Mapping projection neurons originating from male-specific versus ordinary antennal lobe glomeruli in the central olfactory pathway of the moth *Heliothis virescens*

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Content

Sammendrag

Fylogenetisk sett, er de kjemiske sansene de eldste, og alle organismer har utviklet en evne til å detektere kjemisk stimuli fra det eksterne miljøet. Insekter har lange antenner med sensoriske luktenevroner som kan detektere flyktige odoranter fra det omkringliggende miljøet – både feromoner og planteduft er. Lukt-informasjonen blir omformet til nerveimpulser som sendes til det primære luktsenter i insekthjernen, antenneloben. Seksualresponsen til hann-møll er direkte knyttet til luktesansen; å kunne følge duft-sporet sendt ut av en mottakelig hunn-møll, er en forutsetning for hannens reproduktive suksess. De to segregerte, men parallelle luktebanene som man finner i hann-møllens luktesystem, ett for feromoner og ett for planteduft er, gjør nettopp denne skapningen til et velegnet modellobjekt for den som søker å forstå luktesystemets kodingsmekanismer. Ved å sette inn én type fargestoff (dextran tetramethylrodamine/biotin) i det området av antenneloben som er ansvarlig for å prosessere feromoninformasjon og et annet fargestoff (dextran fluorescein/biotin eller Alexa 488) i det området av antenneloben som er ansvarlig for å prosessere planteduftinformasjon, ble de antenno-protocerebrale trakter knyttet til de to systemene visualisert i dette prosjektet. De antenno-protocerebrale traktene består av projeksjonsnevroner som forbinder antenneloben med høyere integrasjonsområder, heriblant mushroom body calyces og det laterale protocerebrum. Resultatene viste fargede projeksjonsnevroner knyttet til både feromon- og planteduftsystemet i alle de tre hovedtraktene. De to nevrontypene viste til dels ulike projiseringsmønstre både i mushroom body calyces og i det laterale protocerebrum. I tillegg til de tre hovedtraktene som er beskrevet før, ble en ny antenno-protocerebral trakt funnet, den såkalte *2nd medio-lateral antennoprotocerebral tract*.

Abstract

Phylogenetically, the chemical senses are considered to be the oldest and all organisms have developed a system for detecting chemical molecules from the external environment. Insects possess long antennae which are covered with olfactory receptor neurons (ORNs) capable of detecting volatile odors from the surroundings environment – both pheromones and plant odors alike. The olfactory information is transduced to nerve impulses that are carried to the primary olfactory center in the insect brain, the antennal lobe. The sexual response of the male moth is directly linked to the olfactory sense; being able to detect the pheromone trail emitted by a calling female, is a prerequisite for reproductive success of the male. The two segregated but parallel olfactory pathways which are found in the male moth, dedicated to pheromones and plant odors, respectively, makes this organism an excellent mini-model for studying the olfactory system. By inserting one dye (dextran tetramethylrodamine/biotin) into the antennal lobe area that is responsible for processing pheromone information and another dye (dextran fluorescein/biotin or Alexa 488) into the area processing plant odor signals, the antenno-protocerebral tracts linked to the two odor systems were visualized in the current project. The antenno-protocerebral tracts connect the antennal lobe to higher integration areas, particularly to the mushroom body calyces and the lateral protocerebrum. The results demonstrate that projection neurons linked to the two olfactory sub-systems, dedicated to pheromones and plant odors, respectively, are present in all the three main tracts. The antennal lobe output neurons tuned to pheromones showed a partly different projection pattern from that displayed by plant odor neuron, both in the calyces and in the lateral protocerebrum. In addition to the three main antenno-protocerebral tracts previously described, a new tract, not formerly found, was discovered – the so-called *2nd medio-lateral antenno-protocerebral tract*.

Introduction

The sense of smell is used for many different purposes. For humans it plays a key role in our experience of food by in many ways being even more important than activation of the taste system; to taste food without access to our olfactory system, as anyone who has experienced to have a cold would know, is a rather boring sensation. Actually, our ability to appreciate *flavor* is one of several characteristics that seems to set us apart from other creatures in nature (Shepherd, 2006). On the other side, there are odorous molecules that we as humans may not be able to respond to. The world of pheromones acting as signals linked to stereotyped sexual responses, is to most researchers believed to be of minimal importance to humans, but extremely important to many insects – exemplified by the upwind flight of an excited male moth tracing the pheromone trail emitted by a female of the same species. For these small creatures, finding a suitable mate by means of olfaction is essential in order to be able to reproduce and thereby secure the survival of the specie. Because of the multitude of the chemosensory detecting ability, which is actually present in all living organisms, it is inspiring that this particular field of neuroscience has experienced a renewed wave of interest over the past decades. One main reason for this may be attributed to the work done by Buck & Axel (1991) identifying the large super family of olfactory receptor genes in mammals. By unlocking basic principles on how olfactory stimuli are detected, they enabled further investigations comprising neural mechanisms linked to processing of odor information. Research on the olfactory system, which not only detects, but also categorizes, identifies and interprets airborne molecules, has also shown that there exist remarkable similarities across different species and that olfactory encoding mechanisms seem to share much of the same logic (Bear, Connors & Paradiso, 2007; Kandel, Schwartz, Jessel, Siegelbaum & Hudspeth, 2013). Because insects and other invertebrates are highly dependent on their sense of smell and rely heavily upon the different olfactory cues provided by the environment they live in, they are favorable model objects for investigations dealing with the olfactory pathways. Besides, their relatively simple and easily accessible nervous system makes these organisms well suited for experimental research in general.

The invertebrate olfactory system

The antennal sensilla and olfactory receptor neurons

The biological information embedded in the odorous molecules sensed by an insect, spans from olfactory cues relevant in mating behavior to odorants emitted by food sources

and oviposition sites. All of these various odorants are detected by functionally different olfactory receptor neurons (ORNs) on the insect antenna. The ORNs are small bipolar neurons that are located in structures termed sensilla (Fig. 1) containing two or more ORNs. The dendritic branch extends inside the sensillum and carries the olfactory receptors. These are different from the G-protein coupled receptors identified in vertebrates, however. Thus, the insect olfactory receptors are heterodimers, constituted of two G-protein coupled receptors (Sato et al., 2008; Wicher et al., 2008). The dendrite is exposed to molecules from the environment through several pores in the sensillum wall. Inside, in the sensillar lymph, numerous odorant binding proteins (OBPs) facilitate the processes of transporting the odor molecules to the receptor (Stengl, Ziegelberger, Boekhoff & Krieger, 1999). The binding of relevant odor molecules, so-called ligands, to the appropriate receptor initiates the transduction process which eventually leads to the generation of action potentials travelling along the axon of the neuron. This axon, being the second branch of the bipolar receptor neuron, projects to the antennal lobe, the primary olfactory center of the brain (Fig. 1). Together, the axons from the ORNs on the antenna form the antennal nerve.

The antennal lobe

The antennal lobe is the first relay station for olfactory information processing in the insect brain. As the long axonal end of the ORNs enter the antennal lobe, they pass through a region termed the sorting zone where they are re-organized before they continue onto small sphere-like structures termed glomeruli. The glomeruli are functional structures, enabling axons from ORNs and dendrites from second-order neurons to form synapses. The number of glomeruli found in the antennal lobe of insects is both species- and gender specific. Because some insects, as for example noctuid moths, rely on pheromone cues in mating behavior, it is often common for males to possess a particular set of glomeruli, the macroglomerular complex (MGC), particularly dedicated to processing of pheromone information (Anton & Homberg, 1999). The MGC, which is situated close to the entrance of the antennal nerve, varies in terms of number of glomeruli. However, it often has one large glomerulus surrounded by two or three smaller units. In the heliothine moth, *Heliothis virescens*, the MGC is made up by four units (Berg, Almaas, Bjaalie & Mustaparta, 1998; Vickers, Christensen & Hildebrand, 1998). The largest, the so-called cumulus, receives input from ORNs tuned to the major pheromone component produced by the female, *cis*-11-hexadecenal (Z11-16:AL). The second largest unit, the dorso-medial glomerulus, receives information about the second component, *cis*-9-tetradecenal (Z9-14:AL). In addition to the two mentioned

units that are tuned to pheromones, two smaller glomeruli located ventrally of the cumulus receive input about substances produced by sympatric females from other related species (Hansson, Almaas & Anton 1995; Berg et al., 1998; Vickers et al. 1998). Thus, each of the four MGC glomeruli is a functional unit receiving information about one particular odor stimulus. Besides, the arrangement of distinct glomeruli being tuned to chemical signals from conspecific females (pheromone components) versus signals from females of other species (interspecific signals), is linked to different behavioral responses in the male. The pheromone blend elicits sexual interest including an intention to locate the female by flying up against the wind in the pheromone plume in a characteristic zig-zag pattern (Willis & Baker, 1984). When components from the pheromone blend of other females, i.e. interspecific signals, hit the male antenna, however, this triggers an opposite behavioral response implying avoidance of the "wrong" female (Vetter & Baker, 1983; Vickers & Baker, 1998). In addition to the MGC glomeruli, the antennal lobe houses numerous other glomeruli tuned to plant odors. These are present both in males and females. In the *H. virescens*, there are 62 so-called ordinary glomeruli in the antennal lobe (Berg, Galizia, Brandt & Mustaparta, 2002; Løfaldli, Kvello & Mustaparta, 2010).

In addition to the input neurons as mentioned above, the glomeruli are formed by the branches of numerous projection neurons (PN) and local interneurons (LN). The PNs are second-order neurons carrying the olfactory information from the primary olfactory center to the secondary olfactory centers in the protocerebrum in the insect brain. There are both male-specific PNs projecting from the MGC and PNs from ordinary glomeruli terminating in areas in the protocerebrum. In most insect species studied so far, moths included, the majority of the PNs are of a uniglomerular type, i.e. they innervate only one glomerulus (Anton & Hombeg, 1999). Some of the PNs are however of a multiglomerular type, meaning that they innervate many, if not all of the glomeruli in the antennal lobe. The second main category of antennal lobe neurons, the LNs, is restricted to the antennal lobe. Most of these are multiglomerular neurons using the inhibitory neurotransmitter GABA; in the antennal lobe of *H. virescens*, for example, there are approximately 330 GABAergic LNs (Berg, Schachtner & Homberg, 2009).

The cell bodies of all antennal lobe neurons are gathered in three different cell clusters enveloping the glomeruli. The lateral cell cluster (LCCl), which is the largest, is located laterally in the antennal lobe. The somewhat smaller medial cell cluster (MC) is positioned

dorsomedially, whereas the tiny anterior cell cluster (AC) is located most anteriorly in the antennal lobe. All PNs have their somata located in one of these three different cell clusters – however, the MC is reported to contain the majority of cell bodies belonging to PNs associated with the MGC (Homberg, Montague & Hildebrand, 1988). The cell bodies belonging to the LNs are located in the LCCl (Homberg et al., 1988). In addition to being linked to the three neuron categories mentioned above, i.e. the sensory neurons, the projection neurons, and the local interneurons, the antennal lobe glomeruli also receive modulatory feedback from higher processing areas via the centrifugal neurons. These neurons usually have their cell bodies located outside the antennal lobe.

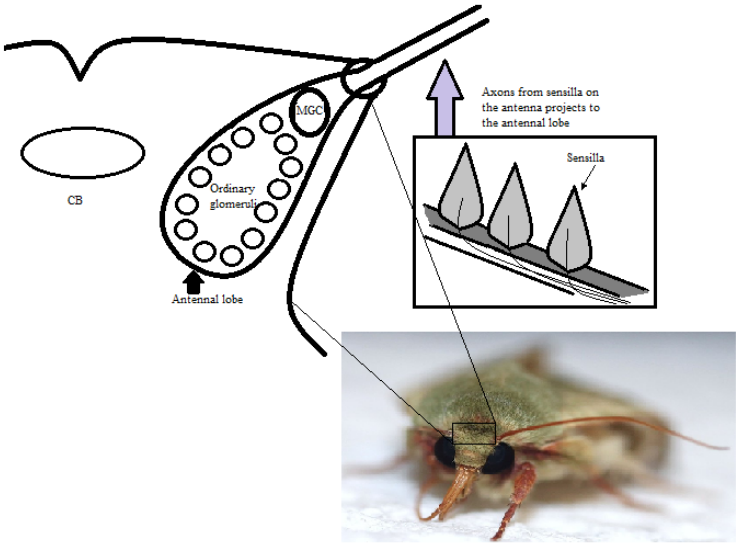


Fig. 1: Schematic overview of the antennal sensilla housing the olfactory receptor neurons. The target regions of the sensory afferents are the glomeruli in the primary olfactory center, i.e. the antennal lobe. MGC: macroglomerular complex, CB: central body.

The antenno-protocerebral tracts

The antennal-lobe projection neurons carry the olfactory information to higher integration centers in the protocerebrum. In moths the projection neuron axons make up three main antenno-protocerebral tracts (APTs) – the medial APT (m-APT), the medio-lateral APT (ml-APT) and the lateral APT (l-APT; Homberg et al., 1988; Rø, Müller & Mustaparta, 2007). This nomenclature, which will be used in the current manuscript, was suggested by Galizia & Rössler (2010). The APTs exit the antennal lobe and project to different parts of the protocerebrum; most notably the mushroom body calyces and the lateral protocerebrum (Fig. 2). The most prominent tract is the m-APT which leaves the antennal lobe dorso-medially and projects posteriorly, bypassing the central body ventrally before turning laterally, running

along the anterior edge of the calyces and sending collaterals into this structure before terminating in the lateral protocerebrum. The m-APT contains axons from primarily uniglomerular PNs (Rø et al., 2007; Homberg et al., 1988). The ml-APT, on the other hand, is reported to contain mainly multiglomerular PNs. This relatively thin fiber bundle follows the m-APT for a short distance before it turns laterally and projects directly to the lateral protocerebrum, without targeting the calyces (Homberg et al. 1988; Rø et al., 2007). The third tract, the l-APT, projects out of the antennal lobe medio-ventrally before turning dorso-laterally into the protocerebrum, terminating in the lateral protocerebrum. Some of the PNs passing in the l-APT also send terminals into the calyces as well as additional protocerebral regions. The l-APT contains both uniglomerular and multiglomerular PNs (Rø et al., 2007; Homberg et al. 1988).

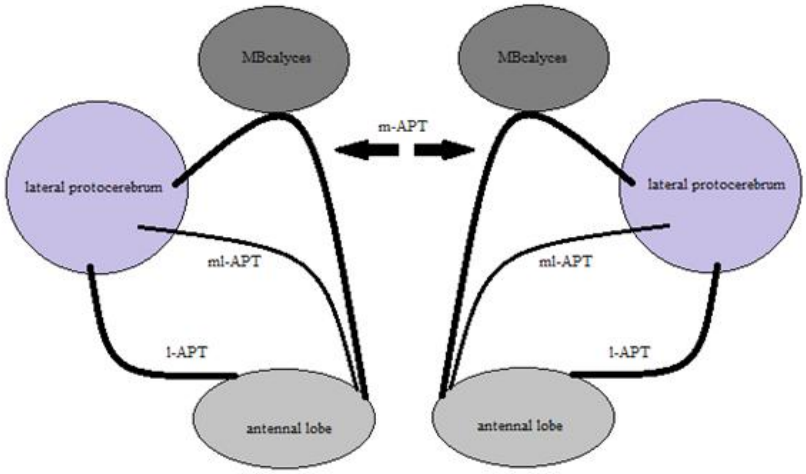


Fig. 2: A schematic overview over the three main antennoprotocerebral tracts and their target areas in the moth brain. M-APT: medial antenna-protocerebral tract, ml-APT: medio-lateral antenna-protocerebral tract, l-APT: lateral antenna-protocerebral tract.

As mentioned above, the antennal lobe contains two main categories of glomeruli linked to pheromones and to plant odors, respectively. It is well known that pheromone and plant odor information, which to a large extent is kept separate also in the antennal-lobe output neurons, is represented in all three main APTs; thus, male-specific PNs in the sphinx moth, *Manduca sexta*, and the silk-moth, *Bombyx mori*, for example, are found in all three tracts connecting the antennal lobe to the protocerebrum, alongside PNs originating from ordinary glomeruli (Homberg et al., 1988; Kanzaki, Soo, Seki & Wada, 2003).

The mushroom body calyces

The mushroom body calyces (MBcalyces) are an outstanding structure of the insect brain located most posteriorly in the protocerebrum. It is identified in all arthropod groups except crustaceans and found in the simplest worms to the most complex social insects (Strausfeld, Hansen, Li, Gomez & Ito, 1998). Structural mutations of the calyces or chemical ablation of this structure have shown to be correlated to defects in olfactory conditioning (reviewed in Heisenberg, 2003). This has led to the suggestion that the calyces are important structures in memory and learning (Strausfeld et al., 1998; Menzel & Müller, 1996). The mushroom body structure is shaped as a stalk (*peduncle*) with cup-shaped protuberances (*calyces*) at the dorsocaudal end and with a lobe-system surrounding the stalk (Heisenberg, 2003). Structurally, the cup-shaped calyx is in some species formed as a single cup as opposed to other species where it is formed as a pair of two cups. A third structural variation is found in moths and flies, where the two cups are partially fused medially (Strausfeld et al., 1998). The mushroom body structure is formed by numerous Kenyon cells (KC) having their cell bodies densely packed around the calyces. The KC dendrites form the calycal structure whereas long and thin axons constitute the peduncle and lobes (Strausfeld et al., 1998; Heisenberg, 2003). Axon collaterals from antennal-lobe PNs passing in the m-APT form one of the main inputs to the KC dendrites in the calyces (Heisenberg, 2003).

The lateral protocerebrum

The lateral protocerebrum is an area of the insect brain which has been described as a possible pre-motoric area (De Belle & Kanzaki, 1999). However, mass-staining experiments performed in *H. virescens* have shown that ventral cord fibers connect only to the ventral area of the lateral protocerebrum (Siri Børø, Master Thesis, 2012). Interestingly, Løfaldli, Kvello, Kirkerud & Mustaparta (2012) have recently described a plant-odor responding neuron in the same species, which projected from the ventrolateral protocerebrum to the ventral cord. In the fruit fly, *Drosophila melanogaster,* a complete pheromone input-output circuit has actually been described (Ruta et al., 2010). Here, third-order neurons in a more dorsally located part of the lateral protocerebrum connect to descending neurons in a particular region of the lateral accessory lobe (LAL), termed the lateral triangle. Also in the silk-moth, *B. mori*, descending pheromone responding neurons are reported to project from the LAL to the ventral cord (Kanzaki, Argas & Hildebrand, 1991). It is however specified that it has not been observed any neurons mediating a direct synaptic contact between the antennal lobe PNs and these descending neurons in the ventro-medial protocerebrum. In general, it is widely accepted that

the lateral protocerebrum as a whole is a region for integration of signals from several sensory modalities. Due to the lack of distinct structures within this area, the knowledge about which purposes it fulfills is still relatively sparse.

The olfactory system in vertebrates

The olfactory epithelium and olfactory receptor neurons

Except for the external structure of the receptor organ, a number of striking similarities have been found in the olfactory pathway of vertebrates and invertebrates, humans and insects included. The mammalian ORNs are also small bipolar neurons making a direct connection between the external world and the brain. The sensory neurons are situated in an epithelium located in the dorsal portion of each nasal cavity (Fig. 3). In humans, this area is about 5 cm^2 in size and it houses around 6 million ORNs in addition to supporting and basal cells (Rosenzweig, Breedlove & Watson, 2005; Bear et al., 2007). Each ORN has an apical dendrite ending up in a knob-like structure which extends 5 – 20 *cilia* into a mucus layer covering the external part of the epithelium tissue. The membrane of these cilia is the site of the receptors detecting the odor molecules. The gene family encoding the odor receptors, which is the largest identified in mammals, were first identified in rats (Buck & Axel, 1991). The detectors are G-protein coupled 7-transmembrane alpha helix receptors with a second messenger system. Odorant binding proteins (OBPs) transporting odor molecules to the receptors on the cilia are also found in the mucus. The various types of receptors have the ability to bind distinct odorants and thereby initiate the transduction process which involves the second messenger system and finally generation of action potentials. The information is conveyed to the brain via the second branch of the bipolar receptor neurons, i.e. the axon. The numerous unmyelinated sensory axons form the olfactory nerve – the cranial nerve I, which projects through a perforated part of the skull, the cribiform plate, before entering the primary olfactory center of the brain, the olfactory bulb (Fig. 3).

The olfactory bulb

In the olfactory bulb, the axons of the ORNs terminate in small glomeruli, similarly to the arrangement in insects. The olfactory bulb in mice is populated by approximately 1800-2000 glomeruli, whereas there are estimated that humans have fewer. Each glomerulus receives input from thousands of ORNs converging upon around 20-50 relay neurons carrying the odor signals to higher integration regions of the brain (Bear et al., 2007; Rosenzweig et al., 2005). The second order neurons responsible for conveying the olfactory information out

of the olfactory bulb are termed mitral and tufted cells and together they form the olfactory tract (Fig. 3). Together with local interneurons, as periglomerular and granule cells, they are responsible for processing the odor information at this particular level of the olfactory pathway (Kandel et al., 2000). How the brain recognizes a particular odor and is capable of categorizing it, has been a subject of different theories. It is generally assumed that that one odor receptor gene is expressed only in one neuron (Buck & Axel, 1991). Furthermore, it is shown that all neurons expressing the same receptor type project to one glomerulus, or a few, in the bulb. As an odor usually consists of several different chemical compounds, these different molecules activate particular receptors which in turn activate specific glomeruli in the bulb. A distinct chemotopical pattern is then created in the bulb, allowing olfactory information to emerge as a map of activated glomeruli (Axel, 1995). This information is then transmitted via output neurons to primary and secondary olfactory centers in the cortex where it is interpreted. The recognition of an odor is therefore based on activation of certain glomerular combinations. This may partly explain why the scent of jasmine and freshly baked bread smells differently. This chemotopical organization of the primary olfactory center is also another similarity identified between different species belonging to vertebrates and invertebrates, respectively (Kandel et al., 2013).

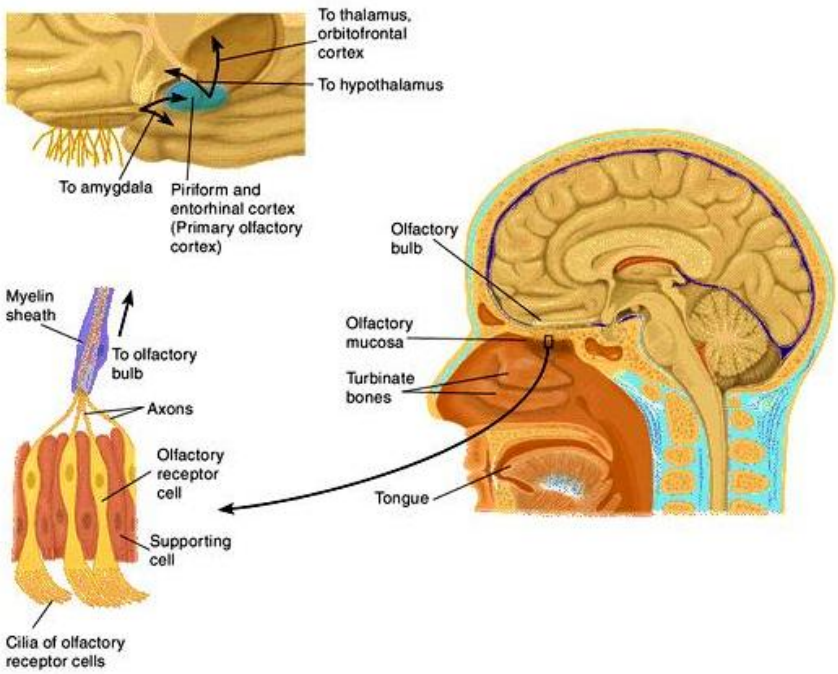


Fig. 3: The mammalian olfactory pathway

The primary olfactory cortex

From the olfactory bulb, the output neurons project directly, via the olfactory tract, to the so-called olfactory cortex. This is a phylogenetically old region of the temporal lobe that partly overlaps with the limbic system (Kandel et al., 2013). The primary component of the regions innervated by input from the olfactory bulb is the piriform cortex (Shepherd, 2007). Interestingly, in mammals all sensory systems but the sense of smell is directed to the thalamus before projecting to the primary cortical region (Kandel et al., 2013). Thus, the fact that the olfactory information bypasses the thalamus before targeting the primary olfactory cortex makes the higher order region dedicated to processing odor information unusually closely linked to the external world. However, the thalamus does play a role in the olfactory processing. Neocortical areas such as the secondary olfactory cortex in the frontal lobe is connected to the temporal lobe region by neural fibers, some of which project through the thalamus (Shepherd, 2007).

Aims of this master thesis

Whereas the neural mechanisms residing at the peripheral and first synaptic level of the olfactory pathway have been relatively thoroughly studied, both in vertebrates and invertebrates, the principles for information processing at the subsequent synaptic levels of the olfactory pathway are still relatively unexplored. The arrangement of the male moth, including antennal lobe output pathways carrying pheromone and plant odor information in distinct neuron types, is ideally suited for investigating how the two categories of odor information are represented in the higher integration centers of the brain. Therefore, the current master project, which deals with projection pattern of second order neurons of the olfactory pathway, has used the moth, *H. virescens*, as a model object.

Main objective

- To understand general processing principles residing within higher integration centers of the brain by investigating the projection pattern of pheromone-specific versus plant-odor specific second-order neurons in the olfactory pathway of the moth *H. virescens*.

Specific objectives

1. To selectively stain male-specific versus plant-odor projection neurons by applying two different fluorescent dyes into the antennal lobe, one in the MGC region and one in the region including the ordinary glomeruli.
2. To explore the projection pattern of the two neuron categories according to the main antenno-protocerebral tracts.
3. To map the target regions of the two neuron categories in the higher brain centers.

Materials and methods

Insects

The insects (*Heliothis virescens*; Lepidoptera; Noctuidae) used in the experiments originated from a lab culture in Basel, Switzerland (Syngenta). Immediately after arrival the insect pupae were sorted by gender, transferred to hatching cages (18 x 12 x 17 cm) and kept separated in two heating cabinets (Refitherem 6E incubator, Struers) at 22-24 °C. The cabinets had a light-dark cycle of 14 – 10h and a humidity of 70 %. After hatching, the insects were transferred to cylinders of plexiglass (18 x 10 cm) and kept in this environment until they were used in experiments, usually 2 – 5 days old.

Ethics

The Norwegian Law concerning animals used in research (Dyrevernloven) includes all vertebrates such as mammals, birds, reptiles, amphibians, and fish, but only invertebrates as decapods, squid, and honey bees (www.lovdata.no). Since the Lepidoptera is not included, there are no restraints regarding the use of this particular order in research. However, the insects in our lab were treated with care; they were regularly fed a mixture of sucrose and water, had paper sheets to climb on, and was housed in a clean environment. In addition, each plexiglass cylinder contained a maximum of 8 moths in order to avoid unnecessary space-related stress for the insects.

Fluorescent dyes

Three fluorescent dyes were used in these experiments, all provided by Life Technologies (www.lifetechnologies.com), MolecularProbes®: 1) Dextran tetramethylrodamine/biotin (3000 MW; micro-Ruby; ex/em: 490/508 nm), 2) Dextran fluorescein/biotin (3000 MW; micro-Emerald; ex/em: 550/570 nm), and 3) Alexa Fluor488 (10 000 MW; ex/em: 495/519 nm). All dyes were stored at -20 °C in a crystallized form. Before being used in experiments, the dyes were kept in the bottles at room-temperature for some minutes in order to prevent the crystals from condensing.

Preparation

A total of 104 male moths were used. The insect was inserted into a plastic tube with utility wax surrounding one end (Kerr Coperation, Romulus, MI, USA). When the head of the moth protruded above the wax, the insect was fastened so it could not escape. The preparation

was then situated under a stereomicroscope (Leica; MZ 12.5) and the head was further fastened by wax. The insect was anesthetized ½ hour by cold, before the experiment started. In order to expose the brain, the cuticula between the eyes was removed with a small knife made of a razor blade in a holder. Then, the muscles and some of the trachea surrounding the brain were removed using forceps. The neural tissue was kept alive during the preparation procedure by continuously applying Ringer's solution with sucrose (NaCl: 150mM, KCl: 3mM, TES buffer: 10mM, CaCl2: 3mM, and 25 mM sucrose) to the brain.

Staining

Application of dye onto specific areas of the insect brain was performed in two different ways. The first approach implied manual handling of the dye via a micro needle (Fine Science Tool) attached to a particular holder. Immediately before applying the crystals into the neural tissue, the brain was temporarily dried off using medical wipes (Kimberly Clark Professional™). Different dyes were used for the two antennal-lobe regions that were stained; tetramethylrodamine (micro-ruby) was applied to the MGC-area and dextran fluorescein (micro-emerald) or Alexa Fluor 488 (Alexa 488) to the area containing ordinary glomeruli. A specific needle was used to apply each dye. After application of the crystals, the brain was covered with medical wipes (Kimberly Clark Professional™) soaked in Ringer's solution with sucrose and then kept in a refrigerator overnight (12 – 18h) at a temperature of 4 °C in order to allow anterograde transportation of the dye. In the second staining approach the insect was moved to a set-up which included a manual micromanipulator (Leitz, Germany) and a high-resolution stereomicroscope (Zeiss; SteREO Discovery.V12). A glass electrode, made by a Flaming-Brown horizontal puller (P97; Sutter Instrument, Novarto, Ca, USA) was used for applying the dye into the brain. The electrode was adapted the staining procedure by slightly damaging the tip. Then the dye crystals were manually placed on the tip of the glass electrode before the glass electrode would penetrate the brain tissue by means of the micromanipulator. The final part of the staining procedure was performed as described above. Some experiments using only one dye (micro-ruby) inserted into the whole antennal lobe region were performed initially, before the two different dyes applied to the two different regions of the antennal lobe, as explained above, was utilized.

Dissection

After 10 – 12 hours in the refrigerator at 4 °C, the brain was dissected out from the head capsule. The insect was placed under a stereomicroscope (Leica; MZ 12.5) and both

antennae were removed before the insect was decapitated using micro-scissors. The head capsule was then transferred to a dissection bowl containing Ringer's solution without sucrose. Forceps were used to remove the proboscis, palps, cuticula, retina, muscle-tissue, and trachea surrounding the brain. Finally, the brain, complete with both eye lobes, was fixated (Roti, histofix) for 2 hour in room temperature or overnight (12 – 18h) in the refrigerator.

Dehydration, mounting and storage

After fixation the brain was dehydrated through a series of alcohol (50 %, 70 %, 90 %, and 96 %) each lasting for 5 minutes. The dehydration process was finished by two series of 100 % alcohol, each lasting 10 minutes. Finally, the brain was removed from the alcohol and stored in a small bottle of glass filled with methylsalicylate. To prepare the brain for the confocal scanning procedure, the preparation was transferred from the glass bottle onto a metal-plate having a circular hole in the middle and one side covered by a thin glass-sheet. The brain was placed into the small circular hole, immersed in methylsalicylate and oriented in an appropriate position before another thin sheet of protective glass was added in order to cover the preparation.

Confocal microscopy

Visualization of the stained neurons was performed by using a confocal microscope (LSM Zeiss 510 Meta Mira 900F) and each successfully stained specimen was scanned as a whole brain preparation. Using dry objectives (10x 0.3 or 20x 0.5; plan-neofluar), a stack of images with a resolution of 1024 x 1024 pixels in the x-y direction and a slice thickness of 2 – 7 µm in the z-axis direction was created. A two-channel scanning including a helium neon laser (wavelength; 543µm) and an argon laser (wavelength; 488µm), was used to excite the tetramethylrodamine and the dextran fluorescein/Alexa Fluor488, respectively.

Image processing

The completed scans were stored as Microsoft Access Database files (.mdb) and were further processed for optimal imaging using different software. First, the Zeiss LSM Image Browser® was used to process the raw data. Secondly, the LSM files were imported to Adobe® Photoshop® CS5 for adjusting contrasts/brightness and if needed, rotating the image to an appropriate orientation. Finally, the images were imported to Adobe Illustrator® CS5 where the figures were edited and figure legends added.

Results

The different staining experiments resulted in successful labeling of the antennal lobe output neurons including their cell body clusters in the antennal lobe, their projection pattern along the various antenno-protocerebral tracts (APTs), and their target regions in the two main higher olfactory centers of the protocerebrum, i.e. the calyces and the lateral protocerebrum.

General projection pattern of the antennal-lobe output neurons

Application of one fluorescent dye into the whole antennal lobe region resulted in staining of the three main APTs, the m-APT, the ml-APT, and the l-APT, as demonstrated in figure 4A. Whereas all successful staining experiments included labeling of the three main tracts, which are previously well described in several moth species (Homberg et al., 1988; Rø et al., 2007), some included staining of an additional antenno-protocerebral pathway. Thus, as demonstrated in figure 4B, there are *two* medio-lateral tracts leaving the m-APT. Similarly to the previously described ml-APT, the new tract projects out of the antennal lobe together with the m-APT. It runs posteriorly and bends off from the prominent m-APT, passing in a lateral direction in parallel with, but posteriorly of the ml-APT (Fig. 4B). This fourth APT branches into three distinguishable fiber bundles. One bundle innervates a region adjacent to the calyces (possibly including this neuropil) whereas the two others innervate the lateral protocerebrum. According to the current findings, I have named the two medio-lateral tracts, i.e. the one previously described and the one discovered here, the first medio-lateral antenno-protocerebal tract (1st ml-APT) and the second medio-lateral antenno-protocerebral tract (2nd ml-APT), respectively (Figs. 4B and 5B).

The most prominent of the antennal lobe output tracts, the m-APT, leaves the antennal lobe and runs posteriorly bypassing the central body ventrally before turning laterally (Fig. 4A-B). The tract then runs along the anterior border of the calyces, sending terminal projections into this neuropil before it continues anterior-laterally into the lateral protocerebrum. The m-APT is also seen in a double-labeled preparation in figure 5A. In figure 6A, the current pathway is shown in a frontally orientated preparation.

The 1st ml-APT is seen as it leaves the antennal lobe projecting postero-medially (Fig. 4A-B, 5A-B and 6A). After following the m-APT for a short distance it turns laterally, at the edge of the central body, and projects directly to the lateral protocerebrum without targeting

the calyces. This tract is considerably thinner than the m-APT and splits in two fiber bundles before terminating in the lateral protocerebrum (Fig. 5A). In figure 6A-B the 1st ml-APT is seen in a frontally orientated brain.

In addition to the tracts mentioned above, the lateral tract appears as a thick bundle leaving the antennal lobe medio-ventrally before turning dorso-laterally and innervating various regions of the protocerebrum (Figs. 6A and 7A). In addition to ventral regions of the lateral protocerebrum, this tract also sends extensive projections into a restricted area located more medially. This area appears as a pillar-like structure in frontally oriented brain preparations, as seen in figure 7A. The heavily stained region can also be seen in the dorsally positioned brain in figure 4B. As demonstrated in some of the figures (Figs. 6A and 7A) the l-APT splits into two neural bundles shortly after leaving the antennal lobe.

In addition to labeling the output tracts, dye application into the antennal lobe resulted in staining of the two main cell clusters of the current brain structure, i.e. the lateral cell cluster, which is known to contain somata of projection neurons and local interneurons (Fig. 5C-D) and the medial cell cluster containing somata of projection neurons only (Fig. 6C).

Double-staining of projection neurons originating from the MGC and the ordinary glomeruli, respectively

Application of one fluorescent dye into each of the two antennal lobe regions, i.e. micro-ruby in the MGC and micro-emerald/Alexa 488 in the ordinary glomeruli, resulted in several successful stainings showing further details regarding projection pattern and target regions of the two types of output neurons.

Double-labeling of the antenno-protocerebral tracts

The double-stainings showed that the three previously described tracts – the m-APT, the 1st ml-APT, and the l-APT – contained projections from both the MGC and the ordinary glomeruli. Thus, as shown in figures 5A-B, 6A-B and 7A-C, these tracts are double-labeled. Of the numerous axons projecting in the prominent m-APT, there seems to be slightly more fibers stained by micro-ruby, originating from the MGC, than by micro-emerald/Alexa 488, originating from the ordinary glomeruli (Figs. 5A-B, 6A-B and 7A-C). The fibers stained by each of the two dyes seem to gather in two distinct bundles within the tract, without any overlap. As compared to the prominent medial tract, the 1st ml-APT shows a somewhat

different staining pattern; thus, the fibers in this particular pathway are to a lesser degree stained by micro-ruby than by micro-emerald/Alexa 488 (Fig. 5A-B, 6A-B and 7A-B). Similarly to the two tracts mentioned above, the l-APT also contained an assembly of fibers stained by the two dyes (Fig. 6A-B and 7A-B). Figure 7A shows a double-labeled preparation where the l-APT is strongly stained by micro-ruby. Whereas this staining provides detailed information about the general path of the lateral tract, it may not be completely reliable concerning fibers originating from the MGC versus ordinary glomeruli. This is because the extensive application of micro-ruby in the dorsal part of the antennal lobe may have labeled a number of ordinary glomeruli in addition to the MGC units (Fig. 6C).

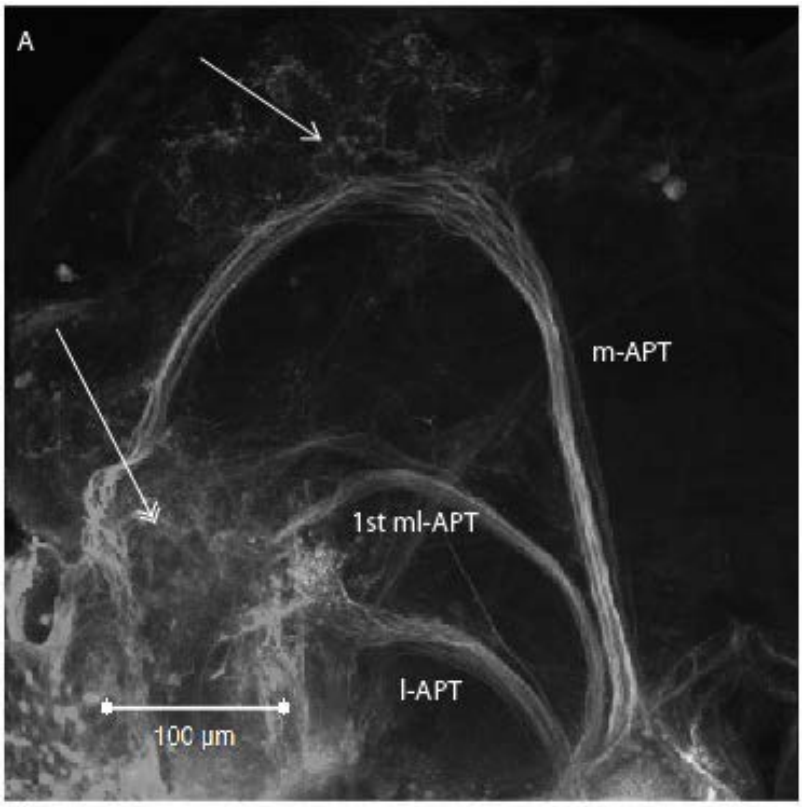
In contrast to the three tracts mentioned above containing fibers from seemingly all antennal lobe glomeruli, the newly discovered tract – the 2nd ml-APT – probably originate primarily (or exclusively) from the ordinary glomeruli, as it is labeled predominantly by micro-emerald/Alexa 488 (Fig. 5B).

Double-labeling of the mushroom body calyces

Application of two different dyes in the MGC and the ordinary glomeruli, respectively, resulted in a unique staining pattern of the calyces. Thus, the projection neurons originating from the MGC, stained by micro-ruby, terminated mainly in the inner parts of the calyces, forming condensed cluster-like structures (Figs. 8A-B and 9A-B). The projection neurons originating from the ordinary glomeruli, on the other hand, which were stained by micro-emerald/Alexa 488, innervated the entire region of the calyces forming a more evenly distributed staining pattern. As shown in figures 8A and 9A, the two types of projection neurons make minimal overlap with each other in the calyces.

Double-labeling of the lateral protocerebrum

The micro-ruby and micro-emerald/Alexa 488 stained PNs in the m-APT contributed significantly to the staining in the lateral protocerebrum (Fig. 7A). As shown in figure 6A-B and 7A-C the two types of PNs passing in this particular tract target slightly different regions, however; the ventral areas of the lateral protocerebrum is innervated by micro-emerald/Alexa 488 stained PNs whereas micro-ruby stained PNs innervated areas located more dorsally. As regards the target regions of the two PN categories passing in the other APTs, it is difficult to evaluate from the current data.



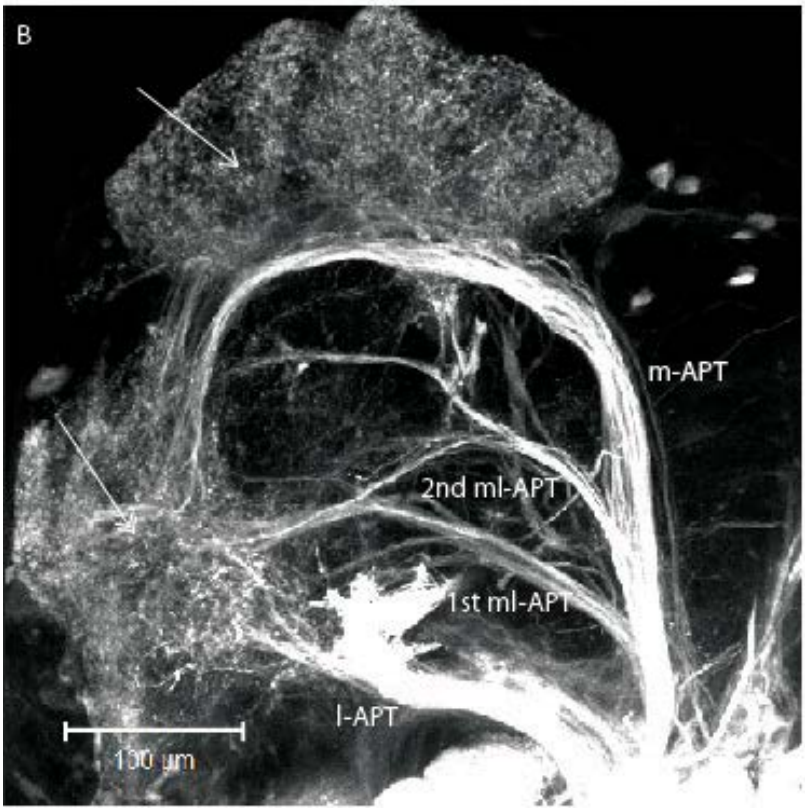


Figure 4: Confocal images of two brain preparations scanned in a dorsal view (right brain hemisphere). **A:** Three-dimensional reconstruction visualizing the main antenno-protocerebral tracts (APTs), the medial APT (m-APT), the 1st mediolateral APT (1st ml-APT), and the lateral APT (l-APT). The target regions of the APTs, i.e. the mushroom body calyces and the lateral protocerebrum, are indicated by a single and double arrow, respectively. **B:** Three-dimensional reconstruction showing the main APTs described above, plus a fourth tract not formerly described, the 2nd mediolateral APT (2nd ml-APT). The characteristic mushroom body calyces, which receive input from terminals of neurons passing in the prominent m-APT, are indicated by an arrow. The lateral protocerebrum is indicated by a double arrow. Both brains were scanned with a 20X objective.

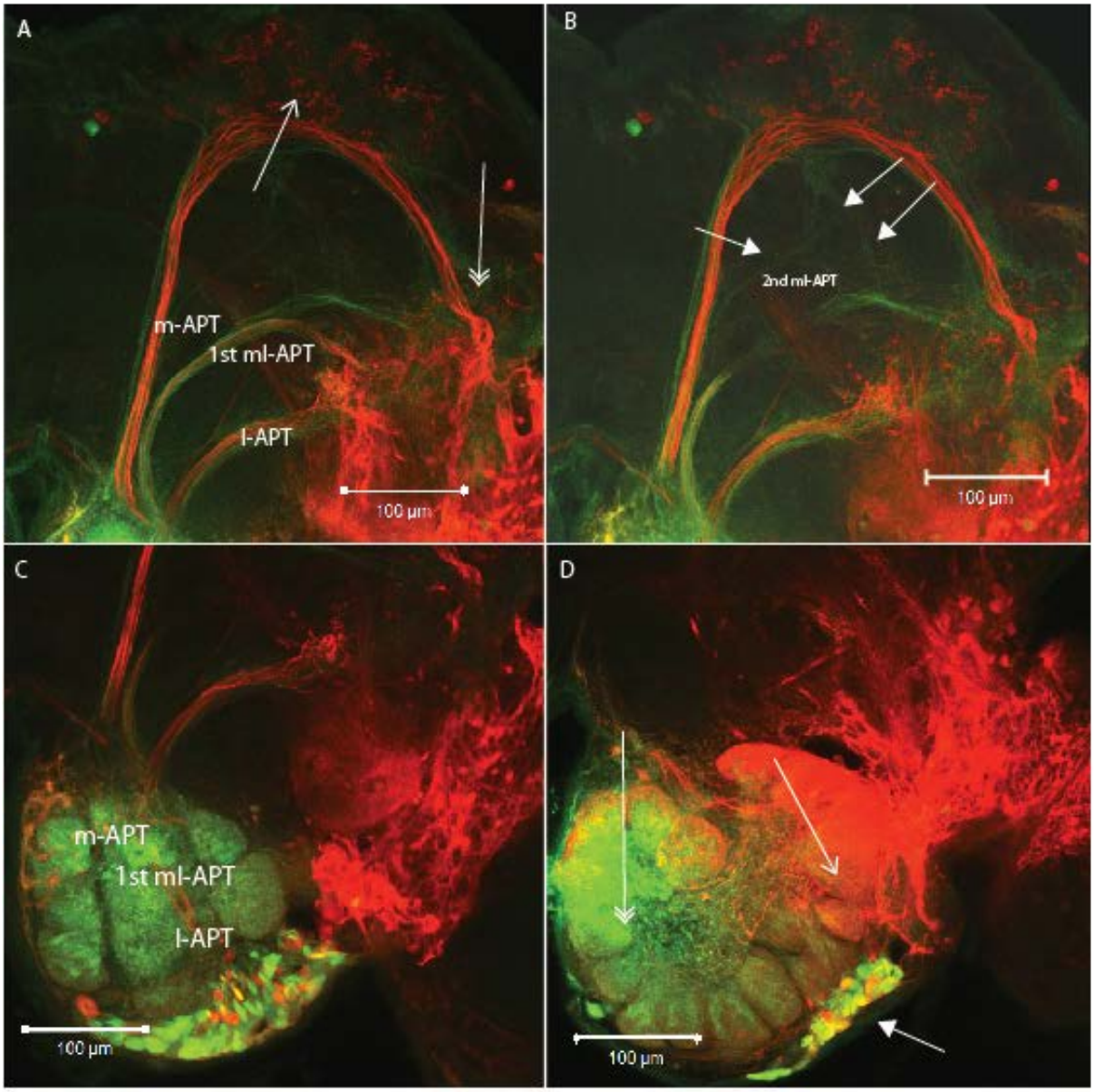


Figure 5: Confocal images of one double-labeled brain, in a dorsal view, showing the projection pattern of antennal lobe output neurons originating from the macroglomerular complex (MGC; red) and the ordinary glomeruli (green), respectively (left brain hemisphere). **A-B:** Two images showing slightly different section stacks; as demonstrated, all three main antennoprotocerebral tracts (APTs) contain projection neuron axons linked to both types of glomeruli (displayed here by red and green fibers, respectively). The second mediolateral APT (2nd ml-APT), however, seems to contain fibers originating from ordinary glomeruli only (B). Whereas the medial APT (m-APT) and the lateral APT (l-APT) seem to contain approximately similar numbers of the two projection neuron categories, the 1st mediolateral APT (1st ml-APT) appears to contain more fibers originating from ordinary glomeruli then from the MGC units. **C-D:** Two images showing different section stacks of the antennal lobe. **C:** Image showing the three main APTs at the exit of the antennal lobe. In addition, the lateral cell cluster can be seen; relatively few cell bodies of neurons linked to the MGC (red) are present as compared to those linked to ordinary glomeruli (green). **D:** Image of a more dorsal part of the antennal lobe indicating the site of application for each fluorescent dye, i.e. micro-ruby in the MGC (single arrow) and Alexa 488 in the ordinary glomeruli (double arrow). The small arrow points at the lateral cell cluster. The preparation was scanned with a 20X objective.

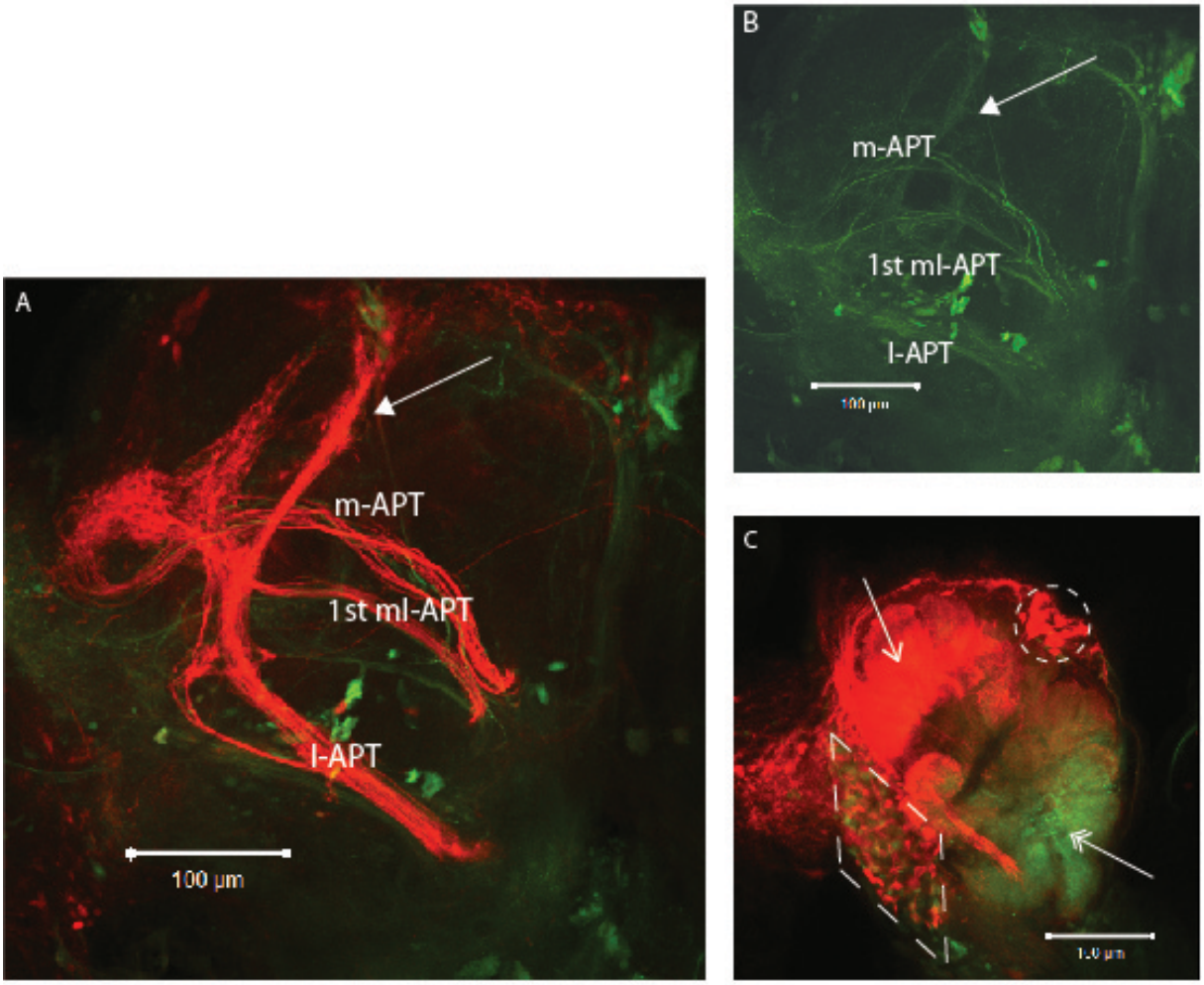


Figure 6: Confocal images of one double-labeled brain, in a frontal view, showing the projection pattern of antennal lobe output neurons originating from the region of the macroglomerular complex (MGC; red) and the ordinary glomeruli (green), respectively (right brain hemisphere). **A:** Double-labeling of antennal-lobe output neurons; as shown, axons stained by micro-ruby (red), which was applied to the MGC region, were strongly stained, whereas axons stained by Alexa 488 (green), which was applied to the ordinary glomeruli, were relatively weakly stained. The three main antennoprotocerebral tracts (APTs) are visualized via stained fibers originating from the MGC region, and the characteristic pillar-like structure of the lateral APT (l-APT) is particularly prominent (arrow). **B:** Same sections as presented in A, but showing Alexa 488-stained fibers originating from ordinary glomeruli only. Again, labeled fibers are present in all three main APTs, but noticeably weaker stained than those filled with micro-ruby. The arrow points to the pillar-like structure of the l-APT. **C:** Confocal image of the antennal lobe indicating the site of application for each fluorescent dye, i.e. micro-ruby in the MGC (single arrow) and Alexa 488 in the ordinary glomeruli (double arrow). The circle encloses cell bodies in the medial cell cluster (MC) and the rectangle encloses cell bodies in the lateral cell cluster (LC). Whereas the cell bodies in the LC constitute a mixture, some being stained by micro-ruby and others by Alexa 488, those in the MC are labeled by micro-ruby only. m-APT medial APT; 1st ml-APT 1st mediolateral APT. The preparation was scanned with a 20X objective.

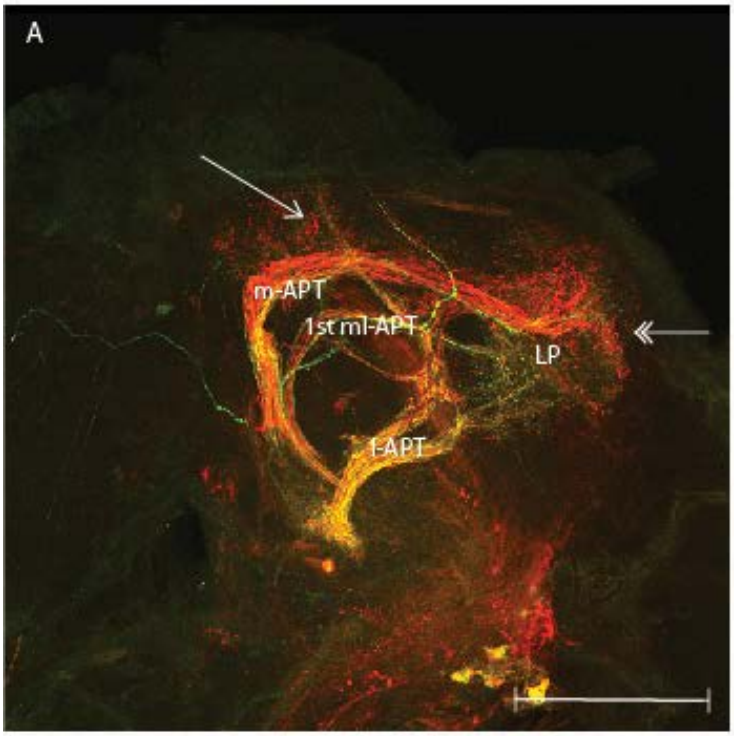
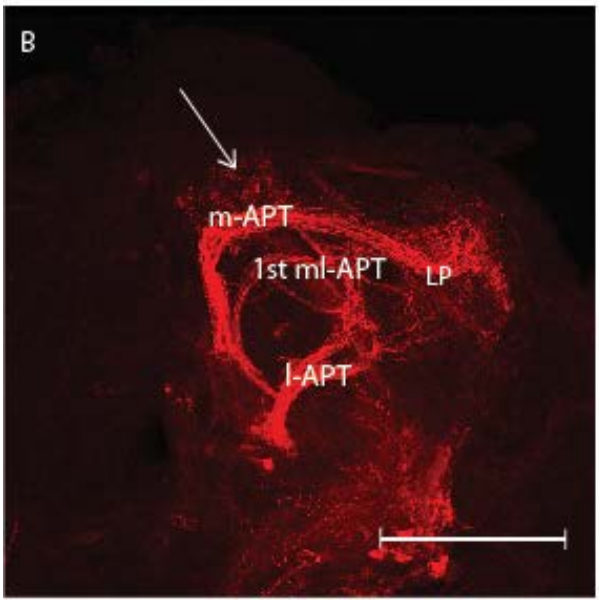
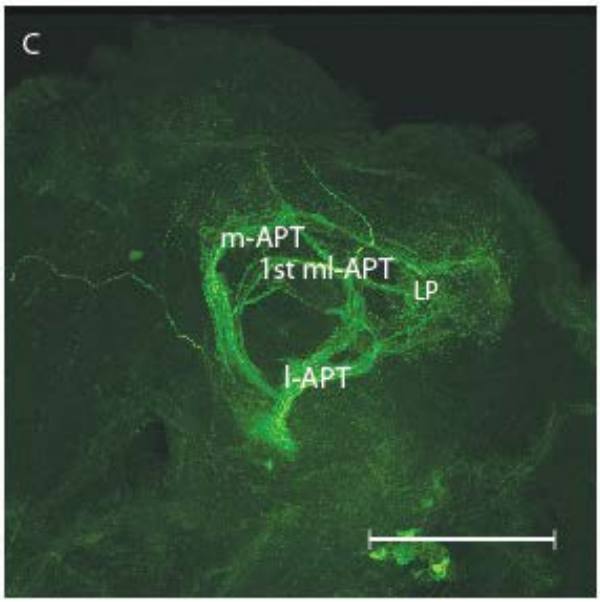


Figure 7: Confocal images of one double-labeled brain in a frontal view, showing the projection pattern of antennal lobe output neurons originating from the region of the macroglomerular complex (MGC; red) and the ordinary glomeruli (green), respectively (left brain hemisphere). **A:** Double-labeling demonstrating the presentence of both fiber categories (i.e. red and green) in each of the main antennoprotocerebral tracts (APTs). The calyces are indicated by an arrow. Slightly non-overlapping target regions in the lateral protocerebrum (LP) labeled by the two dyes are indicated by a double arrow. **B-C:** Two images showing the single stains from the "overlay-image" shown in A; the axons originating from the MGC region, marked red, is shown in B and those originating from the ordinary glomeruli, marked green, is shown in C. m-APT medial APT; 1st ml –APT 1st medi-oloateral APT; l-APT lateral APT. The preparation was scanned with a 20X objective. Scalebar: 100 µm.





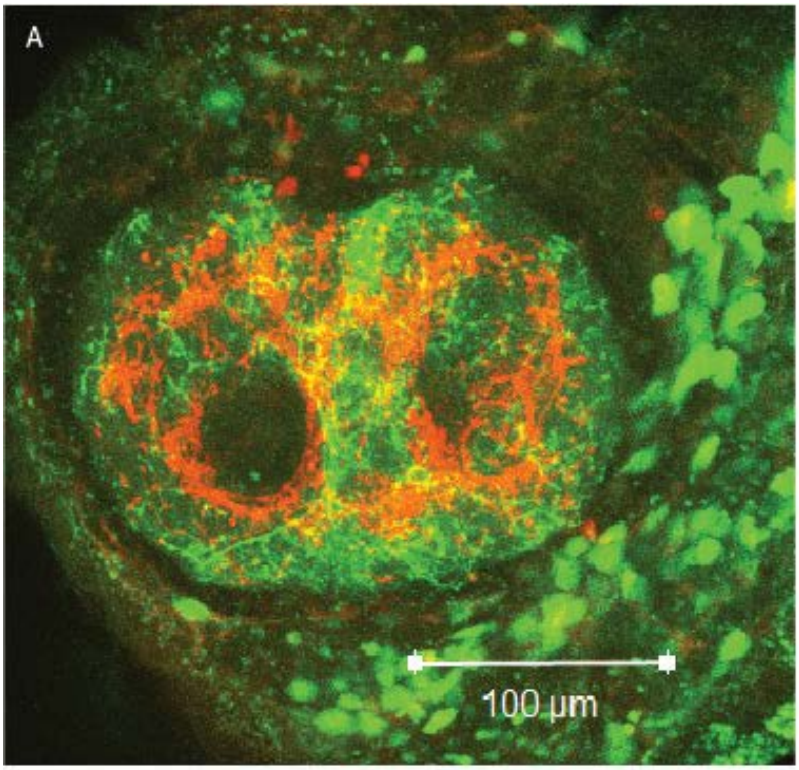
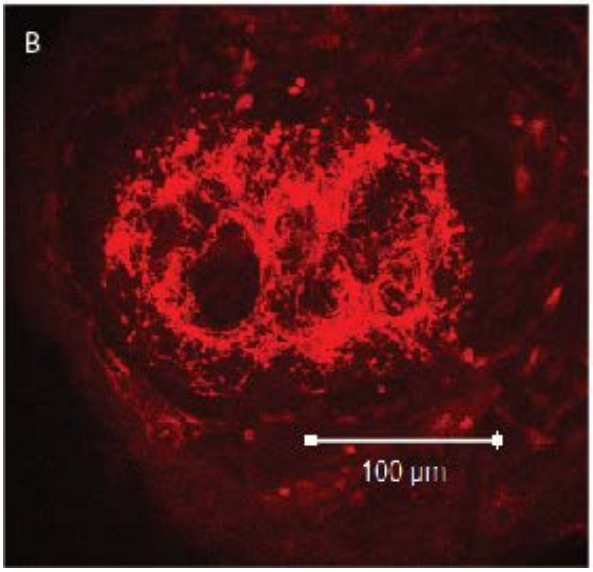
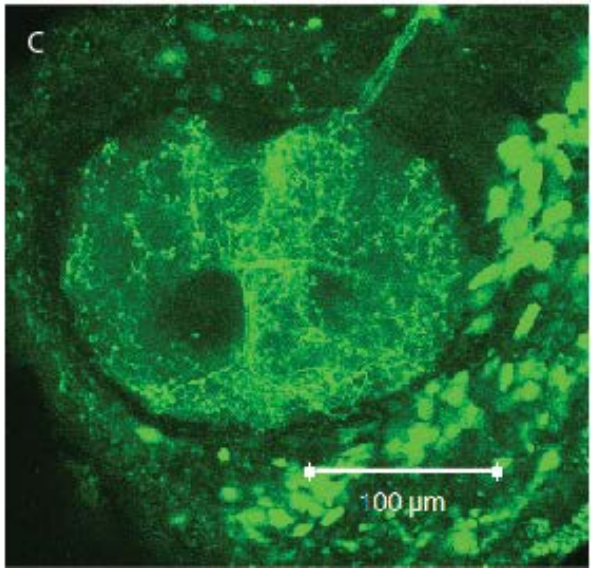


Figure 8: Confocal images of one double-stained preparation showing the calyces (frontal orientation). **A:** Double-labeling showing projections originating from neurons linked to the macroglomerular complex (MGC; red; micro-ruby) and the ordinary glomeruli (green; Alexa 488), respectively. **B-C:** Two images showing the single stains from the "overlay-image" shown in A. As demonstrated from the images, the terminals originating from the two neuron types form markedly different staining patterns and they also show a small degree of overlap. The preparation was scanned with a 20X objective.





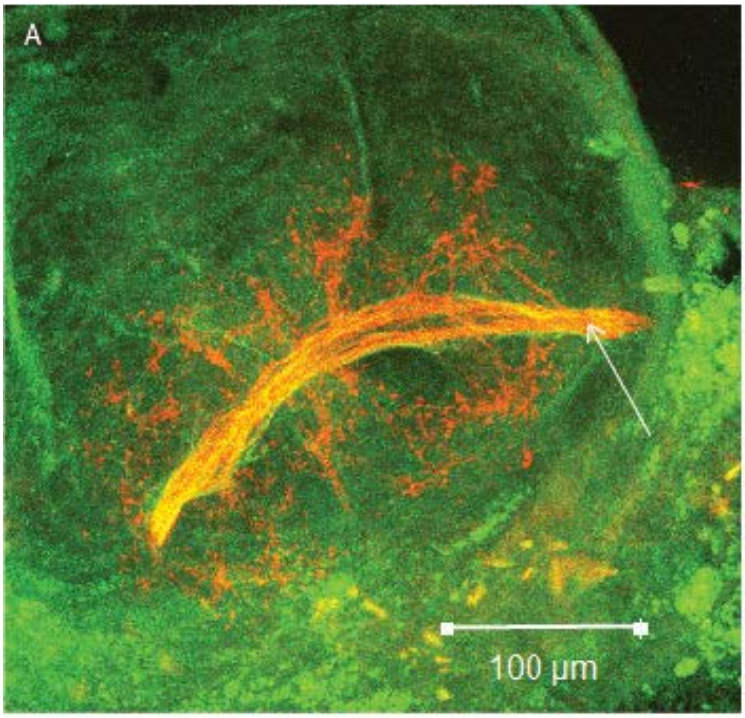
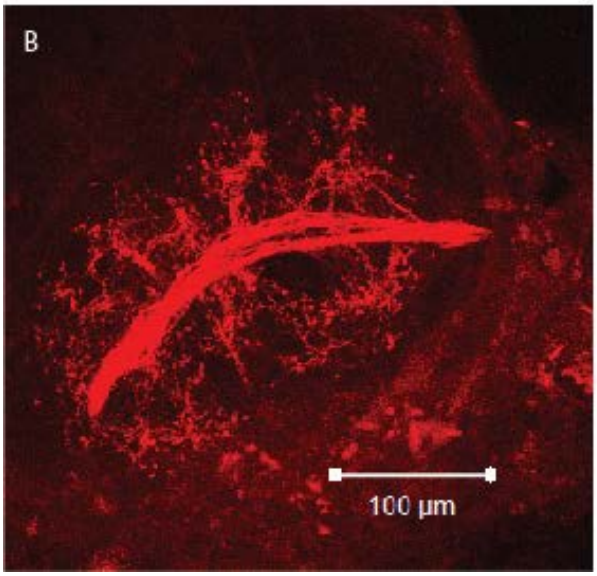
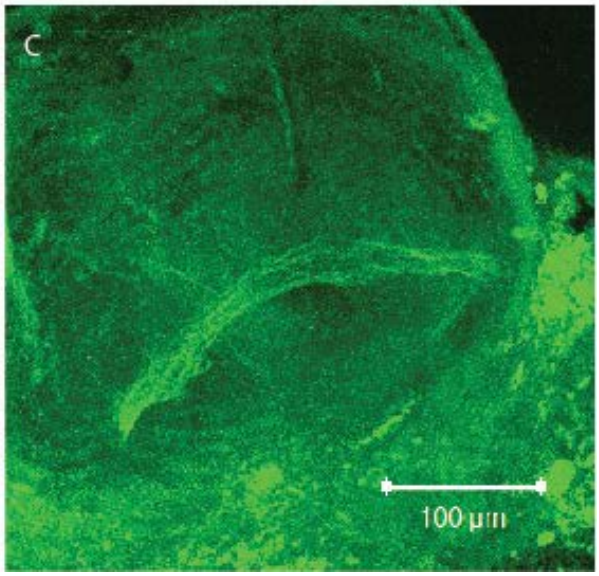


Figure 9: Confocal images of one double-stained preparation showing the calyces in a frontal view. **A:** Double-labeling showing projections originating from neurons linked to the macroglomerular complex (MGC; red; micro-ruby) and the ordinary glomeruli (green: micro-emerald), respectively. **B-C:** Two images showing the single stains from the "overlay-image" shown in A. Like in figure 8, the neurons originating from the MGC, labeled by micro-ruby, formed a distinct staining pattern characterized by condensed regions (A, B). The terminal projections linked to the ordinary glomeruli, here stained by micro-emerald, can hardly be seen in the calyces – even though their axons are clearly visible, as shown in C. The preparation was scanned with a 20X objective.





Discussion

The current master project has established a method for staining functionally different PNs at the second order level of the moth olfactory pathway. Thus, by applying two different fluorescent dyes into distinct regions of the antennal lobe, one in the MGC region and one in the ordinary glomeruli, several characteristics of the two parallel olfactory systems in the moth are described. The stained pathways/structures include four APTs (one of which has not previously been described), the mushroom body calyces, the lateral protocerebrum, and a few additional regions of the protocerebrum. Besides slightly different target regions in the lateral protocerebrum, each of the two functional PN types also formed a specific pattern in the calyces.

The antenno-protocerebral tracts

The current data show that micro-ruby stained PNs from the MGC-area and micro-emerald/Alexa 488 stained PNs from ordinary glomeruli are present in all three main APTs – the m-APT, the 1st ml-APT and the l-APT. This particular organization – where different PNs carrying plant- and pheromone information project in parallel within each tract has previously been described in the sphinx moth, *M. sexta* (Homberg et al., 1988), and the silk moth, *B. mori* (Kanzaki et al., 2003). The fact that the three main APTs were visualized in all successfully stained preparations indicates that each of these tracts is connected to all antennal lobe glomeruli. In general, segregation of information about pheromone versus plant odor stimuli, forming parallel pathways, is typical for the olfactory system, not only in moths but also in other insect species (reviewed by Galizia & Rössler, 2011).

As shown from the current data, the most prominent tract, the m-APT, was strongly stained by micro-ruby and micro-emerald/Alexa 488. The neural fibers labeled by the two dyes clearly create two separate fiber bundles running in parallel throughout the whole tract, from the antennal lobe to the lateral protocerebrum. As most PNs in the m-APT are of the uniglomerular type, it seems likely that the stained fibers are separated and do not display any overlap.

The 1st ml-APT, on the other hand, which is assumed to contain multiglomerular PNs, also showed a relatively limited degree of double-labeled fibers. This relatively thin tract, which projects directly to the lateral protocerebrum, was in some cases stained almost entirely

with micro-emerald/Alexa 488 and in other cases with both micro-ruby and micro-emerald/Alexa 488. However, when both dyes were present, they appeared to occur in distinct fibers, similar to the pattern seen in the m-APT. Thus, even though there are multiglomerular PNs in the 1st ml-APT, there appears to be a separation between plant- and pheromone specific odor information because there are very little overlapping between the micro-ruby and micro-emerald/Alexa 488 dyes. Whether the current staining pattern is due to a real separation, being maintained by distinct neurons, or it is caused by methodological reasons, is an open question.

Regarding the third main APT, the l-APT, it also showed little overlap between fibers stained by micro-ruby and micro-emerald/Alexa 488. In addition, the micro-emerald/Alexa 488 stained PNs seems to terminate mainly in the lateral protocerebrum whereas the micro-ruby-stained PNs to a larger degree target the remaining protocerebral regions, including the more medially area including the pillar-like structure. This characteristic appearance, which ends abruptly anterior to the calyces, seems to have been observed by Homberg et al. (1988) as well. Also, PNs linked to one specific antennal-lobe glomerulus receiving input from CO_2-rceptor neurons in *H. virescens*, were recently reported to include one category, passing in the l-APT, which had terminal endings forming a similar pillar-like structure in the medial protocerebrum (Ingrid Moe Dahl, Master thesis, 2013). Otherwise, from Homberg et al. (1988) it was concluded that the l-APT most likely consist of both uni- and multiglomerular PNs and that each class is either connected to the ordinary glomeruli detecting plant odors or to the glomeruli found in the MGC-area detecting pheromones. Thus, similarly to the m-APT, the lateral tract probably also consists of two functionally different neuron types, linked to pheromones and the plant odors, respectively, which projecting parallel but separated pathways.

The 2nd medio-lateral antenno-protocerebral tract

Whereas a number of previous studies have reported about one ml-APT in moths (reviewed by Galizia & Rössler, 2011) – here named the 1st ml-APT, the current investigation has unraveled a second ml-APT – the 2nd ml-APT. Unlike to the PNs found in the 1st ml-APT, the m-APT, and the l-APT, which all contained a mixture of fibers labeled by both dyes, the neural fibers in the 2nd ml-APT seem to be stained only by micro-emerald/Alexa 488 dye, meaning that the 2nd ml-APT is mainly involved in plant-odor information processing. Why this 2nd ml-APT has been stained in only a few preparations is not clear but one explanation

could be that it is connected to a few glomeruli within the antennal lobe. Whether thee PNs passing in the 2nd ml-APT is uni- or multiglomerular is an open question. The newly discovered tract resembles the 1st ml-APT but it is even thinner, suggesting that there are relatively few axons present in this particular pathway. The fact that the 2nd ml-APT is so extensively stained in only one preparation and only weakly stained in other preparations is also an indication that the relevant glomeruli have to be targeted when intending to visualize it via dye application into the antennal lobe A more specific interpretation of the data might be that the 2nd ml-APT is involved in processing information about CO_2. In the recent master project by Moe Dahl (2013), previously referred to, a partly similar projection pattern as that characterizing the 2nd ml-APT is seen.

The anatomical architecture of multiple ml-APTs, as discovered here, resembles the arrangement found in the honeybee brain where it has been identified three ml-APTs (Kirschner et al., 2006). Investigations on the antenno-protocerebral pathways in the honeybee have revealed that the m-APT and the l-APT consist of uniglomerular PNs and that they constitute a dual olfactory pathway processing olfactory input separately by originating from two different sets of antennal lobe glomeruli and innervating distinct areas in the honeybee MBcalyx and lateral horn (Kirschner et al., 2006; Rössler & Zube, 2006). The three ml-APTs found in the honeybee are however multiglomerular and do not innervate the MBcalyces but rather three areas of the lateral protocerebrum. When looking at the three ml-APT in the honeybee, it is clear that these APTs are less well defined than the m-APT and the l-APT. This is also true for the two ml-APTs stained in this master thesis and it similarly applies to the l-APT. Perhaps the 1st ml-APT, the 2nd ml-APT, and the l-APT could be analogies to the three ml-APTs found in the honeybee. In general, the function of the parallel antenno-protocerebral tracts needs to be further investigated.

Staining pattern in the calyces

As expected, the current data showed that both the PNs originating from the MGC units (stained by micro-ruby) and the PNs originating from ordinary glomeruli (stained by micro-emerald/Alexa 488) innervated the calyces. However, the two neuron types formed distinctly different staining patterns within the calyces. Whereas terminals of PNs linked to the MGC are concentrated at specific areas in the calyces creating a characteristic pattern, the terminal of PNs connected to ordinary glomeruli form a pattern including more dispersed processes throughout this neuropil. Homberg et al., (1988) describes the staining pattern in the

calyces formed by MGC PNs in *M. sexta*; the terminal endings make up 9 – 12 condensations, possibly forming small specializations and lesser divergence onto Kenyon cells than the PNs from ordinary glomeruli. Condensations are also occurring when several PNs from ordinary glomeruli are stained, but these stainings produce a much less pronounced pattern. In moth species, a recent study on *B. mori* (Namiki et al., 2012) has identified concentric zones within the calyces that corresponds to either plant- or pheromone odors. PNs conveying plant odors seem to project to a broader area within the calyces than pheromone-specific PNs. These areas have little overlap.

Male-specific neurons target a region of the lateral protocerebrum different from that targeted by the plant odor neurons

The results presented here show that terminals of PNs originating from the MGC versus PNs linked to ordinary glomeruli target slightly segregated areas in the lateral protocerebrum. As shown in figure 7, it can be seen that the two categories of axons passing in the m-APT end up in partly different regions. This may indicate that the two categories of information are kept separate also at the 3rd order level of the olfactory pathway. In *Drosophila* it has been found that representation of fruit odors are spatially segregated from that of pheromones in the lateral horn; PNs conveying fruit odors terminates in the posterior-dorsal area whereas the pheromone-specific PNs terminates more anterior-ventrally (Jeffries et al. 2007). Whereas the fruit odors seem to show an intermixing pattern in the lateral horn, the pheromones are organized in separated channels from the sensory periphery to the lateral horn.

Methodological considerations

The data presented here, demonstrate that the method of applying two fluorescent dyes into distinct regions of the male moth antennal lobe, i.e. the MGC and the ordinary glomeruli, is a well suited method for studying details regarding the second order level of the olfactory pathway. During the experimental period, a technique enabling relatively precise injection of dye crystals into the two regions was obtained. Having said this, there is also certain restrains linked to the current approach. In many preparations the micro-ruby stained PNs were considerably stronger stained than those labeled by micro-emerald/Alexa 488. This may be caused by different conditions. One possible explanation could be related to the number of glomeruli in the two application sites; thus, the four MGC-units cover a relatively smaller area than the numerous ordinary glomeruli. However, the number of output neurons

originating from the relatively small MGC area is high. When using the mass-staining technique, it is might be “easier” to stain (pheromone) neurons occurring in a small region than (plant-specific) neurons being distributed within a larger area. Another alternative explanation might be related to properties of the two fluorescent dyes; thus, it might be that micro-ruby is more effectively taken up by the neurons and better transported in the axons than micro-emerald/Alexa 488. In future experiments, it could perhaps be useful to test another type of fluorescent dye in combination with micro-ruby. One particular problem with the micro-emerald and Alexa 488 is that they partly overlap with autofluorescens from the moth brain. However, Alexa 488 has an increased photostability and fluorescence output, which makes it superior to micro-emerald. As regards refinement of the staining technique, the use of glass-electrodes rather than micro-needles was important. By using slightly damaged glass-electrodes, it is easier to apply the dye into the neural tissue of the brain. However, one general problem with the current mass staining technique is that you can never be completely sure which neurons are being labeled. Thus, in these double-labeling experiments each of the two dyes utilized may have come, accidentally, into “wrong glomeruli”. The most challenging part of the experiments was perfecting the insertion of the two dyes, i.e. hitting the relevant spots within the antennal lobe. In particular, it should be mentioned that the dye intended for the MGC, in this case micro-ruby, should be applied relatively close (but not to close) to the antennal entrance.

Conclusions

Based on the findings achieved in this master project, four main conclusions can be drawn:

1) The general staining of the antennal-lobe output neurons explored, in addition to the three main antenno-protocerebral tracts, a fourth tract, not previously described in any moth species. The new tract is named the 2nd ml-APT.
2) The double-labeling experiments demonstrated that both micro-ruby- and micro-emerald/Alexa 488-stained PNs are present in the three main APTs meaning that the two neuron types, originating from the MGC and the ordinary glomeruli, respectively, project in parallel, with little degree of overlap, within each tract.
3) The PNs originating from the MGC create a specific pattern of condensed terminal endings in the calyces whereas those linked to the ordinary glomeruli form more distributed innervations. Also, the two types of projections show a minimal degree of overlap.
4) Terminals of the two categories of PNs, i.e. MGC neurons and plant odor neurons, terminate in partially different areas of the lateral protocerebrum; in particular, the two types of PNs passing in the prominent m-APT project in distinct regions located in the dorso-medial and ventro-lateral, respectively.

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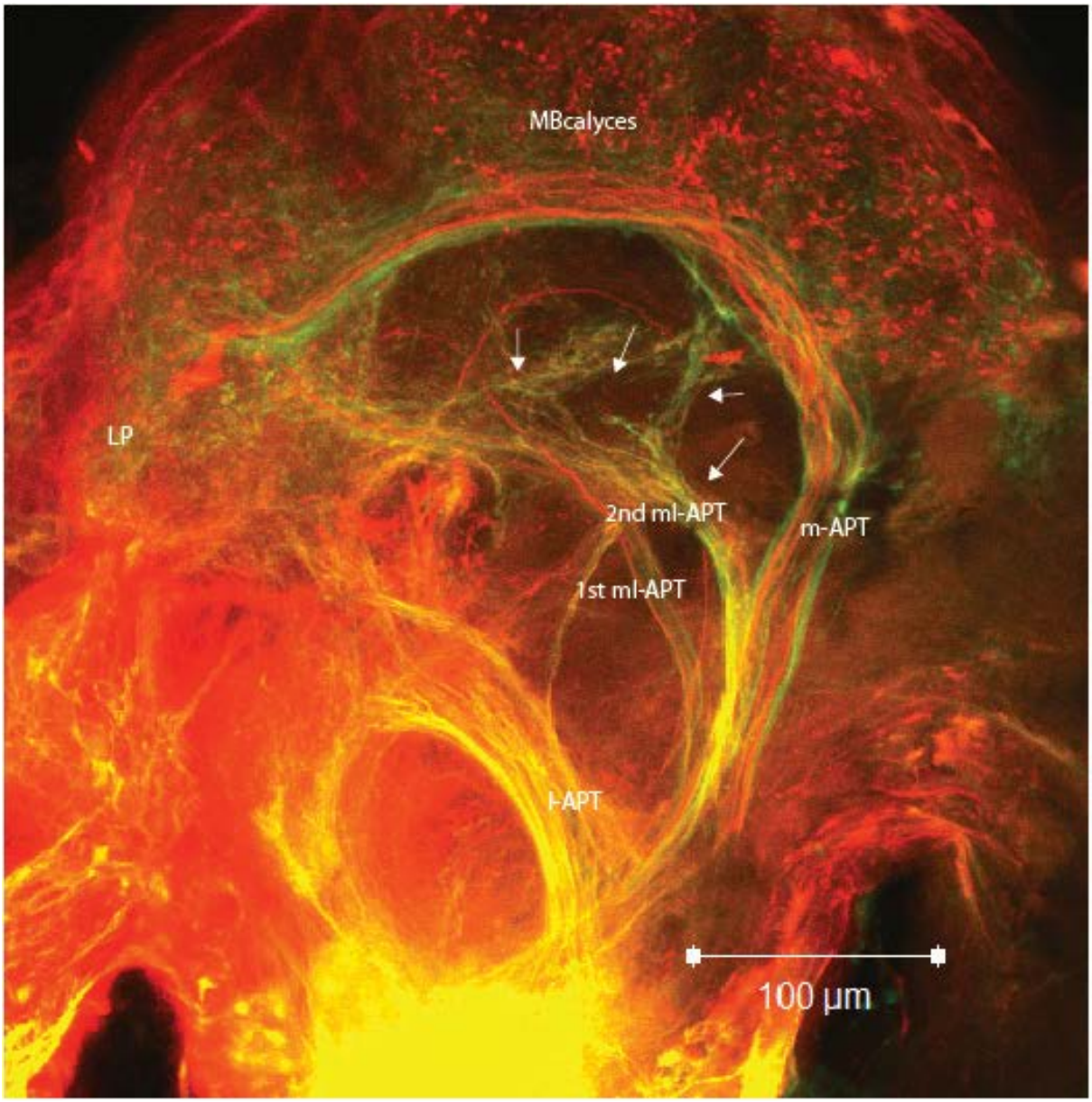
Appendix



Confocal image showing the antenno-protocerebral tracts (APTs) in a dorsal view, stained with micro-ruby and Alexa 488. The 2nd ml-APT is seen projecting along the m-APT adjacent to the 1st ml-APT. m-APT: medial antenno-protocerebral tract; 1st ml-APT: first medio-lateral antenno-protocerebral tract; 2nd ml-APT: second medio-lateral antenno-protocerebral tract; l-APT: lateral antenno-protocerebral tract; LP: lateral protocerebrum; MBcalyces: mushroom body calyces.