Neural organization of olfactory signal pathways in a small model brain

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Acknowledgments

Abstract

The chemical sense is regarded as the oldest sense, and all organisms have evolved a mechanism in order to detect molecules in the environment. Among many other creatures, the moth depends fundamentally on chemical communication for survival. Besides, this insect group possesses an easy accessible olfactory system that shares striking similarities with that of vertebrates. In the project presented here, the moth brain is used as a model for studying the olfactory system; more precisely, the parallel olfactory pathways connecting the primary olfactory center with higher regions of the brain have been investigated. By performing retrograde staining of antennal-lobe projection neurons, five antennocerebral tracts were identified. Stained projection-neuron somata, which are located in the antennal lobe, were found in all three cell clusters: the lateral, the medial and the anterior cell cluster. The stained somata in the lateral cell cluster consisted of two distinctly located groups. The retrograde staining of projection neurons also visualized the glomerular labeling pattern in the antennal lobe. This labeling pattern included a small group of glomeruli that were strongly innervated by fibers from the dorsomedial antennocerebral tract.

Introduction

Smell is an important aspect of the lives of both vertebrates and invertebrates. The chemical sense is regarded as the oldest sense and the most important in early phylogenic development. The way the olfactory system is structured in humans indicates that the development of this system emerged before the newer parts of the cerebral cortex (Brodal, 2007). Humans often regard the sense of smell as aesthetic and associated with what is pleasant and unpleasant. Humans can recognize about 10,000 scents that cause physiological, emotional and cognitive responses (Axel, 1995). Our thoughts, memories and behaviors are strongly connected to the sense of smell; many have perhaps felt how the odor of something can bring back an old memory, or make us react with joy or disgust. For most animals, the sense of smell is the primal sense. Many organisms depend fundamentally on their sense of smell in order to obtain food and find a mate for reproduction. The odors in our environment are a complex mixture of volatiles, and the challenge for all organisms is to differentiate between the molecules that give important information and molecules that are not important. To fully understand how an "internal representation of the external world" (Axel, 1995, p. 155) can be shaped, it is important to study how olfactory information is decoded in the brain.

Nocturnal insects, for instance moths, are well suited as model organisms for exploring chemosensory principles because of their fundamental dependence on chemical communication. In addition, they possess an easy accessible olfactory system that shares striking similarities with that of vertebrates (Hildebrand & Shepherd, 1997). Thus, understanding the nervous system of the insect represents an alternative to exploring the more complex vertebrate system. Although there is a large difference between the morphology of the human nose and the insect antenna, the principles behind odor discrimination involve basic mechanisms that are shared by both vertebrates and invertebrates (Carr et al., 1990, Pelosi, 1996, as cited in Hildebrand & Shepherd, 1997; Hildebrand & Shepherd, 1997).

The moth *Heliothis virescens* relies on a well-developed sense of smell in order to obtain food and reproduce. In general, the olfactory pathways responsible for reproductive behavior have been thoroughly studied in the moth. In addition, several moths are

considered a pest species as they live on economically important plants (Karg & Suckling, 1999).

The current thesis aims to explore basic neural principles underlying anatomical arrangements of the central olfactory pathways. The moth *H. virescens* is used as a model organism, and the thesis intends to uncover neural connectivity patterns between the primary olfactory center and the higher integration centers in the moth brain.

The moth olfactory system

The peripheral system

Insects detect odors in their environment via numerous olfactory receptor neurons located on the antennae. The sensory neurons that are housed inside fingerlike structures called sensilla, are small bipolar neurons with a thin dendrite carrying the receptor protein and an axon projecting directly to the brain (Keil, 1999). Pores that penetrate the cuticle of the sensillum allow odorants to enter the aqueous interior. Usually, each sensillum contains a few receptor cells and accessory cells that are bathed in the sensillum lymph. The aqueous solution contains odorant-binding proteins believed to transport the hydrophobic odorants from the cuticular wall to the dendrite of the sensory neuron (Steinbrecht, 1998). Axons of the olfactory receptor neuron make up the antennal nerve which projects directly into the primary olfactory center of the brain, the antennal lobe, shown in figure 1A and B (Anton & Homberg, 1999).

The antennal lobe: the primary olfaction center in the insect brain

Axons of the olfactory receptor neurons terminate on dendrites of the second order neurons in spherical structures, so-called glomeruli, located inside the antennal lobe (Anton & Homberg, 1999; Rospars, 1988). Each glomerulus functions as a local neuronal network between the axon terminals of the olfactory receptor neurons and three different categories of central interneurons: local interneurons, projection neurons and centrifugal neurons (Anton & Homberg, 1999; Boeck & Tolbert, 1993). Local interneurons are amacrine cells, mainly inhibitory, that are confined to the antennal lobe and innervate many glomeruli (Homberg, Christensen, & Hildebrand, 1989; Hoskins, Homberg, Kingan, Christensen, & Hildebrand, 1986). Projection neurons, on the other hand, send axons into the protocerebrum through one of the antennocerebral tracts. The centrifugal

neurons, which constitute a relatively small population, have dendritic arborizations in various regions of the brain, and send an axon into the antennal lobe (Homberg, et al., 1989).

The male moth has two types of glomeruli in the antennal lobe: so called ordinary glomeruli, receiving input about plant odors; and male-specific glomeruli, receiving input about pheromones and interspecific signals. The latter category makes up the so-called macroglomerular complex (MGC), located dorsally in the antennal lobe at the entrance of the antennal nerve. The MGC in *H. virescens* consists of four separate units (Berg, Almaas, & Mustaparta, 1998; Løfaldli, Kvello, & Mustaparta, 2010). The two largest glomeruli of the MGC receive pheromone information that leads to attraction and sexual behavior, whereas the two smallest receive information about interspecific signals from sympatric species, which disrupts attraction (Berg et al., 1998). The antennal lobe of the male *H. virescens* consists of 63 ordinary glomeruli (Løfaldli et al., 2010), which resembles the number identified in females.
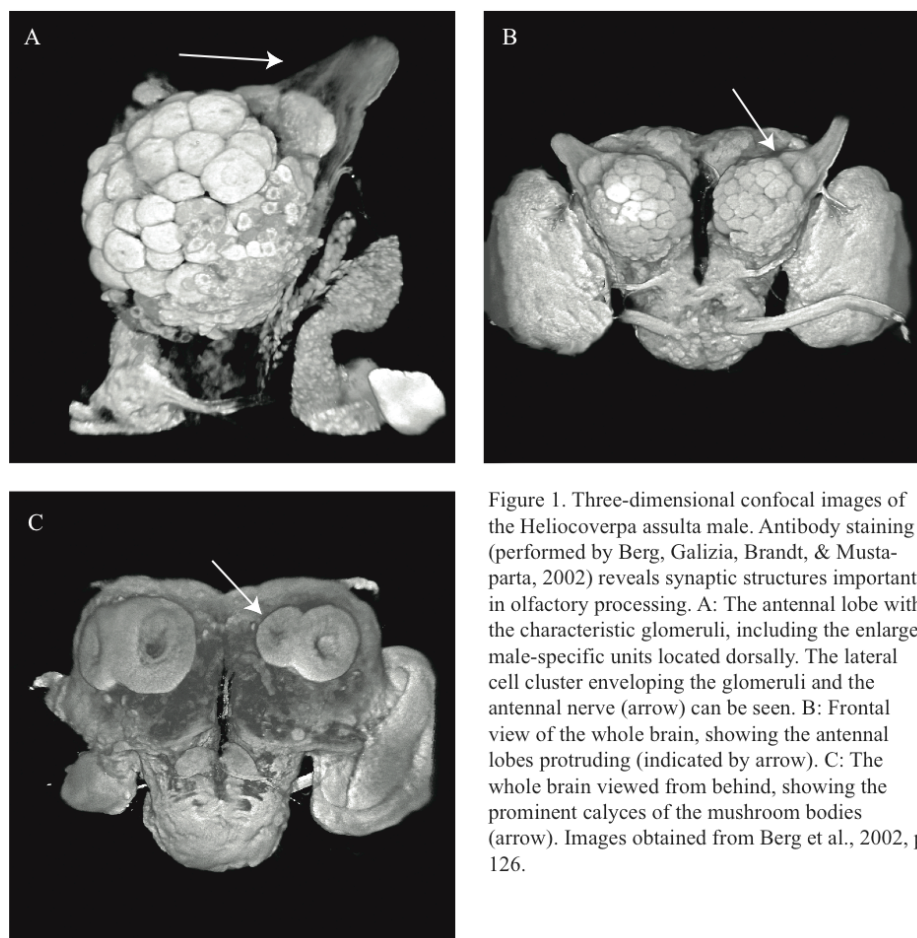
Figure 1. Three-dimensional confocal images of the Heliocoverpa assulta male. Antibody staining (performed by Berg, Galizia, Brandt, & Mustaparta, 2002) reveals synaptic structures important in olfactory processing. A: The antennal lobe with the characteristic glomeruli, including the enlarged male-specific units located dorsally. The lateral cell cluster enveloping the glomeruli and the antennal nerve (arrow) can be seen. B: Frontal view of the whole brain, showing the antennal lobes protruding (indicated by arrow). C: The whole brain viewed from behind, showing the prominent calyces of the mushroom bodies (arrow). Images obtained from Berg et al., 2002, p. 126.

Cell clusters of the antennal lobe

The somata of local interneurons and projections neurons are located in three cell clusters in the periphery of the antennal lobe: a large lateral cell cluster positioned ventro-laterally in the antennal lobe, a smaller medial cell cluster located dorso-medially and a tiny anterior cell cluster located antero-ventrally (Anton & Homberg, 1999; Berg, Galizia, Brandt, & Mustaparta, 2002; Homberg, Montague, & Hildebrand, 1988). Whereas the medial and anterior cell cluster contains projection-neuron somata, the lateral cell cluster comprises both projection neuron and local interneuron somata (Homberg et al., 1988).

The antennocerebral tracts

Three main antennocerebral tracts connect the antennal lobe with the protocerebrum: the inner, the middle and the outer antennocerebral tract (Homberg et al., 1988; Rø, Müller, & Mustaparta, 2007). The antennocerebral tracts consist of projection-neuron axons that transport odor information from the antennal lobe to higher integration areas of the brain.

The inner antennocerebral tract (IACT) exits the antennal lobe dorso-medially and bypasses the central body. In the posterior part of the protocerebrum it turns laterally and gives off neuronal branches to the calyces before it terminates in the ipsilateral protocerebrum (Homberg et al., 1988; Rø et al., 2007). The IACT has two major roots in the antennal lobe: the dorsal and the ventral root, which fuse when they reach the edge of the central body (Homberg et al., 1988). According to Homberg et al. (1988), it seems that the fibers in the dorsal root have their somata in the medial and the anterior cell cluster, whereas fibers in the ventral root have their somata in the lateral cell cluster. The IACT is the most prominent of the antennocerebral tracts, with about 400 fibers in the moth *Manduca sexta* (Homberg et al., 1988). The majority of neurons following the IACT are uniglomerular, meaning that they innervate one single glomerulus (Homberg et al., 1988). However, Rø et al. (2007) found a few neurons in the IACT with multiglomerular arborizations, meaning that the neurons innervate more that one glomerulus.

The middle antennocerebral tract (MACT) joins the IACT for a short distance before it bends laterally towards the lateral protocerebrum. Here, the middle tract divides into several branches that innervate different regions (Homberg et al., 1988; Rø et al., 2007). The MACT, which contains about 120 fibers, bends at the base of the central body and bypasses the pedunculus ventrally. Most axons in the MACT continue antero-laterally to

terminate in a particular region of the lateral protocerebrum, namely the lateral horn (Homberg et al., 1988). Here, the projections of the IACT and the MACT overlap. Some fibers of output neurons passing in the MACT project near the pedunculus, and some more posteriorly in the protocerebrum (Homberg et al., 1988). The somata of the projection neurons following the MACT are located in the lateral cell cluster and are small in size. The arborizations of the MACT neurons in the antennal lobe seem to be multiglomerular (Homberg et al., 1988; Rø et al., 2007). A substantial portion of the neurons following the MACT is GABAergic, meaning that these neurons are inhibitory (estimated to 40-70 fibers in *H. virescens*) (Berg, Schachtner, & Homberg, 2009; Homberg et al., 1988; Hoskins et al., 1986).

The outer antennocerebral tract (OACT) leaves the antennal lobe more ventrally than the IACT and the MACT, and then turns laterally. The main portion of the outer tract continues to the lateral parts of the protocerebrum where it projects to the lateral horn. Some of the fibers then turn dorso-medially and innervate the calyces (Homberg et al., 1988; Rø et al., 2007). The OACT is loosely fibered, and the diameter of the fibers is smaller than those projecting in the IACT and the MACT. Most of the neurons following the OACT probably have multiglomerular arborizations in the antennal lobe, but uniglomerular arborizations have also been reported (Homberg et al., 1988; Rø et al., 2007).

The antennocerebral tracts mentioned above are the three main pathways that connect the antennal lobe with the protocerebrum. However, there are also two additional tracts: the dorsal antennocerebral tract (DACT) and the dorsomedial antennocerebral tract (DMACT). The dorsal antennocerebral tract, which contains about 50 fibers, exits the antennal lobe dorsally. As it leaves the antennal lobe it makes a sharp lateral turn and moves along the antero-ventral margin of the pedunculus to the lateral protocerebrum (Homberg et al., 1988). The other additional tract, the DMACT, exits the antennal lobe dorso-medially and runs along the dorsal surface of the protocerebrum, towards the calyces (Homberg et al., 1988). The fibers in the DMACT, about 15 in total, project to different parts of the protocerebrum, including the contralateral protocerebrum, the inferior median protocerebrum, the lateral accessory lobe and the lateral horn (Homberg et al., 1988).

The mushroom bodies and the lateral protocerebrum

The antennocerebral tracts mentioned above conduct olfactory information mainly into two higher integration centers in the protocerebrum: the mushroom bodies and the lateral protocerebrum (Homberg et al., 1988). The mushroom bodies are paired neuropil structures, found in almost all insects, suggested to play a role in learning and memory (Strausfeld, Hansen, Li, Gomez, & Ito, 1998). The calyces of the mushroom bodies (see Figure 1C), which are cup-shaped structures, are made up of Kenyon cells. The Kenyon cell somata lie on the dorso-posterior surface of the protocerebrum with the dendrites forming the calyces. At the base of the calyces there are two tracts, made up by Kenyon cell axons, together forming the pedunculus that runs through the protocerebral neuropil and branches out into three main lobes: the α, the β and the γ lobe (Rø et al., 2007; Strausfeld et al., 1998). The Y lobe is a second lobe system that projects through the neuropil (Rø et al., 2007). The lateral protocerebrum, one of the main output areas of the insect brain, consists of fibers that receive input from olfactory, taste, visual and mechanosensory centers (De Belle & Kanzaki, 1999; Kvello, Løfaldli, Rybak, Menzel, & Mustaparta, 2009). The input from the olfactory center is mediated via all five antennocerebral tracts (Homberg et al., 1988; Rø et al., 2007).

Processing of olfactory information in the insect brain

The first events of odor detection take place when airborne stimuli hit the sensilla on the antenna of the insect. Odor molecules make their way through the pores in the cuticle of the sensillum. With the help of odorant-binding proteins in the sensillum lymph, odorants interact with the olfactory receptor neurons and initiate a transduction cascade (Steinbrecht, 1998). Recent findings by Sato, Pellegrino, Nakagawa, Nakagawa, Vosshall, and Touhara (2008) have suggested a transduction mechanism different from the indirect pathway previously described in insects (Stengl, Ziegelberger, Boekhoff, & Kriger, 1999). Thus, Sato and colleagues claim that the olfactory receptor is a complex of two subunits having the characteristics of a non-selective cation channel that is directly gated. Similar results are reported by Wicher, Schäfer, Bauernfeind, Stensmyr, Heller, Heinemann, and Hansson (2008), but with an additional metabotropic pathway of the kind previously described. This additional pathway implies that the odorant activates a G-protein that increases cyclic AMP production. The cyclic AMP, in its turn, induces a long-lasting non-selective cation conductance. Nevertheless, the main part of the

transduction mechanism in olfactory receptor neurons of insects seems to involve a dimer, consisting of two seven-transmembrane helical proteins that act as a directly gated ion channel. The transduction cascade causes a change in the membrane potential, eliciting action potentials that travel along the axon of the olfactory receptor neuron (Stengl et al., 1999).

A number of studies, utilizing molecular biological techniques, have demonstrated the odotopical organization of the antennal lobe, which means that one glomerulus receives input from olfactory receptor neurons expressing the same type of odor receptor protein (Axel, 2004; Vosshall, Wong, & Axel, 2000). These findings are in full accordance with those including tracing of physiologically characterized receptor neurons of the male specific pathway of moths (Christensen, Mustaparta, & Hildebrand, 1995; Zhao & Berg, 2010). Thus, the glomeruli seem to act as if a map of the sensory information is formed in the antennal lobe (Axel, 2004).

Relatively little is known about odor processing in higher integration areas, particularly concerning the function of the parallel antennocerebral tracts.

The vertebrate olfactory system

The olfactory organ
The olfactory organ of vertebrates, humans included, is the olfactory epithelium, located at the roof of the nasal cavity. Several million olfactory receptor neurons are embedded in this specialized tissue (Kandel, Schwartz & Jessell, 2000). The olfactory epithelium has three main types of cells: olfactory receptor cells, basal cells and supporting cells (Bear, Connors, & Paradiso, 2007). The vertebrate olfactory receptor cells are continuously renewed and have a cycle that lasts about 4-6 weeks. The basal cells are the source of new receptor neurons. Supporting cells produce mucus in the nasal cavity (Bear et al., 2007).

The olfactory receptor neuron
Like in insects, the vertebrate olfactory receptor neuron is a bipolar neuron directly connecting the brain with the outside world (Kandel et al., 2000). The sensory neuron has a single dendrite with a small knob ending in a thick layer of mucus. Thin cilia, which are

the site for the odor receptor, protrude from the knob. The axons that go through the cribriform plate of the scull make up the cranial nerve number one, the olfactory nerve (see Figure 2B).
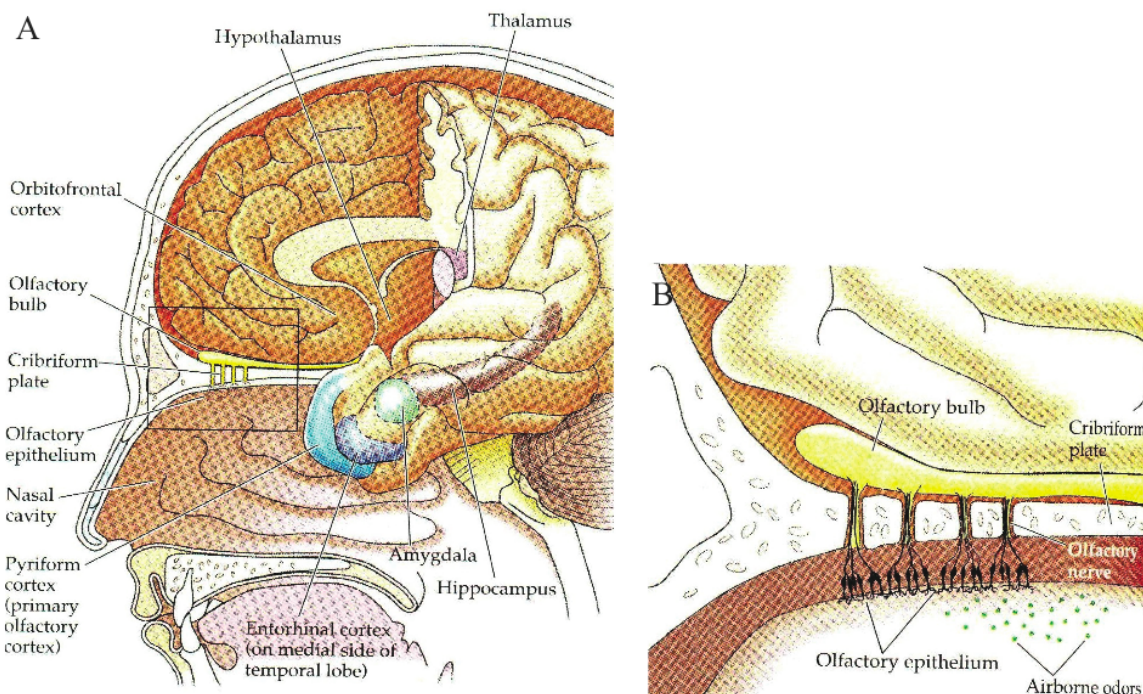
Figure 2. The human olfactory system. A: Central components of the olfactory pathways in humans. B: Enlargement of the box in A, showing the olfactory epithelium with the olfactory receptor neurons and the olfactory bulb. Images obtained from Purves, Augustine, Fitzpatrick, Katz, LaMantia, & McNamara, 1997, p. 264.

The olfactory bulb

The olfactory bulb, which is located above the olfactory epithelium in the frontal part of the brain, is the first level for processing olfactory information. Like in insects, the axons of the sensory neurons terminate on dendrites of second order neurons in spherical structures termed glomeruli (Bear et al., 2007; Purves, Augustine, Fitzpatrick, Katz, LaMantia & McNamara, 1997). Each glomerulus receives input from about 25,000 axons of olfactory receptor neurons that terminate on the dendrites of about 100 second order neurons (Bear et al., 2007). Thus, there is a pronounced convergence in the system as the olfactory receptor neurons are far more numerous than the output neurons they make synapses with (Hildebrand & Shepherd, 1997).

The olfactory receptor neurons synapse with two main neuron types inside the glomeruli: projection neurons and local interneurons (corresponding to the types reported in insects).

The vertebrate projection neurons include mitral cells and tufted cells, and the local interneurons include, amongst others, periglomerular neurons (Hildebrand & Shepherd, 1997). The axons of the mitral and tufted cells constitute the olfactory tract, which targets regions in the temporal lobe, in particular, the phylogenetically old olfactory cortex. The primary dendrites of each mitral and tufted relay neuron are located inside one single glomerulus, whereas the secondary dendrites are located outside the glomerular layer. The periglomerular interneurons make inhibitory synapses with the dendrites of mitral cells inside the glomeruli (Kandel et al., 2000). The different types of olfactory receptor neurons are randomly arranged in the olfactory epithelium, but the order is restored as the axons make their way into the olfactory bulb and the glomeruli. Each neuron expresses one type of receptor, and each type of olfactory receptor neuron connect to one or two invariant glomeruli (Axel, 1995, 2004).

The olfactory cortex

As mentioned above, axons of the olfactory bulb projection neuron make up the olfactory tract that mediates information to higher integration centers in the temporal lobe of the brain; i.e. the anterior olfactory nucleus, the pyriform cortex, parts of the amygdala, the olfactory tubercle and parts of the entorhinal cortex (Kandel et al., 2000). There is also a direct and an indirect pathway, via thalamus, to regions of neocortex, more precisely the orbitofrontal cortex located at the base of the frontal lobe. Finally, there are connections from the amygdala to the hypothalamus and from the entorhinal cortex to the hippocampus. Neural elements in the anterior olfactory nucleus project to the contralateral olfactory bulb (Kandel et al., 2000). Compared to other sensory systems, the anatomy of the vertebrate olfactory system is unique by not passing through the thalamus before it projects to the cortex. Parts of the central olfactory pathways overlap with the limbic system, which plays a major role regarding emotions (Bear et al., 2007; Purves, Augustine, Fitzpatrick, Hall, LaMantia, McNamara, & White, 2008). Brain areas are shown in figure 2A.

Processing of olfactory information in the vertebrate brain

The first step of odor detection involves molecules that bind to receptor proteins placed on the dendritic membrane of the olfactory receptor neuron (Axel, 1995). These receptors are members of a superfamiliy, called G-protein-coupled receptors, and have a seven-transmembrane domain (Buck & Axel, 1991; Mombaerts, 2004). The sensory neuron

converts the chemical energy into electric energy through the transduction process. The interaction between the odorant and the receptor protein leads to an intracellular G-protein mediated cascade reaction, producing the second messenger cAMP (Bear et al., 2007). This causes a depolarization in the olfactory neuron via opening of cyclic AMP dependent ion-channels. Like in insects, action potentials travel along the axon to particular addresses; i.e. distinct glomeruli in the primary olfactory center of the brain (Bear et al., 2007; Hildebrand & Shepherd, 1997).

Similarly to the antennal lobe of insects, the olfactory bulb of vertebrates is organized odotopically, meaning that particular odorants in the environment create distinct glomerular activation patterns in the antennal lobe (Hildebrand & Shepherd, 1997). Thus, the principle of odotopy seems to be universal.

The significance of comparative neurobiology

The interdisciplinary research field of modern neuroscience has often utilized model organisms for studying neural principles. In particular, many basic rules have been revealed by studying invertebrates: the mechanisms of the action potential were investigated in the squid (Freberg, 2006) and principles of neuro-plasticity, relevant for learning and memory, in the snail *Aplysia californica* (Kandel et al., 2000). With regards to understanding of chemosensory signals guiding reproductive behavior, the moth has served as an invaluable source of knowledge. In fact, the first pheromone identified was bombykol, produced by the female silk moth, *Bombyx mori* (Butenandt, 1959, as cited in Kaissling & Kasang, 1978) Thus; in the present study the male moth is used as a model object for investigating the anatomical organization of the olfactory pathways.

Aim of the thesis

Main aim: To gain knowledge about parallel olfactory pathways by performing retrograde staining of antennal-lobe projection neurons in the moth brain.

Specific goals:
1) To establish a technique for retrograde staining of the antennal-lobe projection neurons.

2) To visualize the axons, the dendritic arborizations and the somata of antennal lobe output neurons by combining the fluorescent staining technique with confocal microscopy.

3) To investigate whether the stained projection-neuron somata are distributed or gathered within the lateral cell cluster.

4) To investigate whether the total assembly of antennal-lobe glomeruli is evenly or unevenly innervated by the dendritic arborizations.

Materials and method

Ethics

The tobacco budworm moth *H. virescens* was used as a model organism in this study. All experiments were carried out according to Norwegian law regarding animal rights (Dyrevernloven § 1). Thus, no specific demands were necessary since the moth is an invertebrate. However, the insects were treated well, given reasonable amounts of space and food. All living material was available at the Medical Technical Research Centre, Neuroscience Unit, Norwegian University of Science and Technology.

Insects and preparation

H. virescens (Heliothinae; Lepidoptera; Noctuidae) pupae originating from a lab culture in Switzerland (Syngenta, Basel, Switzerland) were kept in an incubator (Refritherm 6E, Struers, Rødovre, Denmark) on a phase-shifted LD 14 h:10 h photoperiod at 22-23°C. Newly emerged moths were placed in separate plexiglass cylinders, sorted by gender, and allowed to feed on a sucrose solution. The insects were studied when they were 2-10 days old. During the experimental period, which lasted from August 2008 to March 2009, 92 male moths were used in the experiments.

Two different preparation procedures were used in this study. In one procedure the insect was restrained inside a small plastic tube, made from a 1-ml pipette tip from which the tapered end was cut off. The insect was fixed with utility wax (Kerr Corporations, Romulus, MI, USA) so that the head and antennae were exposed over the plastic tube. The scales were removed from the cuticula of the insect by forceps, and the head was tilted maximally forward in order to access the brain regions of interest; i.e. the lateral protocerebrum and the posteriorly located calyces. The brain was exposed by cutting the cuticle between the eyes and removing the mouthparts and the muscle tissue. This was done under a stereomicroscope (Leica Wild M38, Heerbrugg, Switzerland) by means of forceps, micro scissors and a knife made from a razor blade positioned in a razor blade holder.

The other procedure used in this study implied decapitation (separating the head from the body). The insect was refrigerated for a few minutes, to anesthetize it, before the head of the moth was cut off by fine scissors and placed in melted wax so that the posterior part of the brain was facing upwards. Wax was also melted around the head in order to keep the preparation stable during the experiment. The cuticle was cut open so that the brain regions of interest were easily accessible.

Staining procedure

The relevant areas of dye injection; the calyces of the mushroom bodies and the lateral protocerebrum, were carefully damaged with a scalpel or needle in order to allow axoplasmic transportation of the neural marker (Bear et al, 2007). Two staining techniques were used: application of dye crystals by hand and iontophoretic staining by means of a setup for intracellular recordings.

In the first technique, crystals of the dye dextran tetramethylrhodamine (Molecular Probes, Invitrogen, Carlsbad, CA, USA) were picked up by the tip of a small needle and inserted precisely into the current brain region by hand. Ringer's solution with sucrose was applied to the brain in order to keep the insect alive (NaCl: 150mM, KCl: 3mM, TES buffer: 10 mM, CaCl2: 3mM and sucrose: 25mM). The insect was stored over night in a refrigerator (4°C) or at room temperature for 3-4 hours in order to let the dye be transported within the neural axons. Preparations kept at room temperature were supplied with Ringer's solution every 30 minutes in order to avoid dehydration. The brain was then dissected out from the head capsule by use of forceps, micro scissors and a razor blade knife. After being fixed in 4% paraformaldehyde for one hour at room temperature, or over night in a refrigerator (4°C), a dehydration process was administered. The brain was emerged in a series of ethanol: 50%, 70%, 90% and 96% for 5 minutes each and 2x100%, 10 minutes each. After dehydration, the brain was put in metylsalicylate, with the purpose of making the tissue transparent so that it could be imaged through a light microscope and a confocal microscope. The brain was finally placed in methylsalicylate inside a cover slip.

The iontophoretic staining technique implied injection of dye into the brain via a glass electrode attached to a micromanipulator (Leica, Bensheim, Germany). The insect was

placed under a stereomicroscope (Leica MZ APO, Heerbrugg, Switzerland), and the microelectrode was accurately positioned inside the insect's brain by means of the micromanipulator. The tip of the microelectrode was filled with the fluorescent dye, dextran tetramethylrhodamine and 4% neurobiotin, called Micro Ruby (Molecular Probes, Invitrogen, Carlsbad, CA, USA). The glass capillary was back-filled with potassium acetate, a liquid that leads current. An attached amplifier made it possible to feed current into the electrode, for facilitating dye injection. The electrode was kept inside the brain with pulses of current passing through the circuit for 10 minutes. A chloridized silver wire inserted into the eye of the insect served as the indifferent electrode. After the staining procedure, the insect was kept in a refrigerator (4°C) over night, or for 3-4 hours in room temperature before it was dissected out of the head capsule. The brain was then fixed, dehydrated, cleared and mounted in methylsalicylate as described above.

In the first stages of this study, the antennal lobe was stained with crystals of dextran tetramethylrhodamine in order to map the location of the calyces of the mushroom bodies and the lateral protocerebrum. Thus, the staining indicated where to insert the dye in the experiments.

Confocal laser scanning microscopy

The brains were scanned by use of laser scanning microscopy (LSM 510 META Zeiss, Jena, Germany) with 10x, 20x, 40x and 63x objectives (C-Achroplan 10x/ 0,45 W, C-Achroplan 40x/0,8 W, Plan-Neofluar 10x/0,3, Plan-Neofluar 20x/0,5 l and C-Apochromat 63x/1.2 W corr.). The staining obtained from the fluorescence of dextran tetramethylrhodamine (ExMax 550 nm) was excited by the 543-nm line of a helium neon laser (HeNe 1). The interslice distance was 2 µm, and the mean was set to 4. The pinhole size was 1, and the resolution was 1024x1024 pixels.

Data analysis

The visualization software LSM FCS was used to reconstruct the confocal stacks. The three-dimensional images were made using a specialized tool of the LSM software. The software Adobe Photoshop 7.0 served as a tool for adjusting brightness and contrast of the images, and Adobe Illustrator CS4 was used to organize the images into figures.

Results

Injection of dye into the calyces of the mushroom bodies and the lateral protocerebrum, resulted in staining of five antennocerebral tracts: the inner, the middle, the outer, the dorsal and the dorsomedial antennocerebral tract. Furthermore, the dye injection resulted in a characteristic labeling pattern of the antennal lobe, including an array of spherical structures – glomeruli. In addition, the antennal lobe showed labeled somata located in all three cell clusters. The initial experiments, which implied labeling from the antennal lobe, clearly demonstrated the relevant regions for dye application: the calyces of the mushroom bodies and the lateral protocerebrum (see Figure 3).

Staining of the antennocerebral tracts

The inner antennocerebral tract (IACT)
Of the stained antennocerebral tracts, the inner constituted the most prominent one (see Figure 4A and D). All successfully stained preparations visualized the IACT as it passed the central body and projected towards the calyces. In figure 4D the two roots of the IACT, the ventral and the dorsal root, are visible as they exit the antennal lobe before they fuse together and become a coherent IACT. The ventral root connects to somata in the lateral cell cluster, and the dorsal root to somata in the medial cell cluster (see Figure 4D).

The middle antennocerebral tract (MACT)
Some preparations showed the MACT, following the IACT for a short distance. Close to the central body, the middle tract turned laterally and projected towards the lateral protocerebrum (see Figure 4B). As shown in figure 4A and B, a main portion of fibers in the middle tract is fused with the ventral root of the inner tract in the antennal lobe, and thus connects with the lateral cell cluster.

The outer antennocerebral tract (OACT)
The OACT was visualized in some preparations. As demonstrated in figure 4C and F, a loosely bundled gathering of axons leave the antennal lobe more ventrally than the IACT and the MACT, and then projects to the lateral protocerebrum.

The dorsomedial antennocerebral tract (DMACT)

The dorsomedial antennocerebral tract was found in a few preparations. As shown in figure 5, a relatively thin, but strongly stained tract exits the antennal lobe dorso-medially, and continues close to the brain midline towards the posterior protocerebrum. Single fibers of the DMACT could be traced in the antennal lobe, and these relatively thick branches innervated distinct glomeruli located ventrally (see Figure 5D).

The dorsal antennocerebral tract (DACT)

The dorsal antennocerebral tract was identified in one preperation (see Figure 6). A relatively thin bundle left the antennal lobe dorsally and projected laterally. Only a small segment of the tract was visible due to the strongly stained lobes of the mushroom bodies.

Staining of the cell clusters

Three cell clusters enclosing the antennal-lobe glomeruli were found: the lateral cell cluster, the medial cell cluster and the anterior cell cluster (see Figure 7).

The largest cell cluster, the lateral, was located ventro-laterally in the antennal lobe (see Figure 7A, B and C). Stained somata in this cluster consisted of two populations: one that contains a spatially grouped cluster of relatively small cell bodies located ventrally, and the other containing larger somata that were located more dorsally (See Figure 7C). Except for a gathering close to the origin of the dorsal root, the stained somata of larger size were distributed throughout the entire cell cluster and intermingled with unstained somata (see Figure 7B, C). The small somata located more ventrally were grouped (see Figure 7C). As seen in figure 7A and D, the medial cell cluster, positioned dorsally in the antennal lobe, is smaller than the lateral. However, it is more thoroughly stained than the lateral, as most somata therein are labeled. The anterior cell cluster was positioned at the most anterior region of the antennal lobe, medially of the lateral cell cluster and ventrally of the medial cluster (see Figure 7A, C). This small cell cluster consisted of a few somata considerably larger in size than those located in the two other clusters.

Staining of glomeruli in the antennal lobe

The staining pattern in the antennal lobe showed an array of glomeruli appearing as distinct spherical units. The glomeruli were situated at the outer parts of the antennal lobe, with no glomeruli in the mid area (See Figure 8A, B). The inner core contained an

unorganized arrangement of thick neural branches innervating the glomeruli. The four male-specific units of the MGC, located dorsally in the antennal lobe close to the entrance of the antennal nerve – the cumulus, the dorso-medial, the ventro-medial and the ventro-lateral unit – were identified (see Figure 8B). The numerous ordinary glomeruli were clearly separated from the MGC.

Some ordinary glomeruli turned out to be more strongly stained than others (See Figure 5D and 9). In one preparation, three thick fibers from the DMACT could be traced inside the antennal lobe. These branches heavily innervated three ventrally located glomeruli (see Figure 5D). The same preparation contained a few additional glomeruli that were also strongly labeled. However, it was not possible to decide their connection to particular tracts (see Figure 9C, D, E). Another preparation showed a strongly labeled glomerulus, with an unusual thick dendrite that splits in two before innervating one particular glomerulus located anteriorly in the antennal lobe (see Figure 9A, B).

Staining of the mushroom body lobes

Some preparations in this study revealed the lobes of the mushroom bodies. Figure 10A and B show the complex formation of this particular structure.
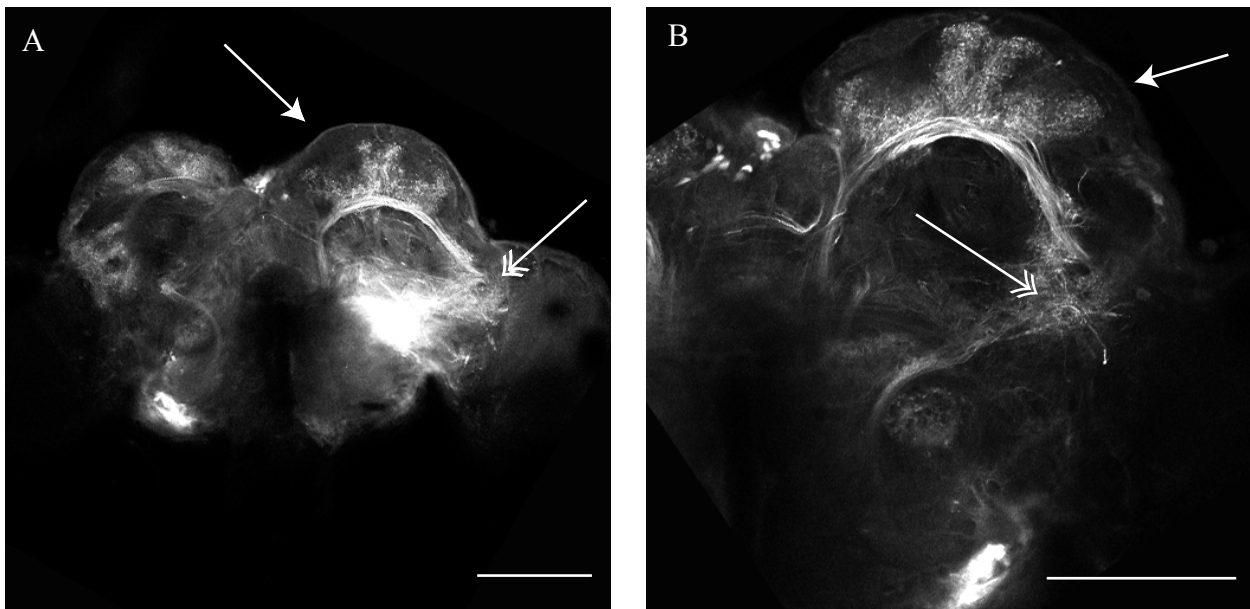
Figure 3. Confocal images of the calyces of the mushroom bodies and the lateral protocerebrum (dorsal orientation). A: The inner antennocerebral tract is visible as it turns laterally in the posterior protocerebrum and gives off projections to the calyces (arrow) before continuing to the lateral protocerebrum (double arrow). The brain is viewed through a 10x objective. B: Image from the same preparation, but viewed through a 20x objective. In addition to the inner tract, fibers of the outer antennocerebral tract are visible. Scale bars: 200 μm.
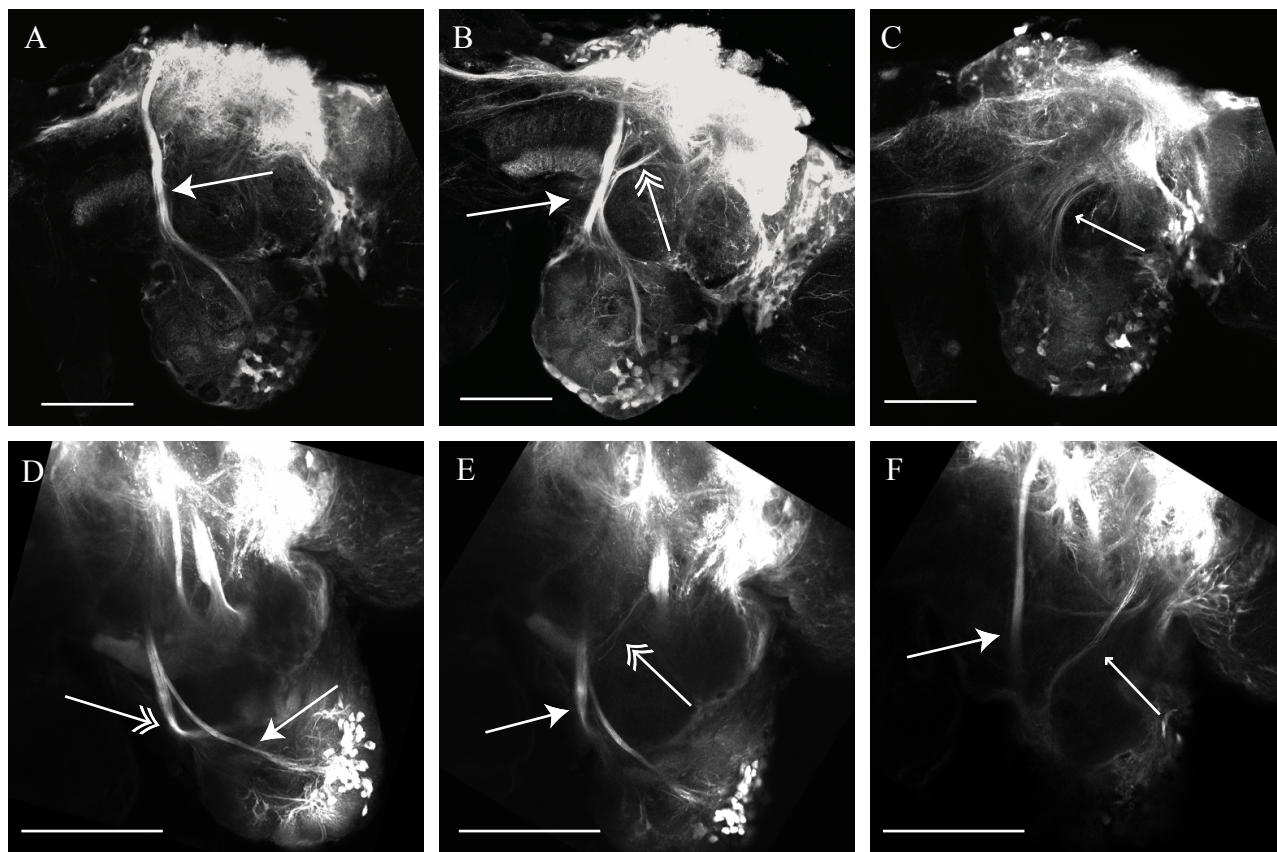
Figure 4. Confocal images of the three main antennocerebral tracts; the inner antennocerebral tract (IACT; single arrow), the medial antennocerebral tract (MACT; double arrow) and the outer antennocerebral tract (OACT; small arrow). A, B and C are from one preparation and D, E and F from another (both dorsally oriented and viewed through a 20x objective). A: Image section showing the IACT with the ventral root connected to the lateral cell cluster. B: Three-dimensional image showing the MACT as it joins the IACT before bending laterally at the central body (double arrow). C: Three-dimensional image showing the loosely fibered OACT that project to the lateral protocerebrum (small arrow). D: Three-dimensional image visualizing the dorsal (double arrow) and the ventral root of the IACT (single arrow). As demonstrated, the ventral root connects to stained somata of the lateral cell cluster. E: Image section showing the two roots of the IACT, again indicated by an arrow. Weakly stained fibers of the MACT are visible as they bend laterally (double arrow). F: Confocal section showing the IACT (arrow) and the OACT (small arrow) as it exits the antennal lobe and then projects towards the lateral protocerebrum. Scale bars: 200 μm.
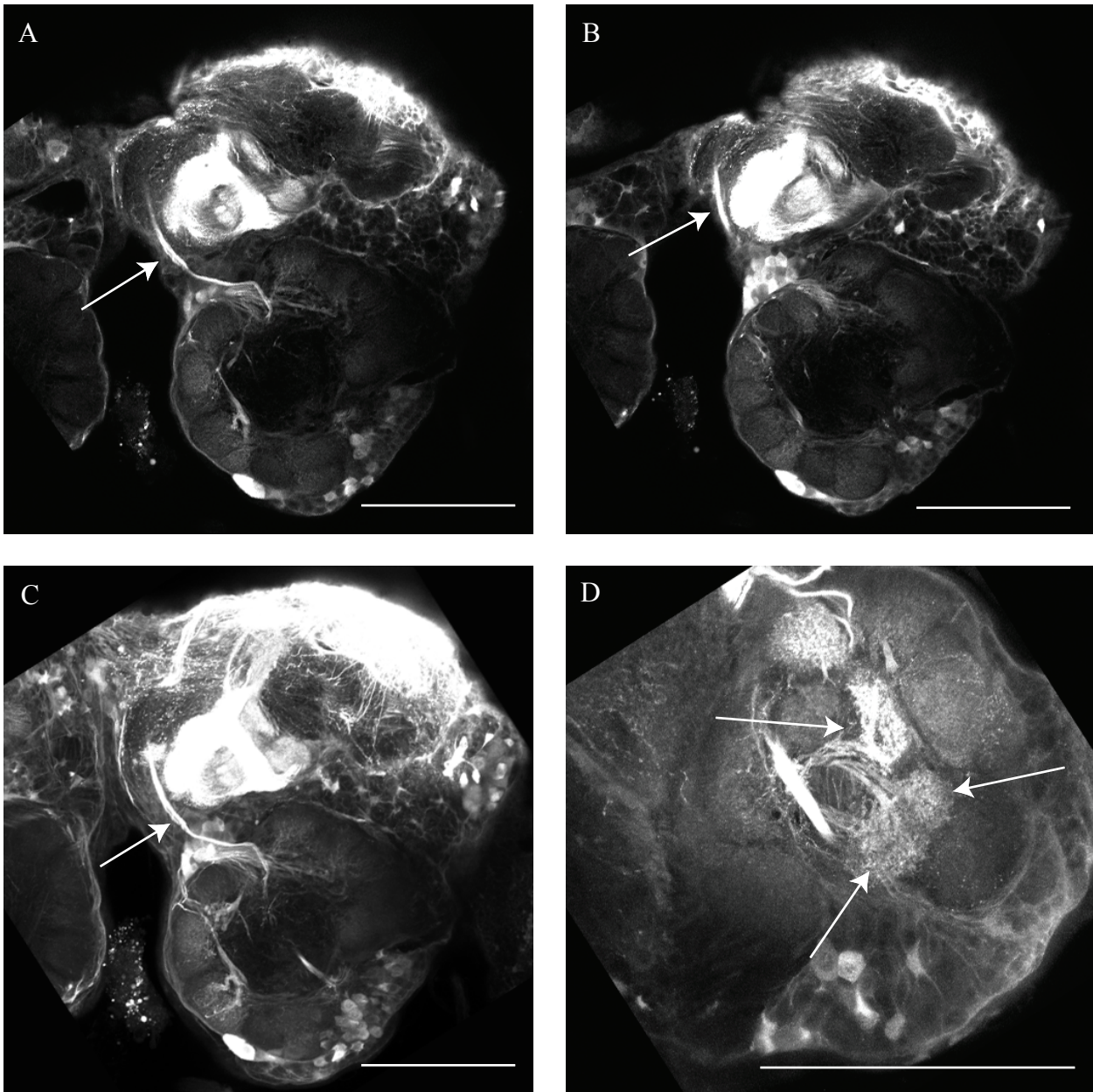
Figure 5. Confocal images of the dorsomedial antennocerebral tract (DMACT). A: One section showing the DMACT as it exits the antennal lobe dorsally before it bends medially and projects posteriorly close to the brain midline (arrow). B: One section showing a thick bundle of the DMACT as it passes the mushroom body lobes near the midline of the brain (arrow). C: Three-dimensional image showing the pathway for the DMACT. A, B and C is obtained through a 20x objective. D: Three-dimensional image of the same preparation as A, B, and C in a higher magnification, visualizing three strongly labeled glomeruli connecting to three fibers of the DMACT, indicated by heavily stained glomeruli (40x objective; arrows). Scale bars: 200 μm.

Figure 6. Confocal image showing a short segment of the dorsal antennocerebral tract (DACT) (20x objective). A thin bundle of fibers are seen exiting the antennal lobe dorsally (arrow). Scale bar: 200 μm.
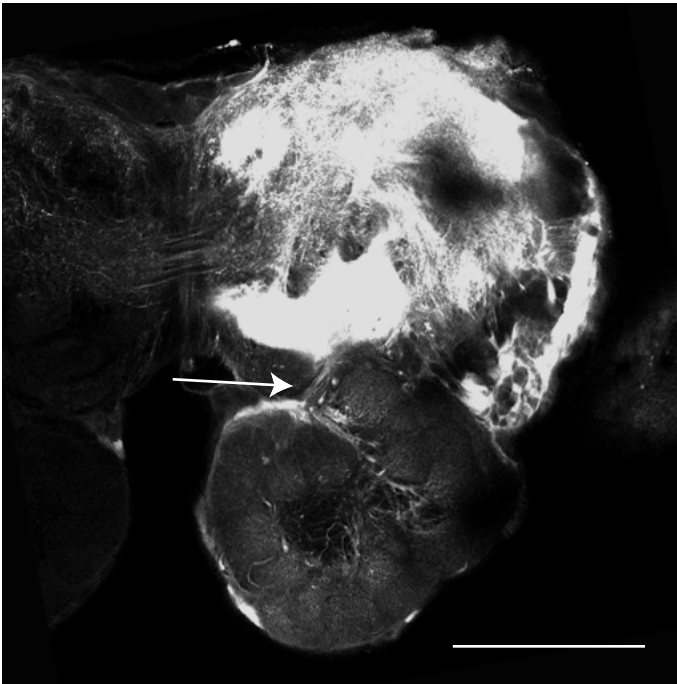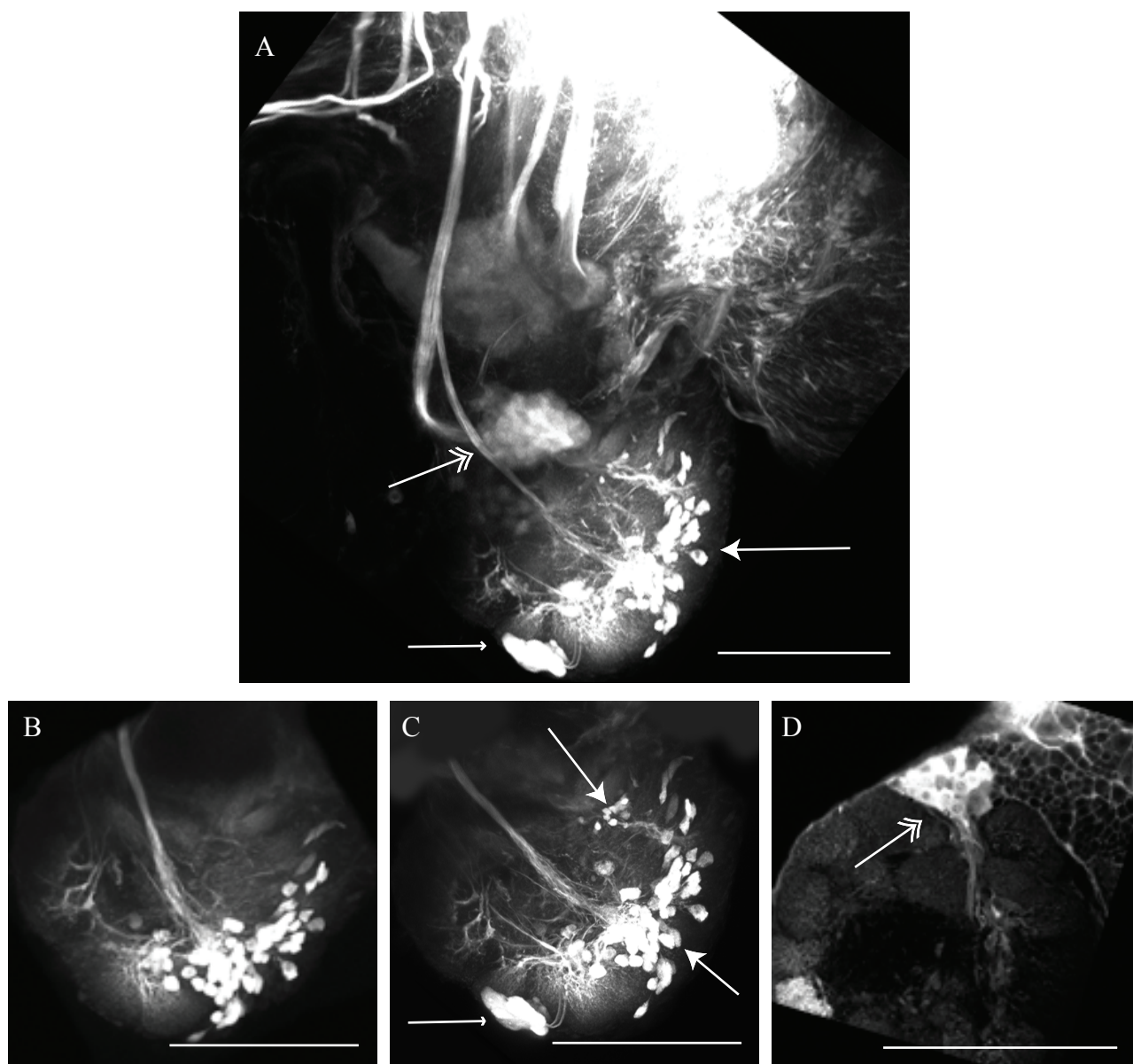
Figure 7. Confocal images of the three cell clusters of the antennal lobe: the lateral cell cluster, the medial cell cluster and the anterior cell cluster. A: Three-dimensional image showing the location of the three cell clusters in the antennal lobe. The lateral cell cluster, located ventro-laterally in the antennal lobe, is the largest (single arrow). The medial cell cluster, located dorsally, is smaller (double arrow). The anterior cell cluster is positioned anteriorly in the antennal lobe and comprises a few large somata (small arrow). Seen through a 20x objective. B: Three-dimensional confocal image (40x objective) visualizing the lateral cell cluster with stained cell bodies and neurites of the ventral root of the IACT. C: Three-dimensional image showing the small anterior cell cluster with large stained cell bodies seen through a 40x objective (small arrow), and the two groups of differently sized somata included in the lateral cluster (arrows). Both A, B and C are from the same preparation. D: Image section from another preparation showing the medial cell cluster seen through a 40x objective. As shown, relatively many of the somata are stained. Scale bars: 200 μm.

Figure 8. Confocal images showing the spherical glomeruli in the antennal lobe (40x objective). A: One section showing glomeruli innervated by thick dendrites. B: One section showing both ordinary glomeruli and the macroglomerular complex: the cumulus (C), the dorso-medial (dm), the ventro-medial (vm) and the ventro-lateral (vl) unit. Scale bars: 100 μm.
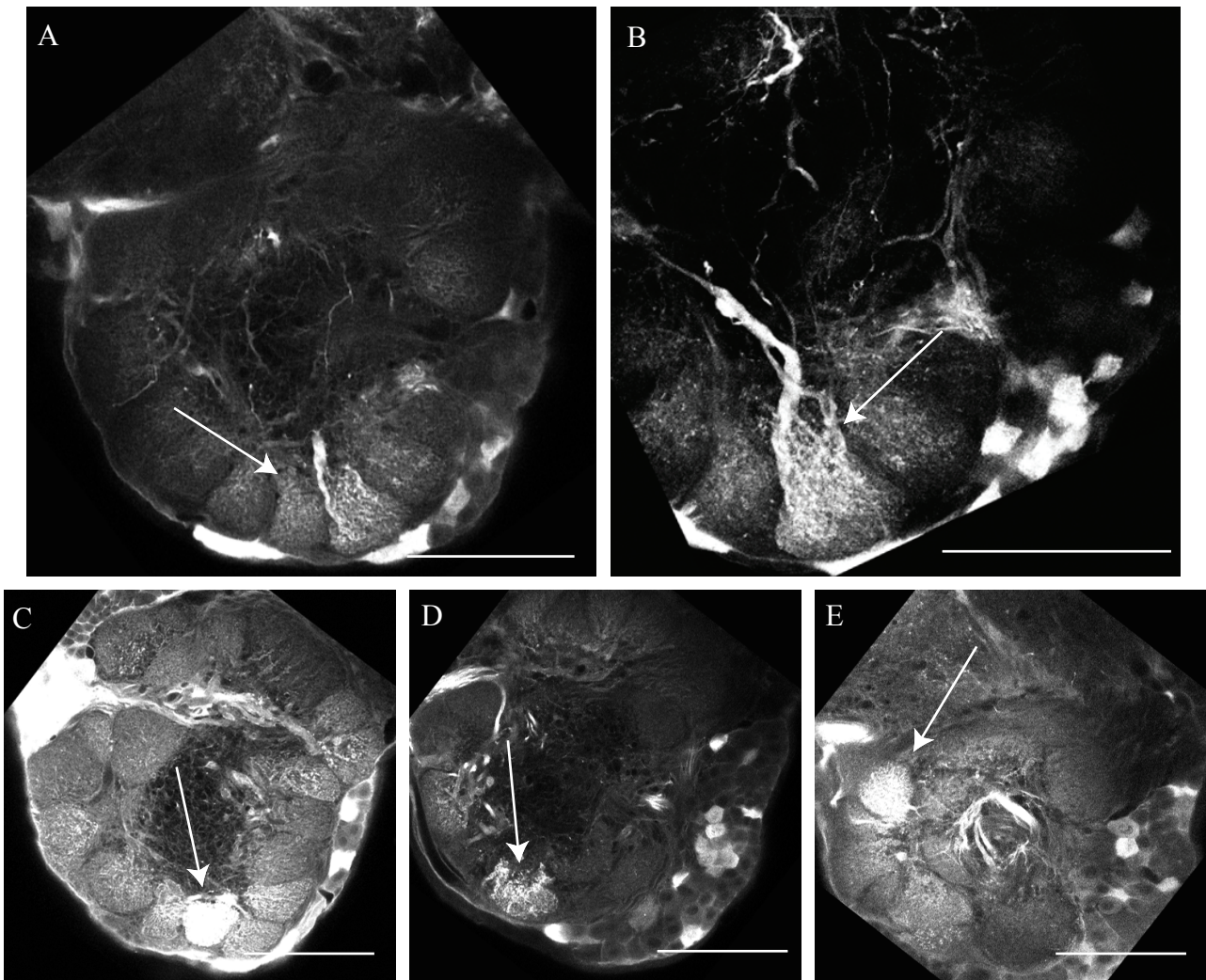
Figure 9. Confocal images of two preparations with densely innervated glomeruli. A: One section showing one densely innervated glomerulus (arrow; 40x objective). B: Three-dimensional image showing the same glomerulus at higher magnification (arrow; 63x objective). The dendrite splits in two before innervating the single glomerulus. C, D and E: Three sections from the same preparation, from dorsal to ventral, showing three strongly stained glomeruli (arrows). Scale bars: 100 μm.
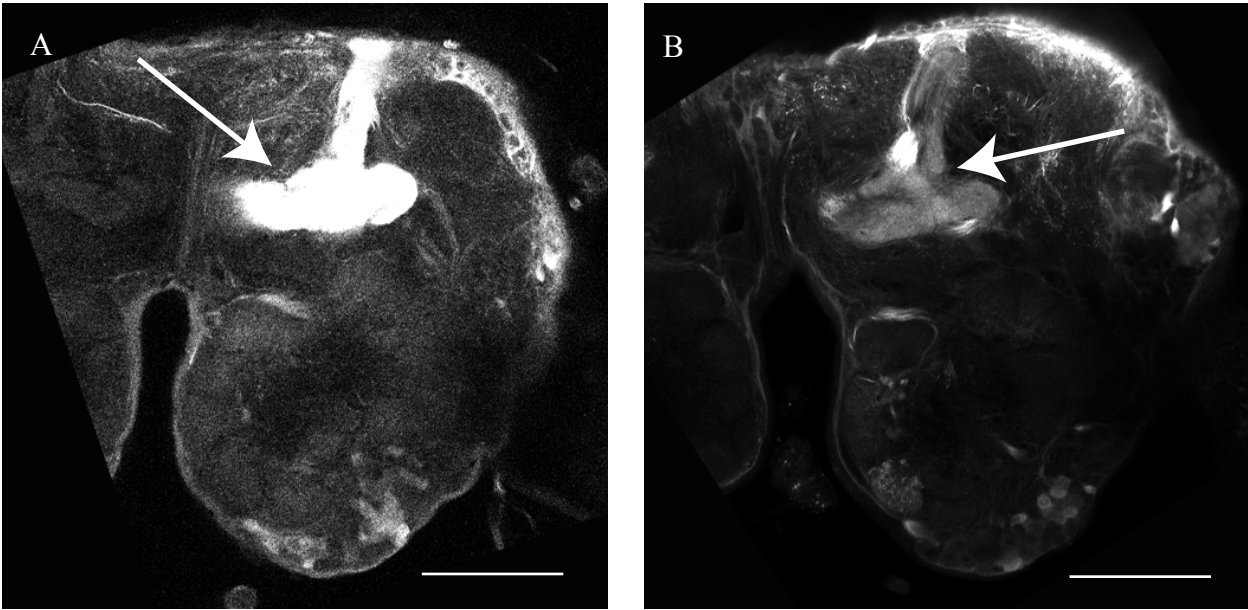
Figure 10. Confocal images of two preparations showing the mushroom body lobes. Three-dimensional images from two preparations showing the complex structure of the mushroom body lobes, in one preparation strongly labeled (A) and in the other more weakly labeled (B). Scale bars: 200 μm.

Discussion

Main findings

Dye injected into the relevant protocerebral regions, the calyces of the mushroom bodies and the lateral protocerebrum, stained projection neurons following five antennocerebral tracts identified as the IACT, the MACT, the OACT, the DMACT and the DACT. Glomeruli and cell bodies in the antennal lobe, which is the final destination for this kind of retrograde axonal transport, were also stained. The labeled somata were located in all three cell clusters; i.e. the lateral, the medial and the anterior cell cluster. In some preparations, the lobes of the mushroom bodies were heavily stained.

Preparation technique and staining procedure

Two different preparation procedures were used in this study. We started using a procedure with the insect restrained inside a plastic tube with the head still attached to the body. By injecting dye crystals by hand it was proven quite difficult to stain the calyces due to their localization in the posterior parts of the brain. In the second technique the head was cut off and placed in melted wax, so that the posterior parts of the brain was facing upward and therefore easy accessible for dye injection. Although this study does not include a systematic investigation of the two preparation procedures, it seems as if the procedure involving decapitation resulted in a more optimal staining of the calyces. Also, two different staining techniques were used in this study: application of dye crystals by hand and iontophoretic staining by a particular setup. Although the latter makes possible a more precise positioning of the dye in the area of interest, the iontophoretic technique seemed to stain the neural structures of interest less effectively than the other technique including crystals of dye.

Anatomical organization of output information from the primary olfactory center

Parallel antennocerebral tracts

The finding of three main antennocerebral tracts, as presented in this study, is coherent with previous reports concerning *H. virescens* (Rø et al., 2007) and *Manduca sexta* (Homberg et al., 1988), as well as a number of other insect species (Galizia & Rössler, 2010). Also, the finding of two additional tracts, the DMACT and the DACT, is in

agreement with former findings. The method used in this study included dye injections into the calyces and the lateral protocerebrum, whereas previous studies by Homberg et al. (1988) and Rø et al. (2007) involved dye injected into the antennal lobe. Therefore, this is the first study that performs retrograde staining of antennal lobe projection neurons in the moth.

Although parallel antennocerebral pathways are thoroughly described anatomically in several insect species (Galizia & Rössler, 2010), the knowledge about the function of this system is scarce. Parallel processing seems to be common in sensory systems, and the visual pathways in mammals are perhaps one of the arrangements best explored (regarding anatomy and function). From retina to the striate cortex there are three parallel pathways: the magnocellular, the parvo-interblob and the blob pathway, each processing different facets of the visual information; namely motion, shape and color (Bear et al., 2007). The olfactory system of the vertebrate crucian carp is also organized in parallel pathways, as the output neurons projecting from the olfactory bulb to the higher integration areas are divided into three bundles that mediate olfactory information related to feeding, reproduction and escape behavior (Hamdani & Døving, 2007).

Studying the system of parallel antennocerebral pathways in insects, it is interesting to pose the question of whether the tracts mediate information about different olfactory stimuli, as reported in the crucian carp, or whether they mediate information about different aspects of the same odor stimulus. Interestingly, male specific projection neurons mediating information about pheromones have been found in all the three main antennocerebral tracts in the moth (Homberg et al., 1988). This may indicate that, at least when it comes to pheromone information, multiple tracts mediate different aspects of the same odor stimulus. Such a system of parallel processing has been suggested by Müller, Abel, Brandt, Zöckler, & Menzel (2002) in their study on the honeybee, *Apis mellifera*. The antennocerebral tracts of the honeybee are somewhat differently organized from the model species used in this study. Whereas the moth has one main tract containing uniglomerular projection neurons, the honeybee has two. The so-called m-ACT and the l-ACT in the honeybee connect to distinct, non-overlapping glomeruli and target the mushroom bodies and the lateral horn; however, it does this in opposite order (Kirschner, Kleineidam, Zube, Rybak, Grünewald, & Rössler, 2006). Müller et al. (2002) suggested that the m-ACT and l-ACT in the honeybee mediate distinct information about the same

odor stimulus, i.e. different properties of that stimulus. More precisely, the authors reported that the neurons following the l-ACT process unspecific information quickly, whereas the m-ACT mediates specified olfactory information with latency. However, based on the finding of a dual pathway in the honeybee, including two tracts connecting segregated glomerular compartments in the antennal lobe, Kirschener et al. (2006) reports that each tract only receives input from one hemisphere of glomeruli. Furthermore, Kirschener et al. (2006) found that projections of the m-ACT and l-ACT were also segregated in the calyces and the lateral horn. Based on the discovery of a dual pathway, the authors point out alternative explanations for the function of the multiple tracts (Kirschener et al., 2006).

In general, many insect species seem to have established more or less corresponding systems that involve multiple output tracts carrying olfactory information from the antennal lobe to the protocerebrum. As mentioned above, the moth's IACT corresponds with the m-ACT and l-ACT in the honeybee. Interestingly, the mediolateral antennocerebral tract (ml-ACT) in the honeybee, consisting of three smaller tracts, may correspond with the MACT in the moth. Both the MACT and the ml-ACT are multiglomerular and target the lateral protocerebrum directly (Kirschner et al., 2006). Additionally, the ml-ACT and the MACT contain GABA-immunoreactive fibers, meaning that the tracts include pathways providing inhibitory information from the antennal lobe to the protocerebrum (*H. virescens*: Berg et al., 2009; *Manduca sexta:* Hoskins et al., 1986; *A. mellifera*: Schäfer & Bicker, 1986, cited in Berg et al., 2009). Even though the function of the GABAergic tract is not understood, it has been speculated that the release of GABA may lead to odor-dependent inhibition of the protocerebrum (Berg et al., 2009).

As previously mentioned, vertebrates and invertebrates share many similarities regarding the olfactory pathways. As described in the introduction, the mitral and tufted cells of the olfactory tract are output neurons that carry olfactory information from the olfactory center to higher integration areas in the brain (Kandel et al., 2000). Thus, the olfactory tract corresponds to the main antennocerebral tracts of the moth, also made up by output neurons transporting olfactory information from the primary processing center to higher integration areas. What is also interesting is that the projection neurons at this particular level of the olfactory pathway have similar arborization patterns; output neurons of both

vertebrates and invertebrates have dense dendritic arborizations forming non-overlapping glomeruli, whereas projections in the higher integration areas are wider and intermingled (Hildebrand & Shepherd, 1997; Wong, Wang, & Axel, 2002). Furthermore, the olfactory pathways are organized mainly ipsilaterally both in invertebrates and vertebrates, humans included. This means that output neurons project from the primary olfactory center to higher integration centers in the same hemisphere (Homberg et al., 1988; Kandel et al., 2000). This contrasts other sensory systems that to a large extent mediate the information bilaterally, like vision and hearing (Kandel et al., 2000).

Cell clusters in the antennal lobe

As mentioned previously, the lateral cell cluster houses somata of both projection neurons and local interneurons. Staining the projection neurons exclusively, as performed here, makes possible a visualization of their location in the lateral cell cluster. As demonstrated in the present study, the lateral cell cluster seems to be divided into two distinctly located subgroups, consisting of cell bodies of different size (see Figure 7C). This division of the lateral cell cluster is supported by Homberg et al. (1988). Some of the stained somata of large size are closely located at the origin of the ventral root, but in general it seems that they are distributed throughout the entire cell cluster (see Figure 7B). However, the group of smaller somata, located ventrally in the antennal lobe, seems to be positioned in near proximity of each other (see Figure 7C). As concerns the medial cell cluster, the more heavily staining of this cell cluster, as compared to the lateral (see Figure 7D), is probably due to the fact that the medial cluster contains projection-neuron somata only, and no local interneuron somata (Homberg et al., 1988). Shown through this study, the tiny anterior cell cluster contains only a few somata, but these are relatively large (see Figure 7C). Research by Homberg et al. (1988) reports that the anterior cell cluster houses approximately 16 cell bodies, which supports our findings.

Glomeruli

The findings of glomeruli appearing as separate, spherical structures inside the antennal lobe (see Figure 8), is in correspondence with a large number of previous publications (Anton & Homberg, 1999; Boeckh & Tolbert, 1993). The arrangement of glomeruli encircling the central core of the antennal lobe, with no glomeruli in the mid parts (see Figure 8), is also supported by previous research (Berg et al., 2002). The complete staining of the central parts of the glomerulus, as demonstrated in the present study, is

also shown in a previous study by Sun, Tolbert, & Hildebrand (1997). This arborization pattern differs from that of the sensory neurons, which seem to innervate the peripheral parts of the gomeruli.

As concerns the glomerular staining pattern, some preparations showed a few glomeruli that were more heavily stained than others (see Figure 5D and 9). This can be due to at least two circumstances: 1) that uniglomerular projection neurons connected to particular glomeruli, for some reason, have been strongly stained; and, 2) that distinct glomeruli contain a more densely packed network of dendrites. The fact that three fibers from the DMACT connected to three strongly labeled units demonstrates that particular glomeruli are more strongly innervated than others (see Figure 5D). Besides, the dendritic staining pattern of the thick DMACT fibers found in the present study suggests that the small population of projection neurons in the DMACT is uniglomerular. The dense uniglomerular arborizations for this neuron category, as reported here, is in accordance with previous findings by Rø et al. (2007), also describing uniglomerular projection neurons in the DMACT.

Concerning the first alternative, it may also be the case that particular projection neurons, for some reason, were strongly stained. In fact, Wong et al., (2002) state that there is a topographic map in higher integration centers of the fruit fly, *Drosophila melanogaster*, and that projection neurons that receive input from a given glomerulus send projections to the protocerebrum in a spatially invariant pattern. This map is different from the map in the antennal lobe in the way that the innervations are distinct, but wider and overlapping (Wong et al., 2002). Results from Marin, Jefferis, Komiyama, Zhu, and Luo (2002) support the findings. Given that a similar projection pattern is present in the moth, we might assume that some of the strongly stained glomeruli (see Figure 9A, B) are a result of staining gathered axonal projections originating from these glomeruli.

Mushroom body lobes
The mushroom body lobes were heavily stained in this study, and compared with the reconstructions by Rø et al. (2007), there is a similarity in both form and position of the lobes obtained (see Figure 10). The strong labeling of these structures is likely due to the numerous Kenyon cells that transport dye into the mushroom body lobes.

Future perspectives

The importance of doing this type of research is to gain new knowledge about how olfactory information is processed at higher levels of the neural pathway. In particular, the combination of anatomical data, as presented here, with data from physiological characterizations of projection neurons passing in the different tracts, may increase our understanding of the parallel antennocerebral pathways. The retrograde staining technique is suitable for studying the arrangement of the antennocerebral pathways; in particular, how projection-neuron somata are organized in the antennal lobe, and the connection pattern between specific tracts and the glomeruli they innervate. Additionally, the data of the present study, i.e. mapping of the antennocerebral pathways, could be adapted and integrated into a standardized brain atlas (Kvello et al., 2009). This would serve as a helpful tool when performing intracellular labeling, as the information about the antennocerebral tracts would illustrate which tract the labeled neuron is following.

Conclusion

The main aim of this thesis was to gain knowledge about the parallel olfactory pathways in the moth brain by means of retrograde staining of antennal-lobe projection neurons. Concerning the specific goals of the project, all issues were fulfilled. 1) A suitable preparation technique for retrograde staining of the current pathways was established. 2) The successfully stained brains visualized five antennocerebral tracts, as previously described. 3) The retrograde staining of the antennal lobe demonstrated that the projection-neuron somata were located in all three cell clusters. The projection-neuron somata of the lateral cell cluster consisted of two distinctly located groups including somata of different sizes. 4) Finally, the retrograde staining of the antennal lobe revealed, in addition to the general glomerular network, a small group of glomeruli that was particularly strongly innervated by fibers from the dorsomedial antennocerebral tract.

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