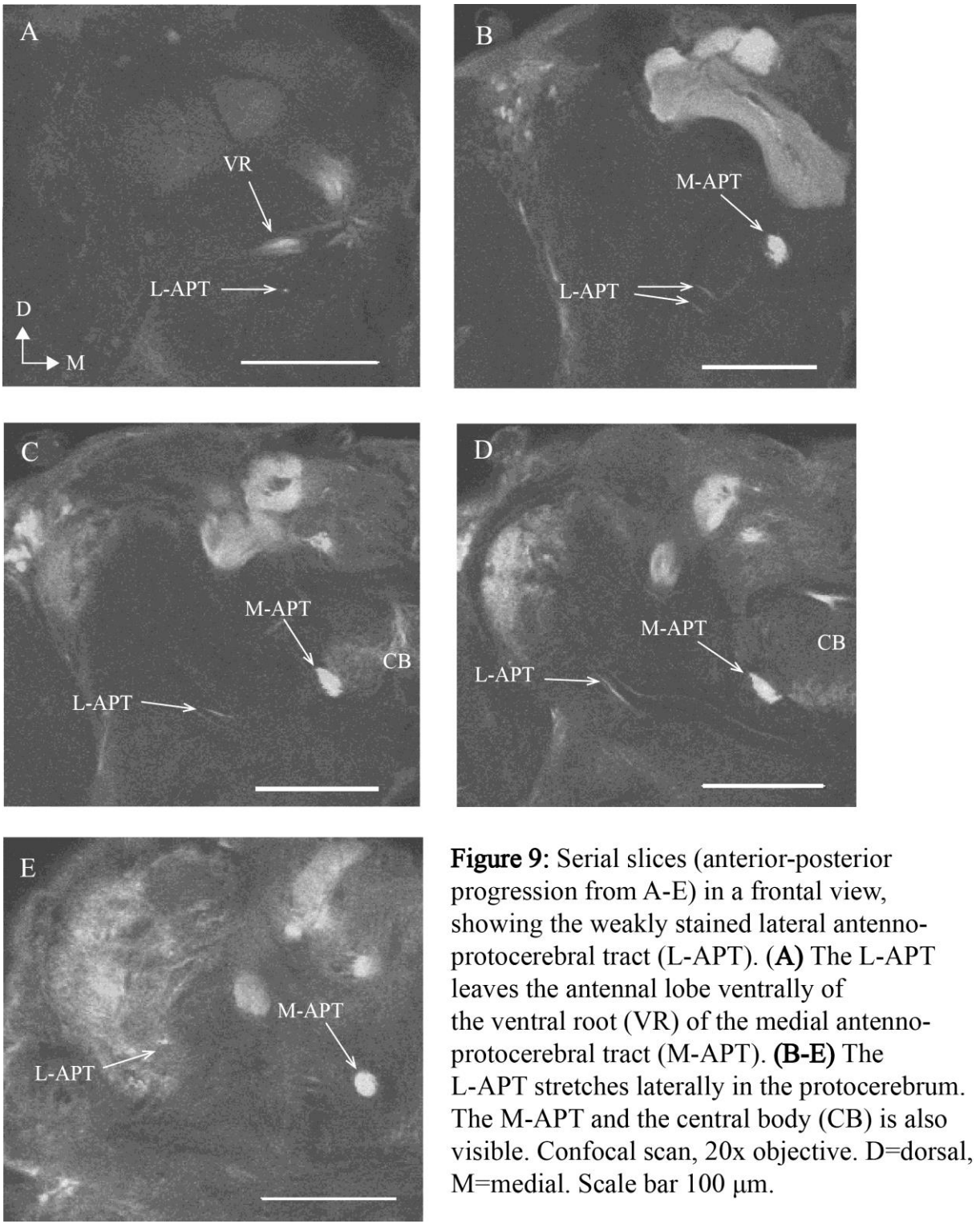


Figure 9: Serial slices (anterior-posterior progression from A-E) in a frontal view, showing the weakly stained lateral antenno-protocerebral tract (L-APT). (**A)** The L-APT leaves the antennal lobe ventrally of the ventral root (VR) of the medial antenno-protocerebral tract (M-APT). **(B-E)** The L-APT stretches laterally in the protocerebrum. The M-APT and the central body (CB) is also visible. Confocal scan, 20x objective. D=dorsal, M=medial. Scale bar 100 µm.

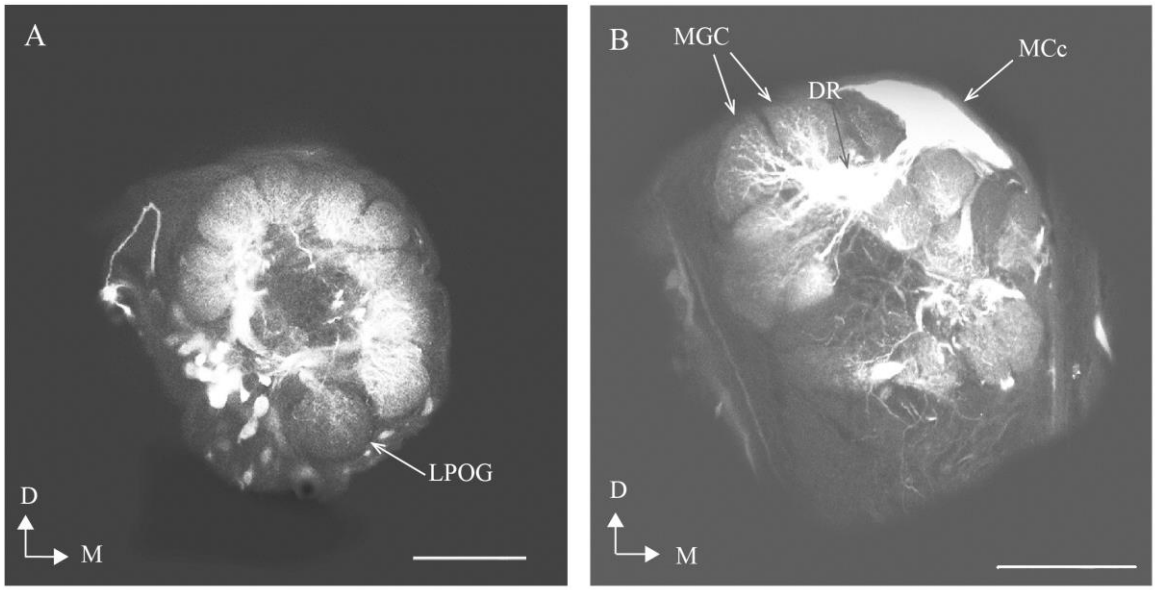


Figure 10: Confocal images showing glomerular arborization pattern of the projection neurons passing in the medial antennoprotocerbral tract (M-APT). **(A)** Frontal view of the right antennal lobe, showing a ventrally located glomerulus believed to be the labial pit organ glomerulus (LPOG). As demonstrated, the LPOG is substantially less stained than the other glomeruli. **(B)** Frontal view of the right antennal lobe, showing projection neurons forming the dorsal root (DR) of the M-APT that branches within glomeruli in the antennal lobe. The macroglomerular complex (MGC) is particularly innervated by this root. As shown, the projection neurons connected to the MGC have their somata in the medial cell cluster (MCc). Confocal images, 40x objective. D=dorsal, M=medial. Scale bar 100 µm.

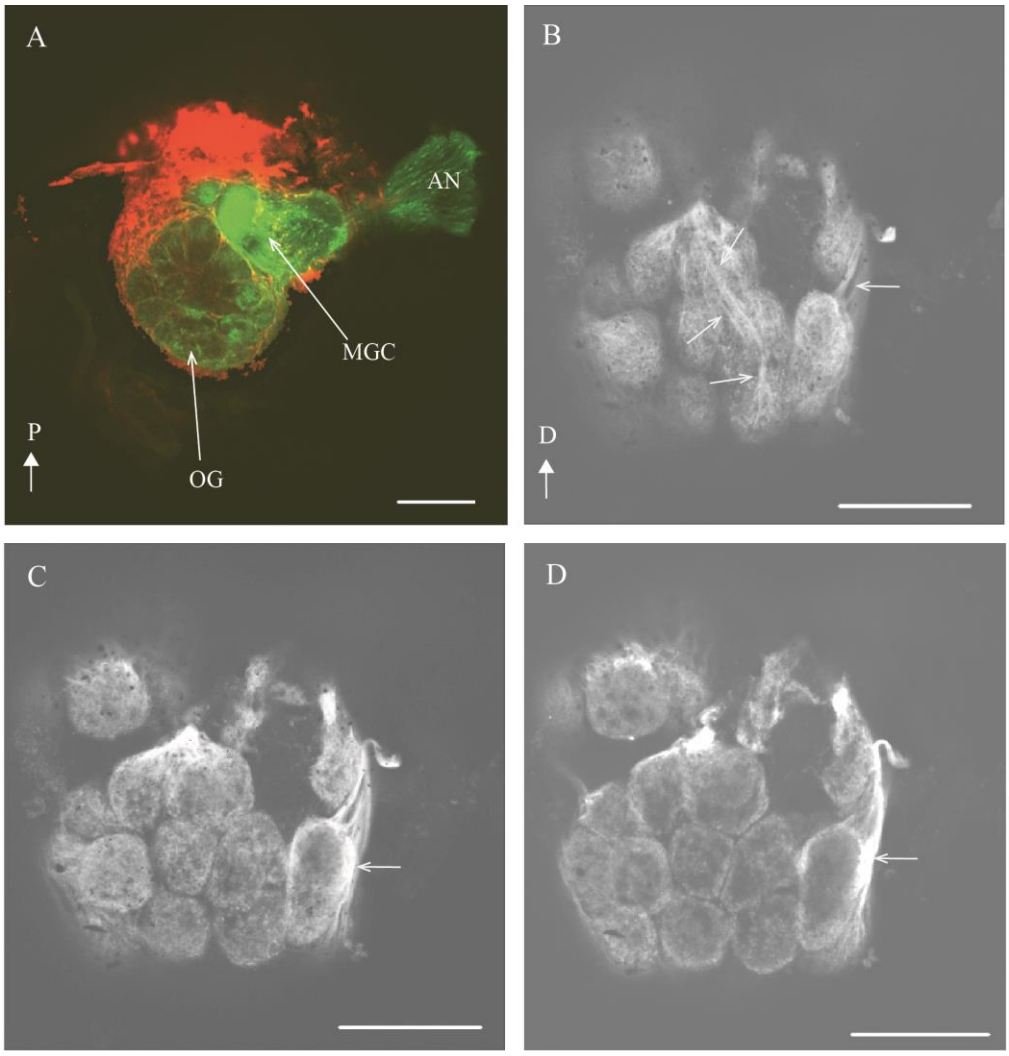


Figure 11: Confocal images showing sensory neuron innervation in the antennal lobe. **(A)** Ventral view of the right antennal lobe demonstrating sensory neuron innervation of the macro glomerular complex (MGC) and individual ordinary glomeruli (OG) from the antennal nerve (AN). **(B-D)** Serial slices (anterior-posterior progression) of the right antennal lobe in a frontal view showing sensory neurons innervating the collection of glomeruli from the periphery of the antennal lobe (arrows). Interval between each slice is 4 μm. Confocal images, 20x objective. P=posterior, D=dorsal. Scale bar 100 μm.

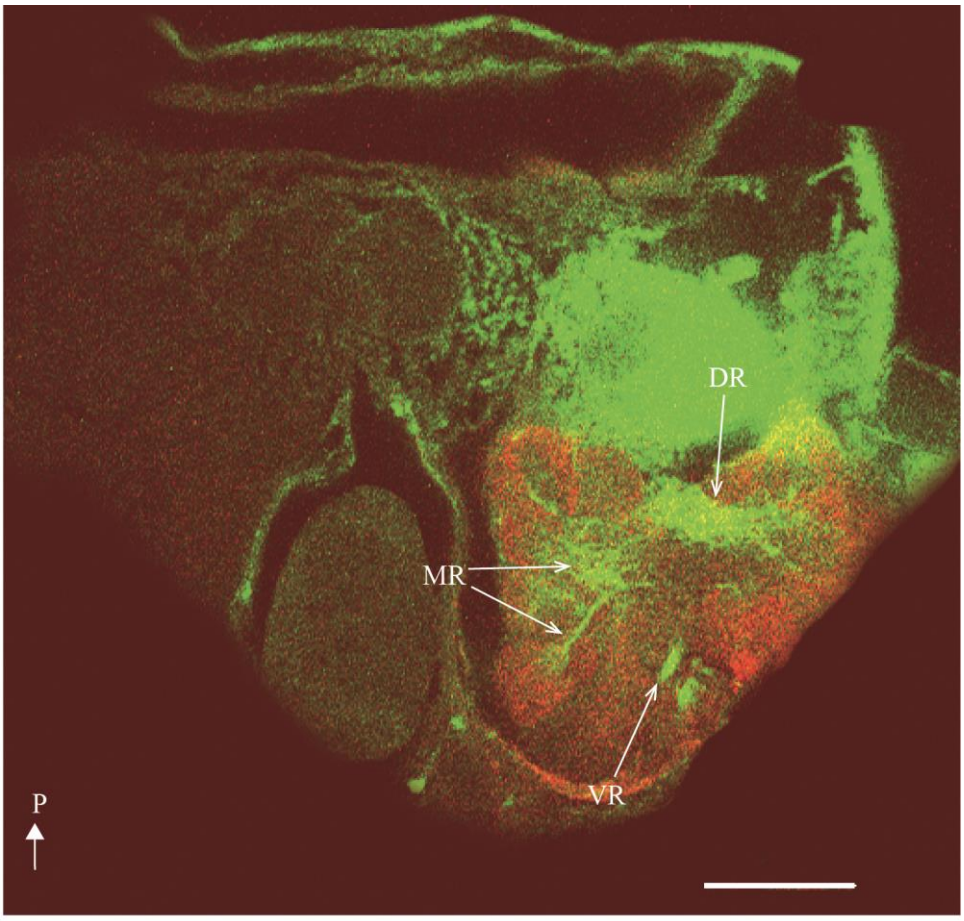


Figure 12: Confocal image demonstrating double labeling of first and second order olfactory neurons. Ventral view of the right antennal lobe showing sensory neuron innervation in the periphery (red), and projection neurons branching within glomeruli (green). Structures visualized through second order neurons include the dorsal root (DR), ventral root (VR), and the medial root (MR) of the medial antenno-protocerebral tract. Confocal scan, 20x objective. P=posterior. Scale bar 100 µm.

Discussion

The present study has morphologically described the primary olfactory center in the moth brain by the means of mass staining sensory and second order olfactory neurons. This resulted in the visualization of neural structures and pathways relevant for processing olfactory stimuli in the insect brain. The main focus has been the antennal-lobe glomeruli, the cell clusters of the second-order neurons, and the most central pathway conveying olfactory information from the primary olfactory center to the calyces, the medial antennoprotocerebral tract (M-APT). In addition to the prominent dorsal and ventral root of this particular tract, connected to somata in the medial and the lateral antennal-lobe cell cluster respectively, the medial root of the M-APT, formerly not described in heliothine moths, was identified and found to be linked to somata in the anterior cell cluster. Also, a fourth cell cluster was discovered, previously not described in any moth species. Due to its modesty, both in collective and individual somata size, it was named the parvus anterior cell cluster (PACc) from the Latin word for small; *parvus*.

Few stained fibers in the L-APT

As is evident from the present study, application of dye into the calyces resulted in a prominent staining of the M-APT. As expected, no stained axons occurred in the mediolateral antennoprotocerebral tract (ML-APT). This tract is reported to project directly to the lateral protocerebrum without innervating the calyces (Rø et al. 2006; Homberg et al. 1988). The nearly total lack of stained axons in the lateral antennoprotocerebral tract (L-APT), on the other hand, was surprising. This tract is reported to project among other regions to the lateral protocerebrum, and is from there believed to target the calyces via numerous fibers. Individual projection neurons passing through the L-APT to the calyces via the lateral protocerebrum are previously described in *H. virescens* (Rø, et al. 2006). Also, a former study on the sphinx moth, *M. sexta*, has reported that one of totally four projection neuron categories passing in the L-APT, terminates in the calyces (Homberg, et al. 1988). However, the specific part of the L-APT connecting the lateral protocerebrum with the calyces has not yet been described in its entirety. Given that a substantial portion of the axons in the lateral protocerebrum projects further to the calyces, it would seem probable to expect a fair amount of stained fibers of the L-APT when putting dye in the calyces. As the present study shows, however, only a few projections in this particular tract were visualized by the current staining

technique (Figure 9). Taking this observation alone into account, it seems as if the majority of the current L-APT projection neurons terminate in the lateral protocerebrum, and that relatively few are actually connected to the calyces. However, it should be mentioned that particular fibers might for unknown reasons have remained unstained in the present study. In order to clarify this issue, further research could be done, for instance by applying one dye in the calyces and another in the antennal lobe. By doing this, a comprehensive and thorough account of the L-APT terminal areas might be obtained.

The M-APT and its connections in the antennal lobe

With the ML-APT being out of the spotlight, and the L-APT virtually not stained in the present study, the visualization of the antennal lobe by retrograde staining from the calyces was done so to speak solely through the prominent M-APT. Hence, glomerular labeling, as shown here, demonstrates that all glomeruli are innervated by projection neurons running through this tract (with one possible exception, which will be commented on later). In general, the organization of axon bundles projecting from the primary olfactory center to higher brain areas via different tracts is highly conserved among various insect species. Most species studied seem to have a M-APT formed by uniglomerular projection neurons (reviewed by Galizia & Rössler, 2010). Along with a L-APT, this organization of tracts, assumingly processing different features of the same chemical stimuli, forms so-called parallel pathways. For instance in the honeybee *Apis mellifera*, similar tracts can be seen. However, its antennal-lobe glomeruli are segregated in axially divided hemispheres such that the L-APT innervates glomeruli in the ventral half of the antennal lobe, and the M-APT innervates glomeruli in the dorsal half. Thus, the two tracts project from the antennal lobe and terminate in the calyces with approximately equal quantities of fibers (Kirschner et al. 2006), making up a so-called dual parallel system. In sharp contrast to this organization, the present study shows that all glomeruli are innervated by the M-APT, and can thus highlight a distinct feature in second order neuron organization that distinguishes the lepidopteran moth from the hymenopterans (which includes bees).

Though the vast majority of fibers passing in the M-APT probably are stained, there is no guarantee that the entire assembly is visualized in the present study. This precaution is noted due to a few fine arborizations of the M-APT, formerly found to terminate in the superior protocerebrum, anterior to the calyces in *M. sexta* (Homberg et al. 1988). Corresponding types of projections, which may be present in *H. virescens*, will thus not be stained if dye is put in the calyces.

Minimal staining in the labial pit organ glomerulus (LPOG)

From the data presented here, there seems to be one exception to the comprehensive retrograde staining of all antennal-lobe glomeruli. Figure 10A shows that the CO_2 associated LPOG is significantly less stained than the remaining glomeruli. That is, this particular glomerulus, receiving signals from the labial palps, seems to be modestly connected to the calyces. A recently completed master study that has managed to selectively stain the output neurons originating from the LPOG found projections in the L-APT and the ML-APT (Ingrid Moe Dahl, 2013, master's thesis), however, not in the M-APT. These data, along with the present findings, thus suggest that the LPOG has no fibers innervating the calyces through the M-APT.

Antennal-lobe roots and cell clusters

As shown in the current study, the three main cell clusters of the antennal lobe appeared as connected to the M-APT via three main sub-bundles, i.e. the roots. Here, the thick dorsal root, connecting dorsally located glomeruli, seemed to be linked to the medial cell cluster. Because of the dense staining, it appears that most somata in the medial cell cluster are labeled in the current study. A significant portion of these stained somata seem to be linked to the male-specific MGC. In this respect, the comprehensive innervation of these enlarged glomeruli situated at the entrance of the antennal nerve, indicates the importance of the MGC structure. The slightly thinner ventral root and the lateral cell cluster, however, are most likely not represented in its entirety in the present study. This is because the lateral cell cluster which is previously found to be the biggest in noctuid moths, *H.virescens* included (Berg, Galizia, Brandt & Mustaparta, 2002), houses not only the somata of the projection neurons passing in the M-APT (being visualized here), but also the somata of all projection neurons passing in the ML-APT and the relatively thick L-APT, plus the somata of all local interneurons (Homberg et al. 1988). As figure 7B and 14 (appendix) shows, the lateral cell cluster covers a fairly large area, but appears to contain fewer stained somata than the medial cell cluster due to their spacious distribution.

The third antennal-lobe root of the M-APT, the medial root, visualized to a various extent in the stained preparations in the present study, seems to be sparsely described in the literature. However, in the study of the sphinx moth *M. sexta*, by Homberg et al. (1988), a bundle of 17-18 large diameter fibers that similarly join the dorsal root before they fuse with the ventral root is described. Thus, the similarity in location and size, relative to the other roots, strongly

suggests that the present data has visualized the corresponding neural bundle in *H. virescens*. The existence of this root, and that it consists of at least 6 main branches has not been previously described in the heliothine moth.

The newly discovered cell cluster, the PACc, was found to be located slightly dorsally of the anterior cell cluster and connect with a few thin fibers that exit the antennal lobe in between the medial and the dorsal root of the M-APT. However, the current image resolution did not allow visualization of exactly where the PACc connectives leave the antennal lobe. Following the thin but distinct primary neurites (figure 8B) posteriorly, they appear to exit the antennal lobe just above the dorsal most branch of the medial root, i.e. between the dorsal and the medial root (as shown in figure 8C). Though the present data cannot distinctly situate these projections within one of the prominent roots, it can with large degrees of certainty be asserted that they leave the antennal lobe within the M-APT. This is because all projections described here fuses in the M-APT when leaving the antennal lobe.

Glomerular innervation pattern

As shown in the present study, dendrites of the second order projection neurons innervated each glomerulus at its base and branched within. The visualization of glomeruli by applying dye into the antenna resulted in stained fibers covering the peripheral part of each spherical unit. This observation correlates with previous studies (Berg et al. 1998; Løfaldli et al. 2010), and was made explicit in the present study by successfully double-labeled preparations. These preparations had one dye visualizing sensory fibers encircling the dendritic branches of projection neurons (in each glomerulus) that were visualized by a second dye. An example of this is shown in figure 11. The fact that antennal projections also reach the antennal lobe in its entirety from the periphery, as shown here, verifies previous reports.

Comparative aspects

When comparing the organization of the insect brain described in the present study with that of corresponding brain areas in vertebrates, the similarities of the neural networks underlying olfaction across species becomes explicitly exemplified. In addition to the similar bipolar sensory neurons in the olfactory organ of insects and vertebrates, analogies in the second order neurons of the olfactory pathway can be drawn. Firstly, the antennal-lobe projection neurons presently described are uniglomerular, as are the mitral and tufted relay neurons of the vertebrate olfactory bulb. Secondly, in vertebrates, these neurons project through the olfactory tract and terminate in cortical regions without passing through the thalamus. The uniglomerular neurons of the moth, which are highlighted in the present study, project directly

to the calyces, a brain region that is suggested to share a common origin with the vertebrate cortex (Tomer et al, 2010). Hence, phylogenetically comparable brain areas processing olfactory input are in both systems connected to the external world via one synaptic level, i.e. the primary olfactory center. Thirdly, the terminals of each projection neuron cover a relatively large area in both vertebrates and insects. Thus, the projection neuron terminals overlap with each other in the higher olfactory centers. Another morphological aspect, similarly organized in vertebrates and most insects, is the ipsilateral arrangement of the olfactory pathway.

Methodological considerations

As seen, second order projection neurons were visualized by the means of retrograde mass staining. Also, sensory neurons and how they are organized in the antennal lobe relative to projection neurons were made explicit in the present study. Thus, the main objectives were successfully achieved. With regards to accessing the whole calycal area for injection of dye, the current procedure of decapitating the preparation turned out to be a useful method for staining the second order projection neurons. In addition, dye application to the cut off bundle of sensory axons making up the antennal nerve, also successfully visualized the antennal-lobe glomeruli as desired. Of the two dyes used, both were capable of staining brain structures in great detail. However, analyzing data with structures visualized by the dye alexa 488 may include a certain disadvantage. When depicting a specimen in a confocal microscope, even if not dyed, the moth brain emits light due to a naturally occurring autofluoresence. When using a dye that overlaps with the emission maximum of the autofluoresence, stained structures have a tendency to blend in with the unstained structures of the brain due to the strong background staining. Thus, of the dyes used in the present study, alexa 488 seems to be the dye most associated with the problem of autofluoresence when interpreting data. For instance, as can be seen from the subesophageal ganglion (SOG) projections, as visualized by injection of alexa 488 into the antenna, in figure 13 (appendix), the staining in this area barely stands out from the contralateral equivalent. The second order neurons however, as visualized by injection of micro-ruby into the calyces, clearly differ from the background. This is because micro-ruby does not overlap, as much as alexa 488, with the emission maximum from that of the auto fluorescent brain. Said otherwise, micro-ruby has the best signal to noise ratio.

Though a substantial amount of projection neurons were visualized from injecting dye into the calyces, the current method does not ensure labeling of all antennal-lobe projection neurons coupled to this structure. It might for various reasons be that neuron categories are

“overlooked” by this procedure. That is, they could for instance be terminating in particular regions of the calyces that were not reached by the current stainings. Having said that, most identified antennal-lobe projection neurons sending projections into the calyces are reported to innervate the whole cup-shaped structure, and precautions regarding this were taken by applying dye crystals into several regions of the neuropil. The mass staining of antennal-lobe projection neurons showed a significantly better quality of the labeling than mass staining of the sensory projections obtained by applying dye into the antenna. Besides the fact that the very nature of these two types of neurons differ from one another, a specific reason for this might be that the very thin sensory axons makes it difficult for the dye to be absorbed into the individual neurons.

Because two dyes were used simultaneously in each specimen during the double-labeling experiments in the present study, the dilemma of which dye to use for what purpose was faced. After trying the two dyes micro-ruby and alexa 488 interchangeably, advice was given by more experienced researchers that micro-ruby was appropriate for staining the sensory neurons. As it turns out, the best stained brain was actually obtained by visualizing projection neurons from the calyces with micro-ruby, and alexa 488 for staining sensory axons innervating glomeruli in the antennal lobe. Because the two ways of staining both gave valuable data, it is on the basis of the present data not possible to say which method is preferable (or why). Though the present study did not focus on this particular aspect of the mass staining, an approach applying different dyes systematically, could conceivably reach further to a conclusion regarding preferable use of dyes.

Overall, the present data provided the means of thoroughly describing organization in the antennal lobe and, not to disregard shortcomings, unforeseen limitations seem mainly to apply to details on a highly specific level. For instance, the very thin PACc primary neurites would need to be visualized in greater detail in order to determine with certainty which root they belong to. Similarly, a finer resolution or a better staining giving more detailed data concerning the projections in the medial root would likely reveal a more accurate number of fibers making up each of the 6 sub-branches described here. Just like the dorsal and the ventral root consists of several densely intermingled finer branches connected to the two bigger cell clusters, the number of fibers in the medial root is likely to correspond to the number of somata in the anterior cell cluster given that all somata stained here connects to this root. As shown in figure 8A and 10B, the strongly stained and densely populated medial and anterior cell cluster makes estimating the number of fibers in the roots, by counting of somata,

not possible. Though the stained somata in the lateral cell cluster are not as densely packed, only a very rough estimate of somata in this cell cluster, and thus the labeled fibers in the ventral root, would be obtainable here. Similarly, determining the amount of fibers stained by studying a particular root itself is not accurately possible from the present data. It seems that more detailed information than that obtained via the mass-labelings performed in the present study, would need to be acquired through more specific staining techniques. In addition, more detailed data depicted with a more magnifying objective than those used here, could improve the image data and gain further information.

Conclusion

- The present data confirms previous findings showing that the antennal lobe has a direct connection to the calyces via the prominent M-APT.
- The results indicate that relatively few output neurons of the antennal lobe project to the calyces via the L-APT.
- Innervation by the dendrites of the fibers in the M-APT seems to be equally prominent in all antennal-lobe glomeruli, with the exception of one large glomerulus situated ventrally, assumingly the LPOG, which is significantly less innervated.
- The data shows a new cell cluster having unusually small somata. The group of cell-bodies, which is located in the anterior part of the antennal lobe, is named the parvus anterior cell cluster.
- The M-APT originates in three main roots in the antennal lobe: the dorsal, the ventral, and the medial root. The medial root subdivides in 6 branches.
- The dorsal root seems to be connected to the medial cell cluster and adjacent glomeruli, particularly the MGC, whereas the ventral root connects to the lateral cell cluster and laterally located glomeruli. The smaller medial root connects with the anterior cell cluster and ventromedially located glomeruli.
- The M-APT seems to be connected with all somata in the medial and the anterior cell cluster, while only connected to a portion of the total amount of somata in the lateral cell cluster. This indicates that projection neurons connected to the L-APT and the ML-APT has somata in the lateral cell cluster only.
- Double-labeling experiments, where projection neurons and sensory neurons were stained in one and the same preparation, showed that the sensory neuron fibers innervate the antennal lobe in a peripheral manner. They also capsule the second order dendrites within the individual glomeruli.

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Appendix

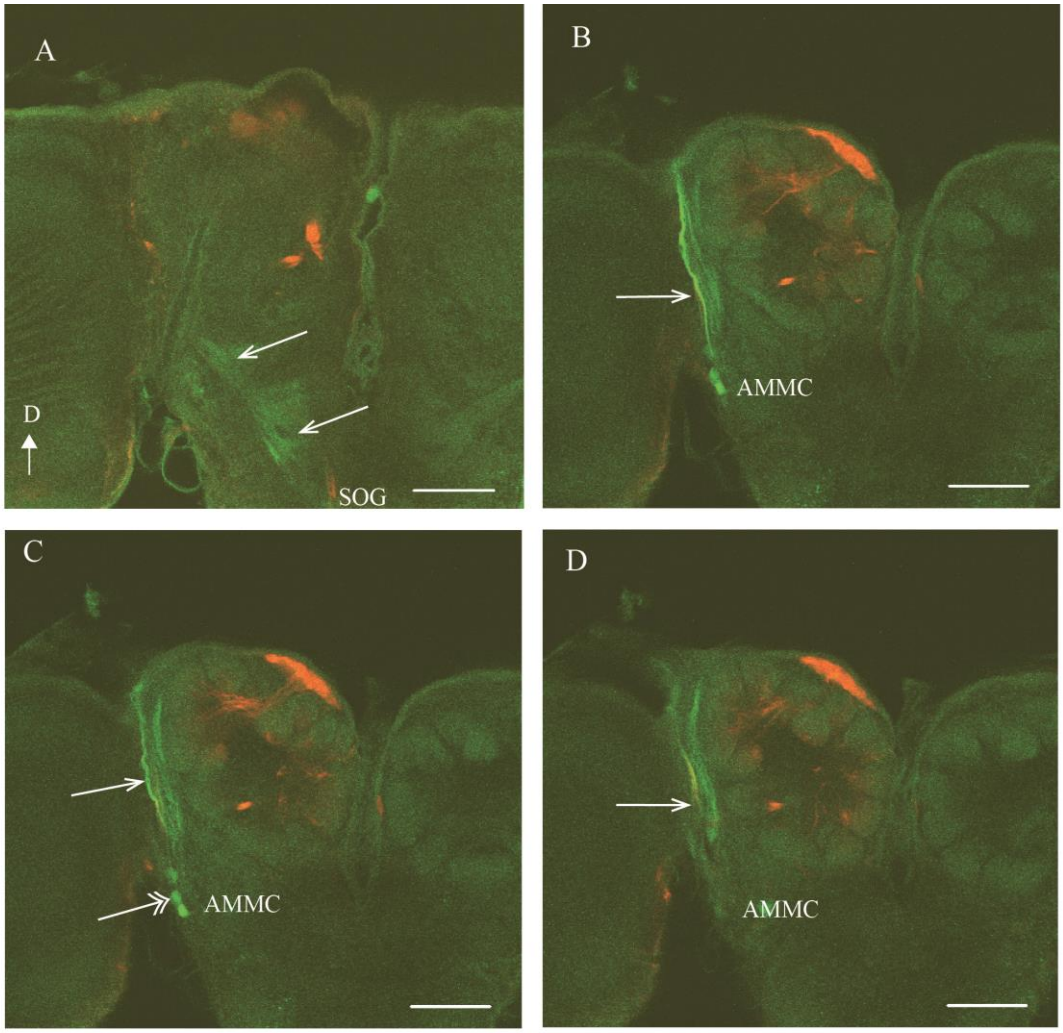

Figure 13: Serial slices of confocal images in a frontal view in a posterior-anterior progression showing particular projections from the antenna in green. **(A)** Antennal projections terminating in the lateral and medial part of the subesophageal ganglion (SOG). **(B-D)** Antennal projections terminating in three big somata (double-pointed arrow) positioned ventrally, close to the antennal mechanosensory and motor center (AMMC). Confocal scan, 20x objective. D=dorsal. Scale bar 100 µm.

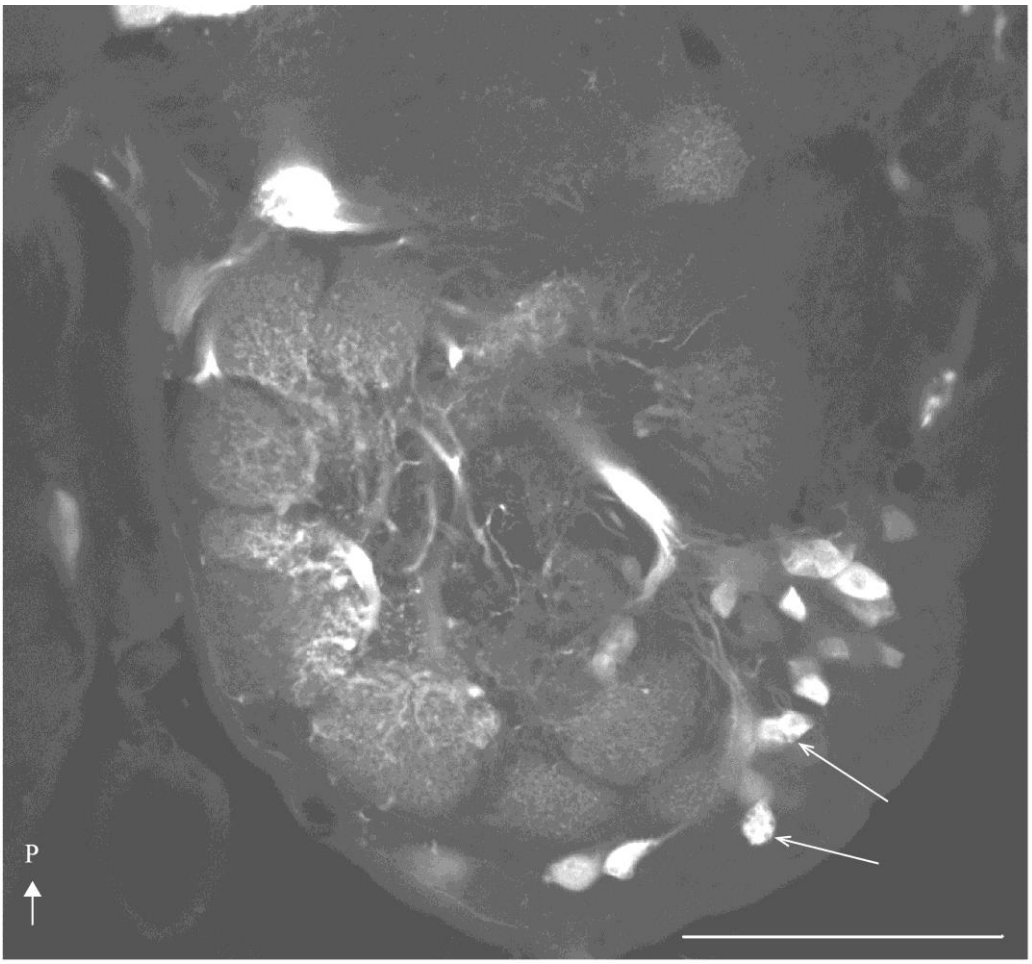

Figure 14: Confocal image of the right antennal lobe in a ventral view. The arrows point to somata containing circular structures surrounding the cell nucleus. Confocal scan, 40x objective. P=posterior. Scale bar 100 µm.

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