Mapping neural networks linked to a higher olfactory center in a model brain

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Preface

This master's thesis has been written at the chemosensory laboratory at the department of Psychology, Norwegian University of Science and Technology (NTNU). Working with this thesis has been truly educational and challenging, and I am thankful for getting the opportunity to immerse myself in such an exciting discipline.

Acknowledgements

First and foremost, I would like to thank my supervisor Professor Bente Gunnveig Berg, for her continuous guidance, dedication and feedback throughout the process of this master's thesis. It has been a privilege to work under such an enthusiastic, patience and knowledgeable person, and I am truly grateful for getting the opportunity to work on such an exciting project! I also wish to thank Elena Ian for teaching me the experimental procedures and helping me with the practical challenges in the lab, always being welcoming and positive. I would also like to give my thanks to all the other people working in the lab during this period: Professor Emeritus Hanna Mustaparta and master's students Andreas L., and Andreas G.

To my family and friends- thank you for believing in me and your encouragement throughout the process! I am grateful for having such supportive people in my life.

Abstract

The sense of smell provides important information about the surrounding environment, affecting a diverse set of behaviors in a wide range of animals. As a vital sense being importantly interconnected with learning and memory, olfaction has a profound impact on the life of many animals, humans included. The organization of the olfactory pathway shows many striking similarities across phyla, opening the possibility for studying general sensory principles using model organisms. With its advanced sense of smell and a relatively simple nervous system, the moth is a favorable model organism, having provided insight in several issues concerning chemosensory mechanisms. While the initial levels of the olfactory pathway have been relatively well explored, the higher levels are still poorly understood. The present study is the first to investigate neuronal networks connected to a higher olfactory center in heliothine moths, using a mass staining technique. Thus, by applying fluorescent dye into the *lateral horn*, distinct regions being directly connected with this neuropil were labeled both in the ipsi- and contralateral hemisphere. In addition to complementing previous studies, particular networks not previously described were characterized. The identification of a new contralateral antennal-lobe tract is considered to be of particular interest.

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Introduction

The sense of smell provides living organisms with rich information about several aspects of the surrounding environment, and the opportunity to respond to these. Olfactory stimuli consist of small, volatile molecules called odorants. In general, odors are critical for finding and selecting food as well as avoiding harmful environments. Also, olfactory signals provide essential information about other individuals' identity and physical state, which is important for social interaction and selecting a potential mate. In essence, all animals use olfactory information in order to survive.

As humans evolved, olfaction may not be as essential for survival as compared to more primitive animals, but no less insignificant in our daily lives. The profound impact of olfaction upon our behavior and overall quality of life is largely underestimated by most people. This may partly be explained by the fact that a substantial portion of olfactory processing resides outside our awareness. Thus, particular odor representations in the brain may be more or less "hidden" from our conscious perception. A second reason for underestimating the role of olfaction in humans is its importance for flavor perception. Thus, one intriguing aspect of olfaction is that what we experience as *taste*, is largely due to smell. The perception of taste is by many mistakenly attributed to be sensed within the mouth. However, it is actually a function of so called *retronasal stimulation*; in short, during food ingestion, molecules released from the food are pumped from the oral cavity to the olfactory epithelium in the nasal cavity, resulting in the perception of taste (Shepherd, 2006). The fact that olfaction is a critical part of our desire to eat becomes obvious if one experiences smell impairments, for example during a cold.

Another interesting characteristic typifying the olfactory system is its ability to trigger memories based on exposure to particular stimuli; the smell of a specific odor can spontaneously evoke a memory of a long-forgotten experience, event, or a person. Common for this type of recalls is that one has previously been exposed to a situation in which the odorant was accompanied by the object of memory; the odorant seems to be learned by classical conditioning. Interestingly, memories triggered by odor stimuli are often accompanied by a strong emotional component.

Similarities in anatomical organization of olfactory pathways across different phyla

Among all sensory systems, the sense of smell seems to be the best preserved system. Thus, research has demonstrated several striking similarities in the organization of the olfactory pathways across a wide variety of taxa, human and insects included (Ache &Young,

2005; Brodal, 2009; Jortner, 2016). The commonalities include the sensory neurons that bind odor molecules in the periphery, as well as central olfactory neurons and their synaptic organization in various centers of the brain. Despite the fact that the chemosensory system has been fine-tuned to meet the needs of highly different organisms being adapted to specific habitats, the anatomical organization of the olfactory pathway is quite similar.

Studying the human olfactory system through the use of in vivo methods is, as of today, fairly limited. However, brain models of various organisms are a valuable tool in exploring basic neural principles underlying processing of sensory information in general. The universal organization of the olfactory pathway makes findings derived from model brains relevant for our general knowledge about chemosensory systems. Insects of different species, such as moths, honeybees, locusts, and cockroaches, have proved valuable knowledge about chemosensory coding principles. There are several advantages of using this group of organisms for studying the olfactory system: 1) Insects have a relatively easy and accessible nervous systems compared to that of mammals. 2) The insect brain is highly devoted to olfaction. 3) The insect brain is small enough for being analyzed in a confocal microscopy as a whole. 4) Insects are not governed by Norwegian law concerning animal protection. Last, but not least, their long evolutionary history, including more than 400 million years, proves their general significance for the history of life on Earth.

In the following sections, a brief overview of both the mammalian and the insect olfactory pathway is presented.

Anatomical organization of the human olfactory pathway

Peripheral pathways: The olfactory epithelium and olfactory sensory neurons

The olfactory organ of vertebrates, the olfactory epithelium, is placed in the dorsal part of each nasal cavity. A significant number of olfactory sensory neurons (OSNs) are embedded in the epithelium. These OSNs are bipolar neurons extending two branches, one dendrite pointing towards the nasal cavity and one unmyelinated axon projecting directly into the brain. The single dendrite of each OSN expands into hair-like structures called cilia at the apical end. The membrane of these cilia holds a large number of specialized olfactory receptor proteins (ORs). ORs belong to the category of G-coupled proteins. The genes which code for these smell receptors are the largest known gene family in mammals, representing approximately 3-5% of the genome (Ache & Young, 2005; Buck & Axel, 1991).

Each OSN exhibit receptor specificity, expressing only one type of OR, but recognizes several different odorants (Buck & Bargmann, 2013; Jortner, 2016). The cilia are enclosed in

the mucus-covered part of the epithelium called the mucosa, which is abundant with different classes of proteins, among these, soluble proteins called odorant-binding proteins (OBPs) (Bear, Connors & Paradiso, 2007; Brodal, 2009; Buck & Bargmann, 2013; Fitzpatrick & Mooney, 2012). Upon entering the nasal cavity, the odorant dissolves in the mucus layer. Considering that most odorants are hydrophobic, it is assumed that OBPs function as passive carriers of odorant molecules to the receptor site, but the physiological significance of the OBPs in the mammalian olfactory system remains unclear (Bear et al., 2007; Brodal, 2009; Tegoni et al., 2000).

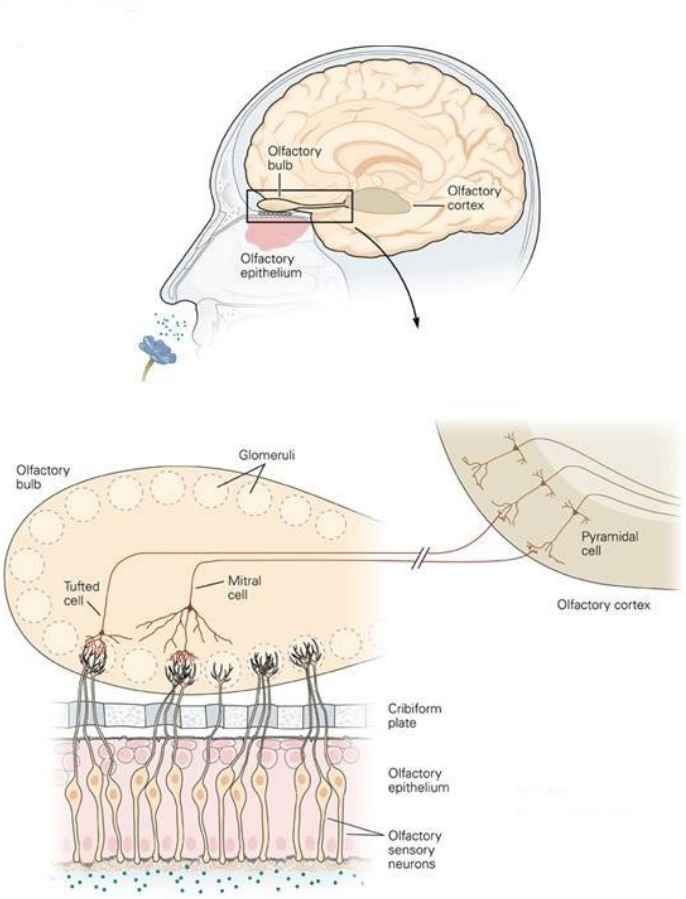

Figure 1. Schematic overview of the human olfactory system. Odor molecules bind to olfactory receptors located on the cilia of olfactory sensory neurons (OSNs). The collection of OSN axons extend through the cribriform plate into the olfactory bulb. The OSNs terminate in neuropilic structures called glomeruli, where they make synaptic contact with second order projection neurons (PNs) called tufted cells and mitral cells. The axons of the PNs leave the olfactory bulb to terminate in several areas of the primary olfactory cortex. (Figure from Buck & Bargmann, 2013, p. 713).

Once an odorant binds to a receptor, a process of transduction is initiated, in which the chemical energy is transformed to a subsequent electrical signal in the OSN. Thus, the odorant information is conveyed as action potentials along the individual sensory axons, which together constitutes the olfactory nerve (cranial nerve 1) (Bear et al., 2007; Brodal, 2009; Fitzpatrick & Mooney, 2012) Each cranial nerve leaves the epithelium and extends

through the cribriform plate, projecting ipsilaterally, to the primary olfactory center in each brain hemisphere, the olfactory bulb.

The olfactory bulb

The olfactory bulb contains a characteristic organization of spherical neuropil structures called glomeruli. Each glomerulus is innervated by axon terminals of about 25 000 OSNs making synaptic contact with the dendrites of approximately 40-100 second- order projection neurons (PNs), mitral- and tufted cells included (Bear et al., 2007; Buck & Bargmann, 2013; Jortner, 2016). Sensory neurons expressing the same type of OR target one or two glomeruli, meaning that specific odorants are represented in distinct glomeruli. This implies that each odorant activates a particular combination of glomeruli, called an *odotopic map* (Buck & Bargmann, 2013; Jortner, 2016). The activation pattern associated with a particular odorant will be transmitted by the PNs to higher olfactory centers and interpreted as a distinct odor quality (Buck & Bargmann, 2013; Jortner, 2016).

The assembly of glomeruli also contains ramifications of local interneurons, which processes are confined to the olfactory bulb, namely GABAergic periglomerular cells. These neurons, as well as granule cells located deep in the bulb, form inhibitory dendro-dendritic synapses with mitral- and tufted cells. Generally, local interneurons are assumed to be important for the synchronization of activity among PNs by enhancing the contrast between populations of strongly and weakly excited neurons, thus modulating the output signal (Aungst et al., 2003; Buck & Bargmann, 2013; Fitzpatrick & Mooney, 2012; Jortner, 2016). The axons of the PNs constitute the lateral olfactory tract, which relay olfactory information from the olfactory bulb to higher brain areas of the temporal lobe, the primary olfactory cortex.

The primary olfactory cortex

Unique for the olfactory system is that the connection of sensory neurons to primary sensory cortex does not involve thalamus. The lateral olfactory tract projects directly to several cortical areas of the temporal lobe, which collectively are defined as the primary olfactory cortex (Brodal, 2009). It comprises primarily the anterior olfactory nucleus, the lateral part of amygdala, the olfactory tubercle, entorhinal cortex and piriform cortex (Brodal, 2009; Buck & Bargmann, 2013; Fitzpatrick & Mooney, 2012; Jortner, 2016). Amygdala is a major component of the limbic system, a network of connected structures in the temporal lobe known to be important for emotions, motivation, and memory. The entorhinal cortex is the main relay to the hippocampal formation, which is involved in forming memory (Jortner,

2016). Piriform cortex, which is regarded as the major olfactory cortical area, is involved in odor recognition, discrimination, and memory. The close contiguity of smell centers and areas related to memory and emotions, explain emotional memories triggered by odors.

Terminal projections of mitral cells are distributed throughout the whole piriform cortex (Buck & Bargmann, 2013; Fitzpatrick & Mooney, 2012; Jortner, 2016). The PNs make synaptic connections with piriform pyramidal cells that mediate odorant information from primary olfactory cortex to a broad range of neocortical areas in the frontal lobe, called the secondary olfactory cortex. Parts of this information are mediated via thalamus. A significant number of projections innervate an area in the frontal lobe called orbitofrontal cortex. Piriform pyramidal neurons also innervate other regions, such as hippocampus and hypothalamus (Brodal, 2009; Buck & Bargmann, 2013; Fitzpatrick & Mooney, 2012).

Anatomical organization of the moth olfactory pathway

Peripheral pathways: The antennae and the olfactory sensory neurons

As in other insects, the main olfactory sensory organ of the moth is the antennae. Each antenna is densely covered with up to 200,000 cuticular hairs called olfactory sensilla, with each sensillum housing a single dendritic process from about 1-30 bipolar neurons of different types (Jortner, 2016; Roth, 2013). Similar to mammals, the OSNs are bipolar neurons with an unmyelinated axon projecting directly into the brain. The insect OSN also expresses only one type of OR, however, these are embedded in the dendritic membrane, thus compartmentalized inside the sensillar lymph. In contrast to the OSN of mammals, the receptor neurons of moths are characterized by a narrow tuning, responding selectively to certain odorants (Berg, Zhao & Wang, 2014; Røstelien, Stranden, Borg-Karlson & Mustaparta, 2005; Stranden al., 2003). Physiological studies of the moth family heliothine have demonstrated that pheromone- and plant odor neurons display high specificity, being most sensitive to one primary odorant and showing weaker responses to a few structurally similar molecules (Berg et al., 2014; Røstelien et al., 2005; Stranden et al., 2003).

The space between the dendritic membrane and cuticular wall is filled with a sensillar fluid containing OBPs and odorant-degrading enzymes (ODEs), an arrangement equivalent to that in the mammalian mucosa (Chang et al., 2015; Galizia & Lledo, 2013; Haupt, Sakurai, Namiki, Kazawa & Kanzaki, 2010; Pelosi, Zhou, Ban & Cavello, 2006; Pophof , 2004). Once an odorant enters the sensillar lymph through the cuticular pores perforated in the sensillar wall, the OBPs bind and transfer the molecule over the aqueous lymph to the OR (Galizia & Lledo, 2013; Pelosi et. al., 2006). Studies of the moth species *Chilo suppressalis* have shown

that appropriate combinations of OBPs and ORs improve odorant discrimination, thus, OBPs may have an essential role in odorant detection and discrimination in addition to serving as a general carrier protein (Chang et al., 2015; Pophof, 2004). After the activation of ORs, odorants are rapidly inactivated by ODE, which is assumed to be important for the ORs in maintaining high selectivity to subsequently incoming stimuli (Haupt et al., 2010).

The olfactory information is transmitted via the assembly of descending OSN axons, which together constitutes the antennal-nerve. As in mammals, it projects directly to the primary olfactory center of the brain, which is called the antennal-lobe (AL) in insects (Galizia & Lledo 2013; Jortner, 2016).

The primary olfactory center: the antennal-lobe

The AL is located anteriorly in the insect brain (Berg, Galizia, Brandt & Mustaparta, 2002). Similar to the mammalian olfactory bulb, the AL contains glomeruli, sites of synaptic contact between the axon terminals of OSNs and dendrites of antennal-lobe projection neurons (AL PNs). The somata of the AL PNs are gathered in one of three cell cluster situated peripherally in the AL; the lateral cell cluster, the medial cell cluster, and the anterior cell cluster (Homberg, Christensen & Hildebrand, 1989; Rø, Müller & Mustaparta, 2007). The dendritic arborizations of the PNs are either multiglomerular or uniglomerular, that is, innervating either one or several glomeruli.

In male moths, the organization of glomeruli is divided in two subunits; the macroglomerular complex (MGC) located dorsally, at the entrance of the antennal nerve, and the ordinary glomeruli (OG) (Berg et al., 2002; Homberg et al., 1989; Zhao et al., 2014). The MGC constitutes three to four units that receive axon terminals from male-specific OSNs responding to pheromones released by both conspecific and interspecific females (Berg, Almaas, Bjaalie & Mustaparta, 1998; Galizia & Lledo 2013). The more numerous OGs, also present in females, receive axon terminals from OSNs responding to general odorants, like plant odors. The number of antennal-lobe glomeruli in moths has previously been estimated to approximately 65 (Berg et al. 2014). However, recently 78-80 glomeruli were found in the male moth *Helicoverpa armigera* – a number which probably applies to other moth species as well (Zhao et al., 2016). Studies of the moth species *Heliothis virescens* and *Manduca sexta* have identified two female-specific glomeruli, located near the entrance of the antennal nerve (Berg et al., 2002; Rössler, Tolbert & Hildebrand, 1998).

In addition to the PNs, the AL glomeruli are innervated by two other categories of second-order neurons, namely local interneurons (LNs) and centrifugal neurons. LNs are

intrinsic cells of the AL, with their cell body gathered in one of the three cell cluster described above. These local neurons interconnect the individual glomeruli by multiglomerular dendritic arborizations, providing an internal circuitry in the AL (Galizia & Lledo, 2013; Haupt et al., 2010; Homberg et al., 1989; Martin et al., 2011; Rø et al., 2007). Similar to the local neurons of the mammalian olfactory bulb, the majorities of the LNs in insects are GABAergic (Berg, Schachtner & Homberg, 2009; Galizia & Lledo, 2013; Jortner, 2016) In the same manner, the inhibitory LNs induce odor-released synchronization of PNs activity in the AL, modulating the output signal before it reaches higher brain areas in the protocerebrum (Christensen, Waldrop, Harrow & Hildebrand, 1993; Jortner, 2016; Martin et al., 2011).

Centrifugal neurons usually have protocerebral origin, with their dendrites and somata residing outside the AL (Homberg et al., 1989; Rø et al., 2007; Zhao & Berg, 2009). These neurons are believed to provide modulatory feedback, affecting the signaling in AL output neurons according to information from other sensory modalities (Dacks, Christensen & Hildebrand, 2006; Zhao & Berg, 2009).

The antennal-lobe pathways

The AL PNs forward olfactory information from the AL glomeruli to several higher brain areas in the protocerebrum, by means of several antennal-lobe tracts (ALTs**)** (Homberg et al., 1989; Rø et al., 2007; Zhao et al., 2014). Three main ALTs are identified in several insect species; the medial ALT (mALT), the mediolateral ALT (mlALT), and the lateral ALT (lALT) (terminology adopted from Ito et al., 2014). The primary targets of the ALTs are the calyces of the mushroom bodies (calyces) and the lateral horn (LH).

The PNs of the mALT have mainly uniglomerular dendritic arborizations (Zhao et al., 2014). In the AL, these neurons give rise to a ventral- and a dorsal root; thus, PNs with somata in the lateral cell cluster leave through the ventral root, and PNs with somata in the medial cell cluster exit through the dorsal root (Homberg et al., 1989; Rø et al., 2007). The two tracts fuse as they leave the AL, and run posteriorly in the protocerebrum. The thick fiber bundle bypasses the lateral edge of the central body (CB) ventrally, and continues laterally toward the calyces (Homberg, Montague & Hildebrand, 1988; Rø et al., 2007). The mALT sends off branching terminals to the calyces before it continues to the lateral parts of the median protocerebrum and innervates the LH (Homberg et al., 1988; Rø et al., 2007; Zhao et al., 2014).

The PNs of the mlALT have multiglomerular ramifications, and their cell bodies are located in the lateral cell cluster of the AL (Homberg et al., 1988; Rø et al., 2007). This

relatively thin tract leaves the AL postero-medially and follows the mALT for a short distance. It turns laterally at the edge of the CB and projects directly to the LH, without innervating the calyces (Homberg et al., 1988; Rö et al., 2007). A substantial amount of the PNs confined to the mlALT are GABAergic (Berg et al., 2009).

The PNs constituting the lALT have both multi- and uniglomerular aborizations, and somata located in the lateral cell cluster. The relatively thick lALT leaves the AL more ventrally compared to the mALT and mlALT. Several of the lateral-tract axons project directly to the LH (Rø et al., 2007). It has been assumed that a considerable number of PNs following this tract proceeds further to the calyces. However, it is shown that they are quite few in number (Ian, Berg, Lillevoll & Berg, in revision).

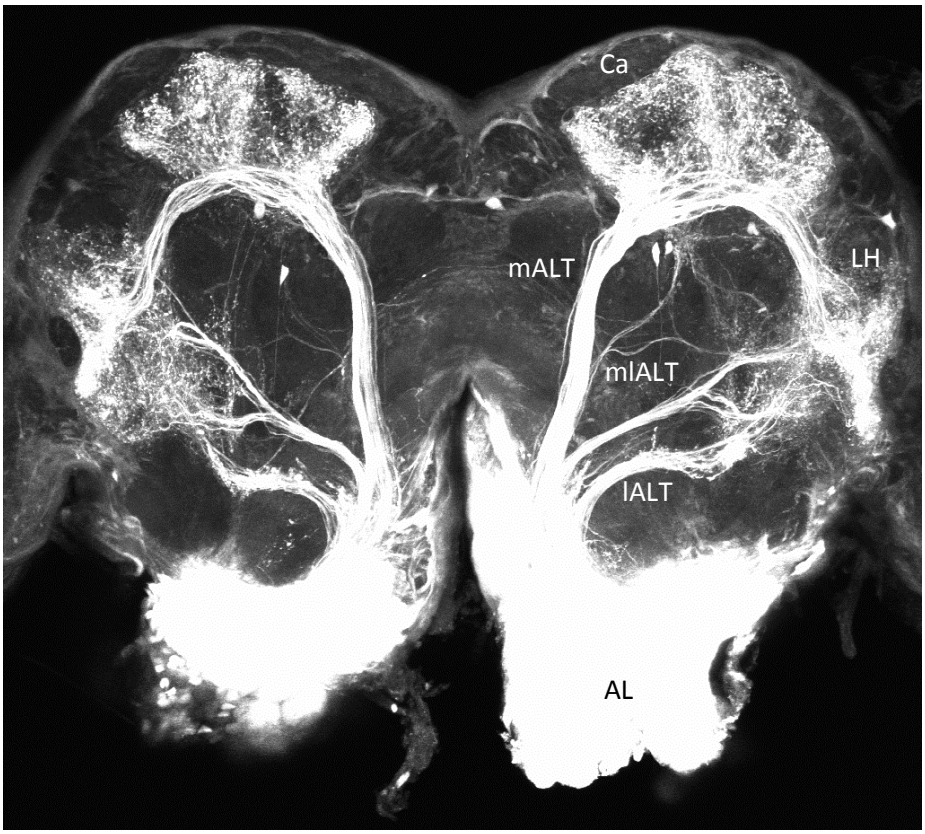

Figure 2. Confocal image displaying the projection pattern of the three main antennal-lobe tracts (ALTs) of the moth brain, in a dorsal oriented preparation. The medial ALT (mALT) projects to the calyces (Ca) and continues to the lateral horn (LH). The mediolateral ALT (mlALT) and the lateral ALT (lALT) project directly to the LH. (Xin-Cheng Zhao, unpublished data).

Higher olfactory centers: The calyces and the lateral horn

The calyces are prominent neuropilic structures with a recognizable architecture, located posteriorly in the protocerebrum. These are generally referred to as multisensory integration centers, recognized as dominant structures in the establishment of odor learning and memory in insects (Belle & Heisenberg, 1994; Fahrbach, 2006; Galizia & Rössel, 2010;

Menzel, Leboulle & Hammer, 1997). The calyces take the form of two cup-shaped structures called the medial and the lateral calyx, constituting the primary calyces. These are constructed by the dendrites of so called Kenyon cells (KCs), and constitute the input area of the mushroom bodies (Fahrbach, 2006; Galizia & Lledo, 2013; Jortner, 2016; Rø et al., 2007). As mentioned, PNs following the mALT innervate the calyces and thus provide input to the large number of KCs. The cavities of the cups are occupied by KC somata, which axons fuse to form the peduncle and lobes, constituting the output node of the mushroom bodies (Fahrbach, 2006; Galizia et al., 2013; Jortner, 2016; Rø et al., 2007).

As the only folded structure in the insect brain, the calyces have interestingly been compared to the olfactory cortex of mammals/humans (Heisenberg, 1998; Jortner, 2016; Strausfeld, Hansen, Yongsheng, Gomes & Ito, 1998). As mentioned in pervious sections (p. 4-5), several structures of the mammalian olfactory cortex are essential for odor memory processes. Similarities in structure and function to parts of the mammalian cortex, makes calyces a promising network model for studying brain processes associated with learning and memory (Heisenberg, 1998).

Interestingly, the separation of pheromone and plant odor information is reported to be maintained in the higher olfactory centers of the male moth; here the PNs originating from the MGC and the OG show different projection patterns both in the calyces and the LH (Zhao et al., 2014).

The LH is a region of the lateral protocerebrum, defined by the terminal arborizations of the AL PNs (Galizia & Rössel, 2010; Ito et al., 2014; Zhao et al., 2014). Compared to the calyces, less is known about the function of the LH in olfaction, although some possible roles have been suggested. It is generally regarded as a multimodal integration center that is more closely connected to the motoric system than the calyces. Furthermore, the brain region is suggested to mediate innate behavioral responses (Galizia & Lledo, 2013; Jortner, 2016; Ruta et al., 2010; Zhao et al., 2014). Innate preferences for signals allow the animal to rapidly and efficiently respond to biologically relevant stimuli (Galizia & Lledo, 2013; Menzel, 2013). Many innate odor responses are assumed to be initiated via this route, e.g. orientation behavior towards pheromones released by a conspecific female (Galizia & Lledo, 2013; Namiki, Iwabuchi, Kono & Kanzaki, 2014; Ruta et al., 2010). In fact, studies have identified specific circuits for pheromone orientation, demonstrating the close connection between the LH and motoric neurons involved in eliciting this behavior (Namiki et al., 2014; Ruta et al., 2010).

Also, the LH is assumed to mediate bilateral integration of sensory information; while information received from the OSNs at the initial levels of the olfactory system is mainly processed ipsilaterally, this information is thought to be bilaterally integrated in the LH (Gupta & Stopfer, 2012; Namiki et al., 2014). Furthermore, a study by Gupta and Stopfer (2012) on the loctus brain, suggests that LH plays an important role in extracting general stimulus features, such as odor intensity, thus, contributing to concentration coding. The same study revealed a profound diversity of LH neurons, comprising at least 10 distinct morphological classes. This kind of diversity indicates that the LH may play a role in processing information originating from other sensory modalities (Gupta & Stopfer, 2012).

As mentioned above, a division of the LH is made according to the distinct projection pattern of the neurons originating in the MCG and the OGs, respectively; studies of several moths species, including the heliothine subfamily, have shown that axons of pheromone specific neurons terminate in an area located slightly more medially, anteriorly, and dorsally to that innervated by PNs transmitting information about general odorants (Homberg et al., 1988; Zhao et al., 2014). This kind of sub division has also been identified in the silk moth, *Bombyx mori*. Here, the region receiving pheromone information is referred to as the delta area of the inferior lateral protocerebrum (ΔILPC), and that receiving information from plant odorants is more generally referred to as the LH (Namiki et al., 2014). Different innervation patterns of pheromone specific versus fruit odor specific neurons in the lateral protocerebrum are also found in the fruit fly, *Drosophila* (Jefferis et al. 2007). Thus, it is evident that signals evoking different behavioral responses are represented in partly distinct subfields of the LH (Berg et al., 2014; Namiki et al., 2014; Zhao et al., 2014).

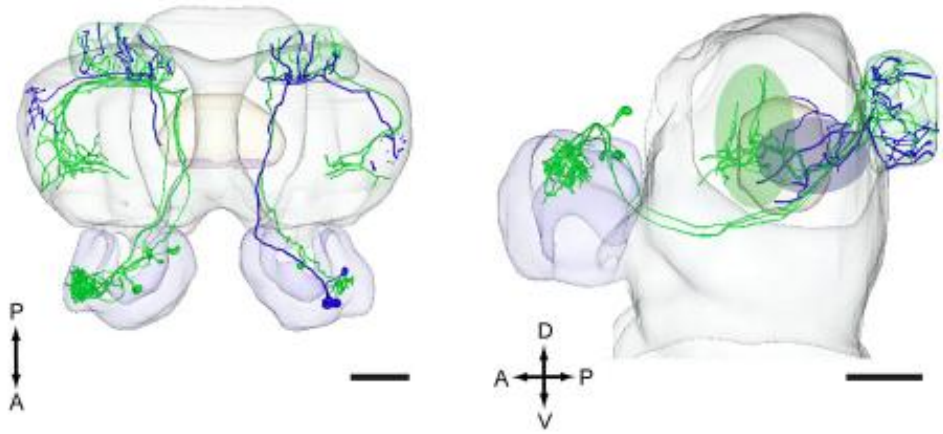

Figure 3. Standard brain atlas of the *Heliothis virescens,* in a dorsal and sagittal view, displaying the segregation of pheromone information and plant odor information in distinct regions of the lateral horn (LH). Pheromone projection neurons originating from the MGC (green) innervate a region of the LH located more medially and anteriorly to that innervated by plant odor neurons originating in the ordinary glomeruli (blue). (Figure from Zhao et al., 2014, p. 10).

Main aim of the thesis

In order to fully understand olfactory mechanisms, it is necessary to address each level of the olfactory pathway. Whereas the two first levels of the olfactory pathway have been relatively thoroughly studied, little is known about the subsequent paths. In moths, for example, knowledge about third order olfactory neurons is particularly scarce. Therefore, the present project intended to study the neural connections to a prominent secondary olfactory center in the heliothine moth brain.

Principal goal: To explore the olfactory neural networks linked to a higher olfactory center in the moth brain.

Specific goals:

- To map the dendritic arborizations of ipsilateral and contralateral antennal-lobe projection neurons targeting the lateral horn.
- To identify termination regions of third-order neurons projecting out from the lateral horn.

Materials and methods

The insects

Moths of the species *Heliothis virescens* (Lepidoptera; Noctuidae, Heliothinae) and *Helicoverpa armigera* (Lepidoptera; Noctuidae, Heliothinae), both males and females, were used in the experiments. The *H. virescens* originated from a lab culture bred in Bayern (Crop science, Germany), and was delivered to our lab as larvae. Here, the larvae were kept in a climate incubator (BINDER, GmbH, Tuttlingen, Germany) at 27°C and 66% air humidity, and fed an artificial diet up until pupation. *H. armigera* originated from China and arrived to our lab as pupae. The insect pupae from both species were kept separated and sorted by gender immediately after pupation/arrival, and were further placed in distinct hatching chambers (18 x 12 x 17 cm). The hatching chambers were placed in two separate climate cabinets (Refitherem 200, Struers-Kebolab, Albertsund, Denmark) with a reversed day and night photoperiod cycle (14 hours light: 10 hours dark), a temperature of 22-24°C and humidity of 70%. Hatched insects were transferred to a cylindrical Plexiglas container (18 x 10 cm), and kept in the same climate cabinets up until the preparation procedure. Both hatching cages and the Plexiglas containers were equipped with a sucrose solution (10%) and paper sheets.

Ethical considerations

The Norwegian Law of animal welfare (Dyrevernloven) does not comprise Lepidoptera, thus there are no formal restrictions regarding use of this order for experimental purposes. Irrespective of legislate guidelines, the life of any organism should be respected in their own right, however low in order or utility value for humans. The insects in our lab were well cared for and inspected daily. They were held in proper conditions of temperature and light, and had access to a large quantity of sucrose solution, which was replaced regularly. The hatching chambers and Plexiglas cylinders were equipped with paper sheets for insects to climb on. The cylinders were cleaned at least once a week, with dead insects being removed. Each cylinder contained a maximum of 8 moths in order to avoid space-related stress.

Selection of fluorescent dyes

One fluorescent dye, dextran tetramethylrhodamine/biotin (3000 MW; Micro-Ruby; ext/emis: 490/508 nm), was used in the experiments. The dye was stored in a freezer at -20^{o}C, thus retained in its crystalline form. Ahead of the staining procedure, the closed glass bottle containing the dye crystals was kept in darkness, at room temperature, for a short period before being opened in order to prevent condensation of the fluorescent crystals. The

fluorescent dye used in this study was provided by Life Technologies (www.lifetechologies.com).

Preparation of the insects

Prior to the preparation procedure, the insect was sedated in a refrigerator for 5-10 minutes. It was then restrained inside a narrow plastic pipette with the tapering end cut off, leaving the thorax and wings inside the pipette and the head exposed on the outside. The head was further immobilized by means of dental wax attached around the opening of the pipette. Under a microscope (Leica; MZ 12.5), hair and cephalic scales were removed with forceps, and remaining hair was removed by wiping a wet medical paper over the insect's head capsule. Next, one of the two antennae was cut off by using a micro-scissor. This was done in order to simplify the process of removing the cuticle, when making an incision crossing the area of the removed antenna. Although it is important to leave the brain as intact as possible, this was necessary in order to provide a better overview of the brain, making the lateral horn more accessible by creating sufficient space for targeting this brain area.

In dorsal view, an incision was first made posteriorly, followed by an incision crossing the area of the removed antenna. The antenna on the opposite side was left intact by creating an incision medially to the antenna. Finally, an incision was made between the eyes, detaching the cuticle from the rest of the insect's exoskeleton and exposing the brain. Ringer's solution (NaCl: 150mM, KCl: 3mM, TES buffer: 10MM, $CaCl^2$: 25mM sucrose, pH: 6.9) was applied immediately after picking up the cuticle with forceps, in order to keep the neural tissue alive and hydrated. Next, the preparation was placed such as the brain was in a dorsal, slightly frontally tilted orientation. The tracheas and intracranial muscles were removed to eliminate movements, and to further expose the brain. Fluids were soaked up using a medical paper, in purpose of revealing the thin neuronal sheet covering the brain. The neuronal sheet was thereafter removed by fine forceps, in order to ease the penetration of the staining electrode. Ringer's solution was applied continuously up until and throughout the dissection procedure.

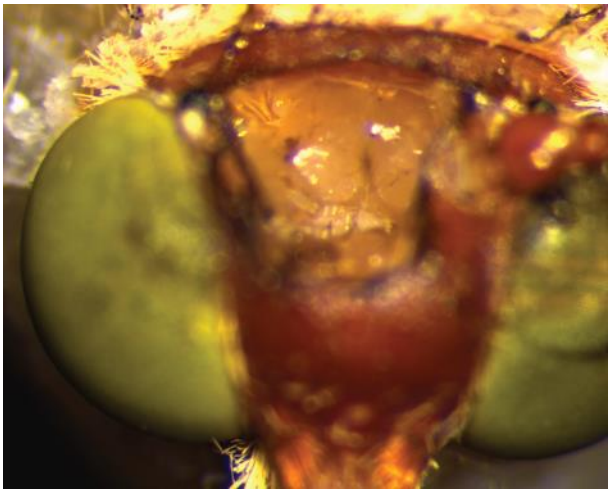

Figure 4. Display of the exposed brain.

Staining of the lateral horn

A micro needle was used to pick up crystals of the fluorescent dye (Vaseline on the tip of the needle was used in some staining experiments). Immediately before inserting the dye, fluid was soaked up using a medical wipe, temporarily drying the brain. The tip of the micro needle was then inserted manually into the LH. Ringer's solution was applied after dye injection, and thereafter soaked up by using a medical wipe. This was done with the purpose of removing excess dye, and in order to reveal whether the area of interest was stained. To make sure that the brain got sufficient nutrition and was kept hydrated up until the dissection procedure, a medical wipe soaked in Ringer's solution was placed on top of the brain. The preparation was then sited in a lightproof and moist container, either in room temperature for 2 hours if the dissection was performed the same day or in a refrigerator at 4^{o}C if dissection would take place the following morning. This procedure gives the dye sufficient time to be transported.

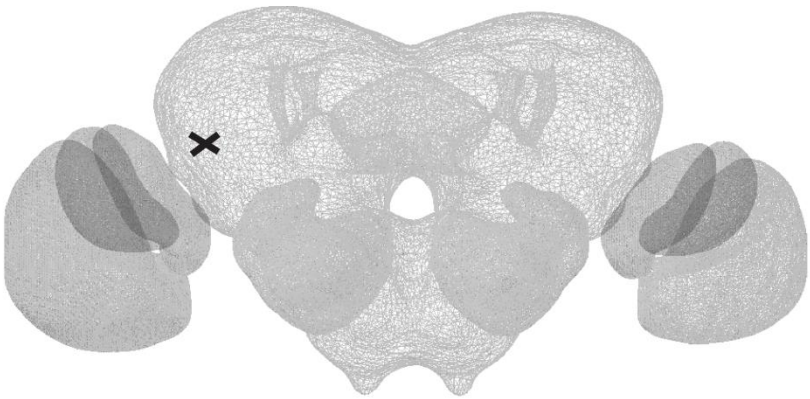

Figure 5. A standard brain model of the *Heliothis virescens*, indicating the approximate site for dye application (cross) during mass staining. The moth brain was positioned in a dorsal, slightly frontally tilted orientation during the staining procedure.

Dissection and fixation

The insect was placed under a microscope, and the paper tissue covering the brain was removed. The proboscis and neck were cut off with a micro-scissor, and the head was picked up with forceps. A piece of dental wax, which was placed in a dissection bowl, was heated using a soldering iron, quickly followed by placing the head in the wax before it congealed. This was done in order to facilitate the dissection, by restraining the head in wax when dissecting the brain out from the exoskeleton. First, the corneal facet lens was removed by using a sharp razor blade knife. Next, each segment of the exoskeleton was cut with a micro-scissor, detaching each part from each other. These were carefully removed by forceps, detaching the brain from the head capsule. The dissected brain was transferred to another glass dissection bowl containing Ringer's solution, and the remainder of tracheas and eye matter were removed with fine forceps. Following dissection, the brain was immediately transferred to a small glass bottle containing 4 % paraformaldehyde (Roti, Histofix pH 7), and was placed on a rotator, in room temperature for 1-2 hours. This was done to fixate brain structures and to prevent neuronal degradation.

The fixed brain was washed in phosphate buffered saline (PBS; NaCl: 684mM, KCl: 13mM, Na2HPO2: 50.7mM, KH2PO4: 5mM, pH 7.4) for 10 minutes to remove the fix. Next, it was dehydrated in a series of ethanol; 50%, 70%, 90%, 96%, and 2 x 100%, for 10 minutes during each step. The brain was then mounted on an aluminum plate in methyl salicylate (methyl 2-hydroxybenzonate), sealed between two cover glasses on each side of the plate. The preparations were stored in a refrigerator at 4^{o}C up until the analyzing procedure in the confocal microscope.

Visualization of stained processes using a confocal laser scanning microscope

All preparations were first examined under a light microscope equipped with a mercury lamp and fluorescent filters (Leitz Aristoplan, Wetzlar, Germany), in order to reveal whether the dye injection and dehydration/mounting had been successful. This was evaluated it terms of visible staining, and whether the brain was seemingly intact. Preparations that fulfilled these criteria were brought to a confocal microscope (LSM 510 Meta Mira Zeiss 900F, Jena, Germany) for further analysis. All preparations were scanned using two dry-lensed objectives; a 10x objective (10x/0.3 Plan-Neofluar) was used for an overview of the brain as a whole, and a 20x objective (20x/0.5 Plan-Neofluar) was used for finer details of the staining process. A helium neon laser (HeNe1, 543 nm) was used to excite the tetramethylrodamine/Cy3, and so to illuminate the mass staining. A resolution of 1024 x 1024 pixels, a pinhole size of 1 airy

unit, and a section distance of 2-4μm was used in scanning the preparations. The stacks of images were saved as Zeiss image files (.lsm).

Intensification of brains

Some of the preparations that were successfully stained according to the objectives of the project were intensified for further analysis. In addition to tetramethylrhodamine, micro-ruby contains biotin, an element being visualized only if the brain is intensified. Streptavidin-Cy3 binds to biotin and has the same wavelengths of maximum absorption and emission as tetramethylrhodamine, thus, it was used in the intensifying process, which was carried out as follows: The brain being cleared in methyl salicylate was rehydrated in a decreasing series of ethanol: 2 x 100%, 96%, 90%, 70% and 50%, for 10 minutes during each step. Next, it was washed in PBS for 10 minutes, followed by being incubated in Streptavidin-Cy3 (1:200, in 0.1M PBS Ph 7.2) for 2 hours in room temperature. The brain was again washed in PBS for 10 minutes, and subsequently dehydrated in an increasing series of ethanol: 50%, 70%, 90%, 96%, 2 x 100%, 10 min each. Finally, it was cleared and embedded in methyl salicylate.

Data analysis and image processing

Confocal scans of all successfully stained preparations were analyzed by visual inspection. Brains were classified as successfully stained when the three main ALTs; the medial, the mediolateral, and the lateral, ALT, were visible in the ipsilateral hemisphere. Confocal images of the successfully stained preparations, including single optical sections and projection views, were stored in Photoshop CS5 (Adobe Systems, San Jose, CA) before the final figures were edited in Adobe Illustrator CS5.

Results

A total of 193 preparations were made in this study, of which 57 were scanned by means of confocal microscopy. Totally, 32 preparations were successfully stained according to the criterion concerning retrograde tracing of the three main ALTs, which was used as an indicator of successful dye application in the LH. Confocal images from 7 different brain preparations are presented in the results. Figures supporting the present finding are also presented in Appendix I, which contain data from 5 additional preparations.

Cataloguing

All preparations are numbered according to the sequential order of the specimen. Both gender and species were noted to keep track of possible gender- and species differences appearing in the findings. The following results show data from the species *H. armigera* (2 males and 6 females) and *H. virescens* (1 male and 3 females). In general, no sex-specific patterns connected with the LH were observed. Labeling of two commissures projecting from ipsilateral to contralateral hemisphere were present in both genders, however, their innervation patterns were evident only in males.

Overview of labeled neuronal networks in the ipsilateral and contralateral hemisphere

Application of fluorescent dye in the LH resulted in extensive labeling of neuronal networks in both the ipsilateral and contralateral hemisphere. The labeled areas in the contralateral hemisphere included both the AL and the LH (Fig. 6A), plus weak staining in the calyces. More specifically, the AL included several stained PNs passing along a distinct fiber bundle, whereas the LH was innervated by two stained commissures (CI and CII) targeting slightly different areas. The labeled areas in the ipsilateral hemisphere comprised the AL, including all cell body clusters, the three classical ALTs, and the calyces (Fig. 6B). In addition, the two commissures, CI and CII, emerging from the site of dye application in the LH were also visualized. Finally, the calyces of the ipsilateral hemisphere showed strong labeling. The following part of the results presents the data in more detail, in which figures referred to in the text are presented on page 21-34.

Labeling of contralateral AL PNs passing along a distinct tract

Dye injection in the LH resulted in retrograde labeling of a particular population of PNs in the contralateral AL. As shown in figure 7, stained processes innervated several glomeruli located in the posterior region of the AL. The somata of these PNs were gathered in the lateral

cell cluster, and their axons were confined to a tract not previously discovered; this new antennal-lobe tract is named the *contralateral ALT* (cALT) (Fig. 8).

The contralateral PNs forming the cALT innervated not only distinct glomeruli, but also the central non-glomerular core of the AL (Fig. 7). Their axons were connected with the stained somata in the lateral cell cluster via the ventral root. As shown in figure 7C and D, the labial pit organ glomerulus (LPOG), located most ventrally in the AL, was among the glomeruli targeted by this PN population. The axons of the PNs leave the AL along the course of the mALT (which is not visible here in the contralateral hemisphere), forming a distinct and prominent fiber bundle (Fig. 8A). When the tract approaches the lateral edge of the CB, it turns medially and passes along the lower part of the CB as it crosses the midline (Fig. 8B). In the contralateral hemisphere, the cALT projects laterally towards the lateral protocerebrum, where it meets with the mALT at the lateral edge of the CB. From here, it continues further laterally, seemingly along the course of the mlALT, towards the LH (Fig. 8C).

The connection between the LH and the contralateral AL was also demonstrated in other brains, one of which turned out to be particularly interesting. In this preparation, presented in figs. 6 and 9, the LPOG was distinctly stained. Nine small cell bodies in the lateral cell cluster, which were connected to the LPOG were identified as well (Fig. 9E). Furthermore, single fibers innervating the LPOG were labeled. These projected postero-medially in the AL, but unfortunately, the ascending trajectories of these were not possible to identify (Fig. 9D). In addition to the 9 small cell bodies which were specifically connected to the LPOG, a collection of approximately 51 bigger somata was stained (Fig. 9C). These were also located in the lateral cell cluster, but more dorsally situated compared to the aforementioned group. However, it was not possible to see the innervation pattern of the PNs connected to these somata. All these somata, including those linked to the LPOG as well as the others, are probably connected to PNs passing along the cALT.

Projection pattern of commissures connecting the LHs in the two hemispheres

Dye application to the LH resulted in staining of two commissures connecting the LHs in the two hemispheres. These commissures were observed in both male and female preparations. Two brains, both from males, displayed the more distinct innervation pattern of the commissures (preps. No.7; *H. armigera* and 11; *H. virescens*). As previously mentioned, the two commissures are referred to as commissure I (CI) and commissure Π (CΠ) in the present study. Figure 10 shows a schematic overview of the two commissures' trajectories in the heliothine brain, in a dorsal, frontal, and sagittal position.

In both males and females, CΠ projected more dorsally and anteriorly than CI. As shown in figure 11A, CΠ passed along to the posterior edge of the CB. In the more ventral section, CI also appeared, seemingly from a more posterior and ventral part of the LH (Fig. 11B). CΠ continued its course towards the contralateral hemisphere following the posterior edge of the CB, whereas CI projected slightly more posteriorly and ventrally (Fig. 12).

The two commissures maintained their positions relative to each other in the contralateral hemisphere. As shown in figure 13, CΠ terminated in an area of the LH located dorsally and anteriorly to the area targeted by CI. Thus, as CΠ targeted its termination area, it coalesced and innervated the anterior and dorsal region of the LH (Fig. 13A). This innervation pattern is also demonstrated in figure 14A. Following single slices from dorsal to ventral position, the trajectory of CΠ can be followed in reverse: The tract split close to the lateral edge of the CB and targeted a large region of the LH (Fig. 13A-D). This projection pattern is also shown in figure 14A-B. The other commissure, CI, divided and targeted a relatively small area located more posteriorly and ventrally in the LH (Fig. 13E-F). The innervation pattern of CI is also displayed in figure 14C-D.

Staining of the ipsilateral ALTs

All three main ALTs, the mALT, the mlALT, and the lALT, were labeled in the ipsilateral hemisphere (Fig. 15). In addition, the tALT was labeled in a few preparations. The mALT and the mlALT appeared as the most prominent tract in all the preparations presented here. The lALT was systematically more diffusely stained.

The mALT projected dorsomedially from the AL and bypassed the CB before it turned laterally and innervated the calyces. It then continued antero-laterally and terminated in the LH (Fig. 15A- B). The substantially thinner mlALT followed the mALT as it exited the AL. At the lateral edge of the CB, it turned laterally and projected directly to the LH (Fig. 15A) The lALT exited the AL more ventrolaterally and projected directly to the LH as well, however, in a more ventral position as compared to the mlALT (Fig. 15C). The tALT, which occurred occasionally, bifurcated from the mALT just posteriorly of the mlALT (Fig. 15C). Figure A1 in Appendix I shows a sequence of confocal images from 2 different preparations visualizing the ALTs.

Labeling of the ipsilateral AL

Seemingly all glomeruli of the ipsilateral AL were stained. As shown in figure 16, both the MGC and the numerous ordinary glomeruli were innervated by stained processes. Strong labeling was seen in all three cell clusters, including the large lateral cell cluster, the midsized

medial cell cluster, and the small anterior cell cluster (Fig. 16B,D). In addition to stained processes innervating the glomeruli, extensive labeling was observed in the non-glomerular core of the AL (Fig. 16C-D). Figure A2 in the Appendix I supports the present findings, displaying the ipsilateral AL of 8 individual preparations.

Staining of the calyces in the two hemispheres

The ipsilateral calyces displayed extensive labeling throughout the whole structure, In addition to the confocal images from 4 different preparations shown in Fig. 17, this is also emphasized in Fig. A3 in Appendix I. The calyces in the contralateral hemisphere, on the other hand, were weakly stained. As demonstrated in figure 18, a few stained processes forming a scattered innervation pattern can be seen in the confocal images.

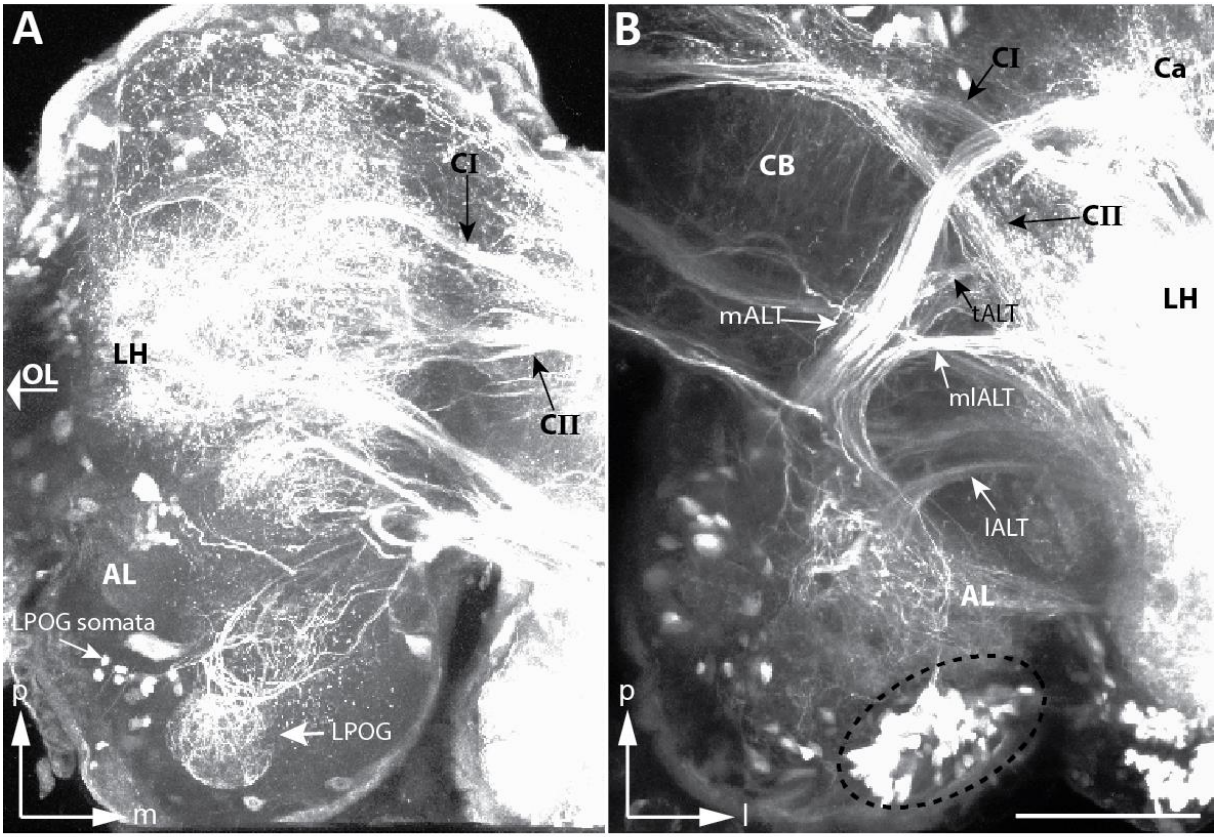

Figure 6. Confocal projection images displaying an overview of olfactory neural networks in the contralateral (A) and ipsilateral (B) hemisphere labeled by dye applied into the lateral horn (LH; preparation No.7). The composite consists of 8 confocal stacks, presented in a dorsal orientation. **A:** The labeled areas in the contralateral hemisphere include both the LH and the antennal-lobe (AL). Two commissures, commissure I (CI) and commissure II (CII) innervate different areas of the contralateral LH. In the contralateral AL, one glomerulus, the labial pit organ glomerulus (LPOG) is strongly stained. Nine somata connected to the LPOG, all located in the lateral cell cluster, can be seen (arrow). (Some commissures connected to other systems than the olfactory were stained as well). **B:** In the ipsilateral hemisphere, CI and CII projecting from the LH and crossing the midline posterior to the central body (CB) can be seen. In addition, four antennal-lobe tracts are stained: 1) The most prominent, the medial antennal-lobe tract (mALT), projects from the AL to the calyces of the mushroom bodies (Ca). 2) The mediolateral ALT (mlALT) follows the mALT from the AL, but then turns laterally and projects directly to the LH. 3) The lateral ALT (lALT) projects directly to the LH antero-ventrally of the mlALT, and 4) the transverse ALT (tALT) bifurcates from the mALT just posteriorly of the mlALT. In the ipsilateral AL, numerous cell bodies located in the lateral cell cluster are stained (indicated by dashed circle). Optic lobe (OL), posterior (p), medial (m), lateral (l). 20X objective. Scale bar: 100 µm.

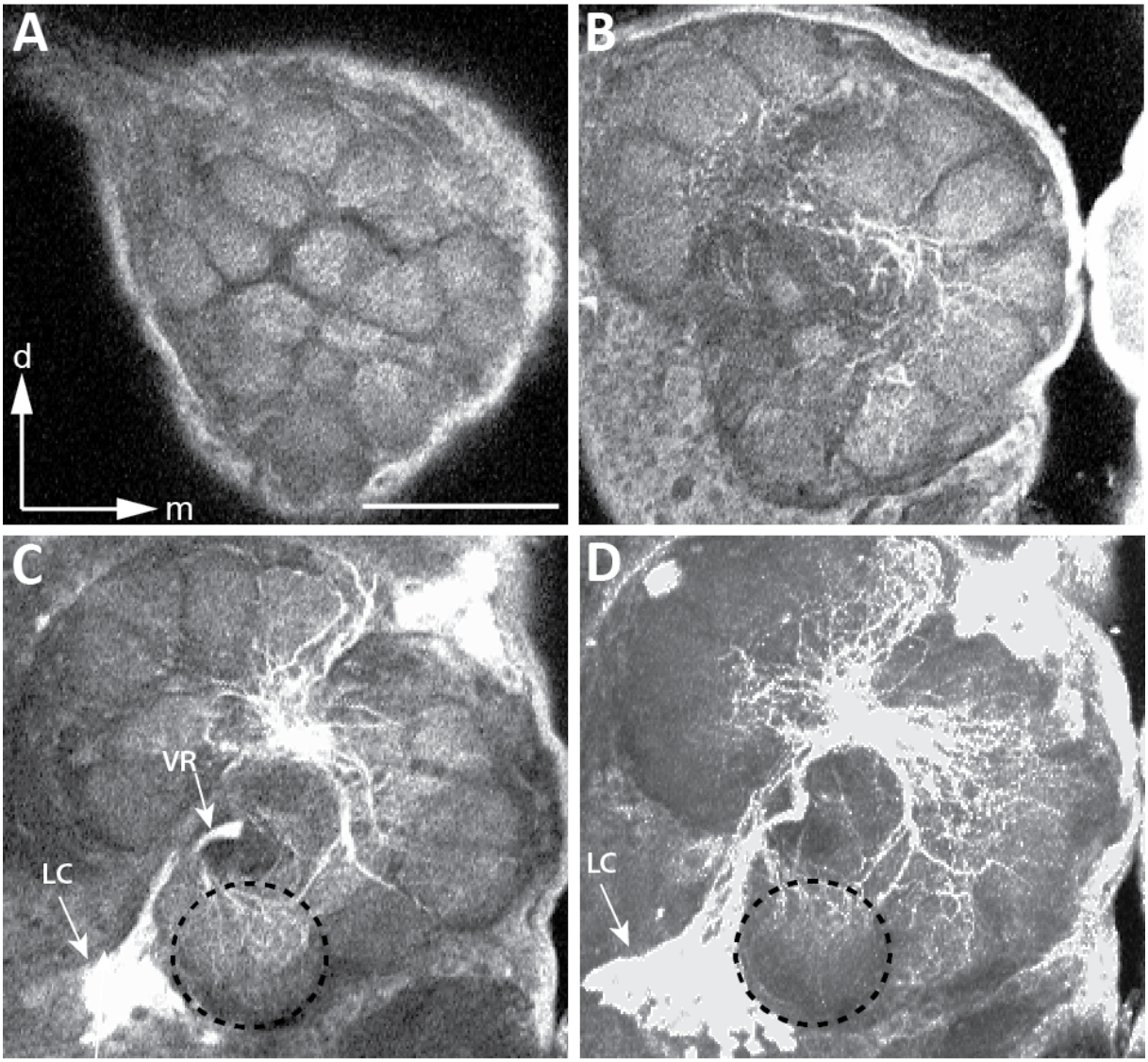

Figure 7. Single-slice confocal images (A-C) and projection image (D) showing labeled structures in the contralateral antennal-lobe (AL) of preparation No.193 (female) (frontal orientation, from anterior to posterior). **A:** Glomeruli located most anteriorly in the AL are weakly stained. **B:** In more posterior sections, labeling can be seen in the non-glomerular region located centrally in the AL. **C-D:** Extensive labeling in even more posterior parts of the AL. As shown in C and D, several glomeruli including the labial pit organ glomerulus (indicated by dashed circle) are innervated by stained processes. The axons of the stained projection neurons leave the AL through the ventral root (VR), and project along the course of the medial antennal-lobe tract before turning and passing to the contralateral hemisphere (see figure 8). A group of strongly stained somata, located in the lateral cell cluster (LC), is also visible. 20X objective. Scale bar: 100 µm.

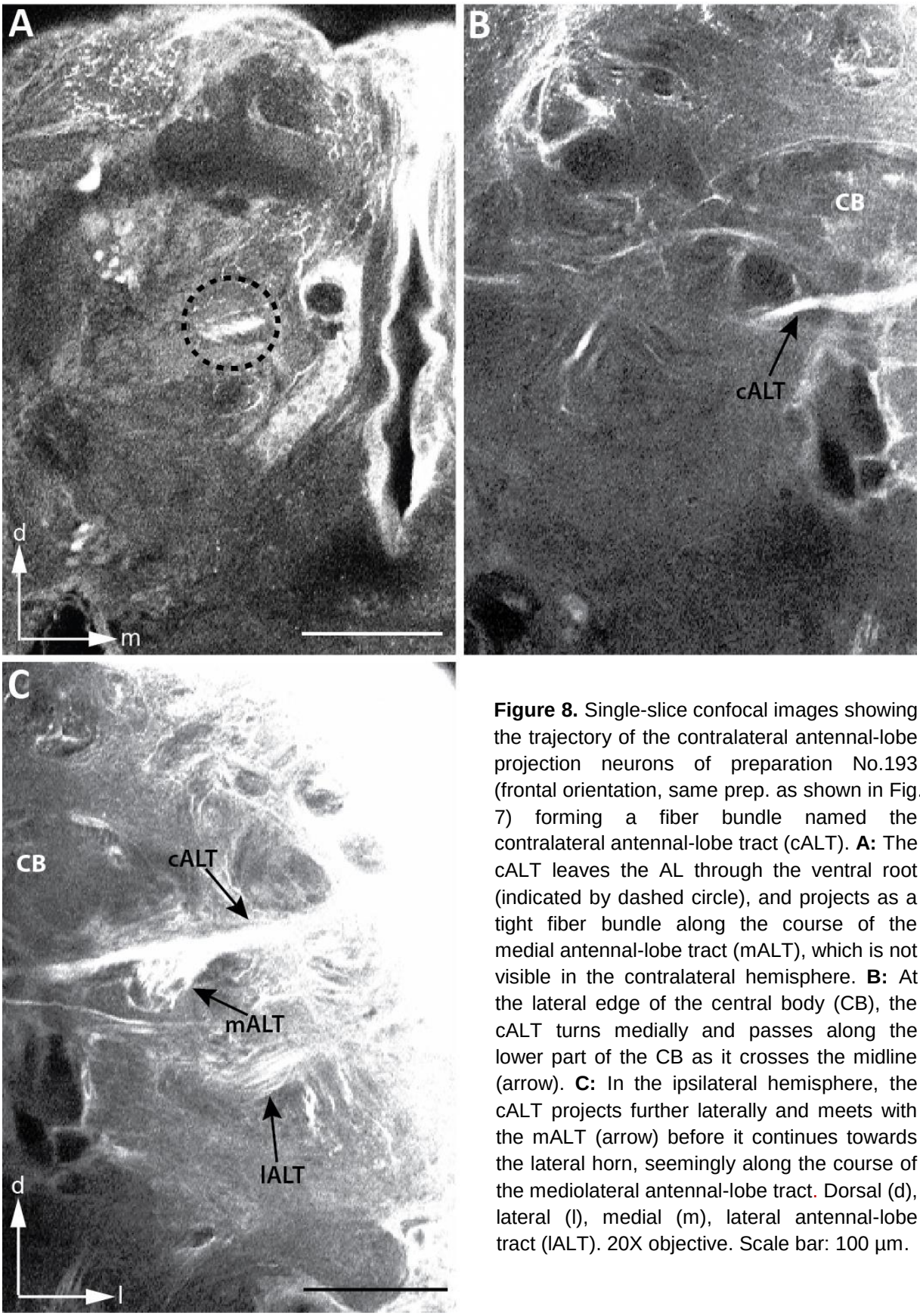

Figure 8. Single-slice confocal images showing the trajectory of the contralateral antennal-lobe projection neurons of preparation No.193 (frontal orientation, same prep. as shown in Fig. 7) forming a fiber bundle named the contralateral antennal-lobe tract (cALT). **A:** The cALT leaves the AL through the ventral root (indicated by dashed circle), and projects as a tight fiber bundle along the course of the medial antennal-lobe tract (mALT), which is not visible in the contralateral hemisphere. **B:** At the lateral edge of the central body (CB), the cALT turns medially and passes along the lower part of the CB as it crosses the midline (arrow). **C:** In the ipsilateral hemisphere, the cALT projects further laterally and meets with the mALT (arrow) before it continues towards the lateral horn, seemingly along the course of the mediolateral antennal-lobe tract. Dorsal (d), lateral (l), medial (m), lateral antennal-lobe tract (lALT). 20X objective. Scale bar: 100 µm.

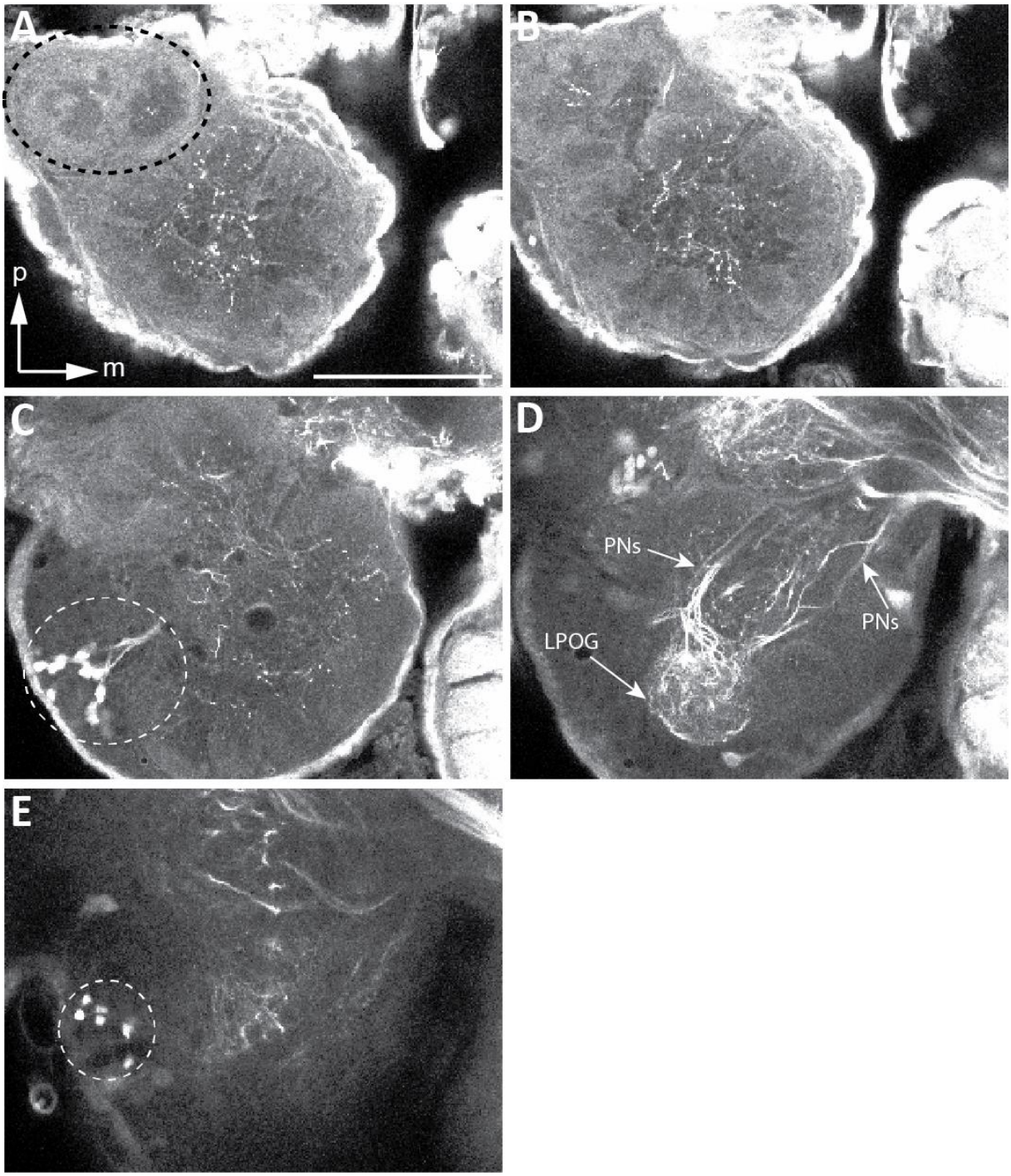

Figure 9. Single-slice confocal images showing labeled structures in the contralateral antennal-lobe (AL) of preparation No.7 (from dorsal to ventral, same prep. as shown in Fig. 6). **A-B:** Scattered innervations can be seen in two enlarged glomeruli of the macroglomerular complex (MGC) (indicated by dashed circle in A) and numerous ordinary glomeruli located dorsally in the AL. **C:** A large group of stained somata (51 counted) located in the lateral cell cluster can be seen (indicated by dashed circle). **D:** The labial pit organ glomerulus (LPOG) located ventrally in the AL, is strongly stained. Single fibers innervating the LPOG are also labeled (PNs). **E:** A small group of cell bodies located most ventrally in the lateral cell cluster all connect to the LPOG (six out of totally 9 somata are shown here). Posterior (p), medial (m). 20X objective. Scale bar: 100 µm.

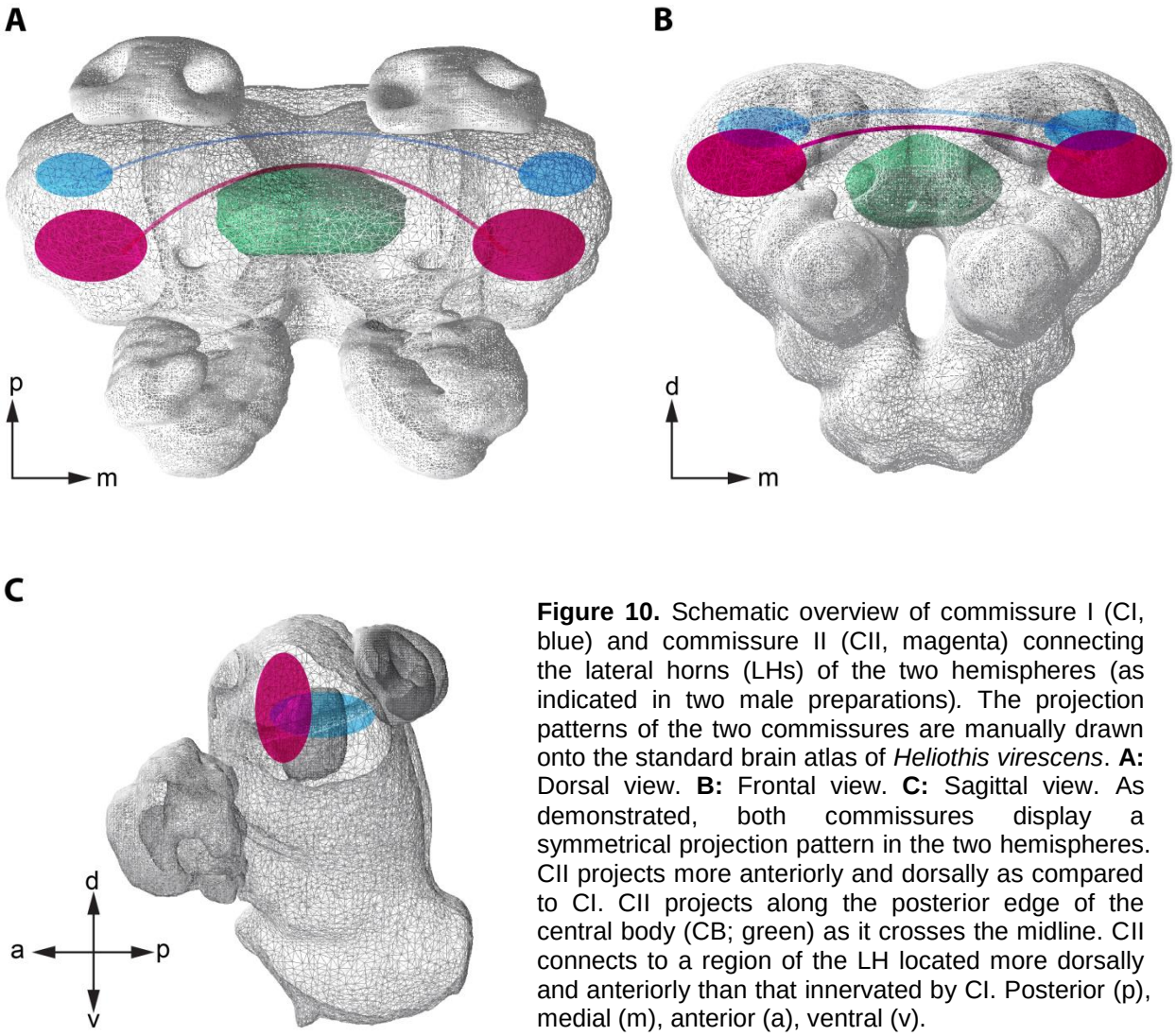

Figure 10. Schematic overview of commissure I (CI, blue) and commissure II (CII, magenta) connecting the lateral horns (LHs) of the two hemispheres (as indicated in two male preparations). The projection patterns of the two commissures are manually drawn onto the standard brain atlas of *Heliothis virescens*. **A:** Dorsal view. **B:** Frontal view. **C:** Sagittal view. As demonstrated, both commissures display a symmetrical projection pattern in the two hemispheres. CII projects more anteriorly and dorsally as compared to CI. CII projects along the posterior edge of the central body (CB; green) as it crosses the midline. CII connects to a region of the LH located more dorsally and anteriorly than that innervated by CI. Posterior (p), medial (m), anterior (a), ventral (v).

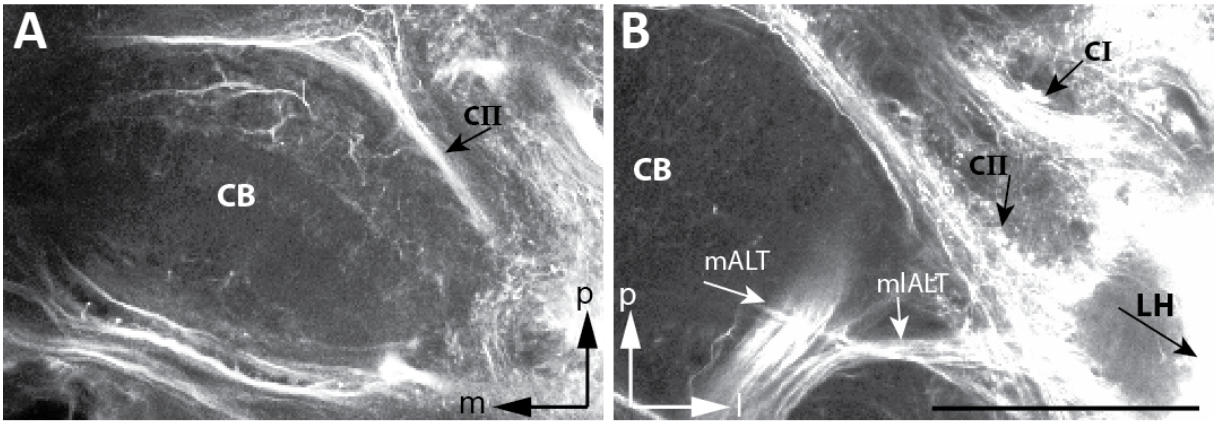

Figure 11. Single-slice confocal images displaying two labeled protocerebral commissures in the ipsilateral hemisphere of preparation No.7 (male) (from dorsal to ventral). These commissures make connections between symmetrical regions of the lateral horns (LHs) in the two hemispheres. **A:** Commissure II (CII) projects close to the posterior edge of the central body (CB). **B:** The two commissures emerge from different areas of the LH. Commissure I (CI) projects posteriorly and ventrally of CII. Medial antennal-lobe tract (mALT), mediolateral antennal-lobe tract (mlALT), posterior (p), medial (m), lateral (l). 20X objective. Scale bar: 100 µm.

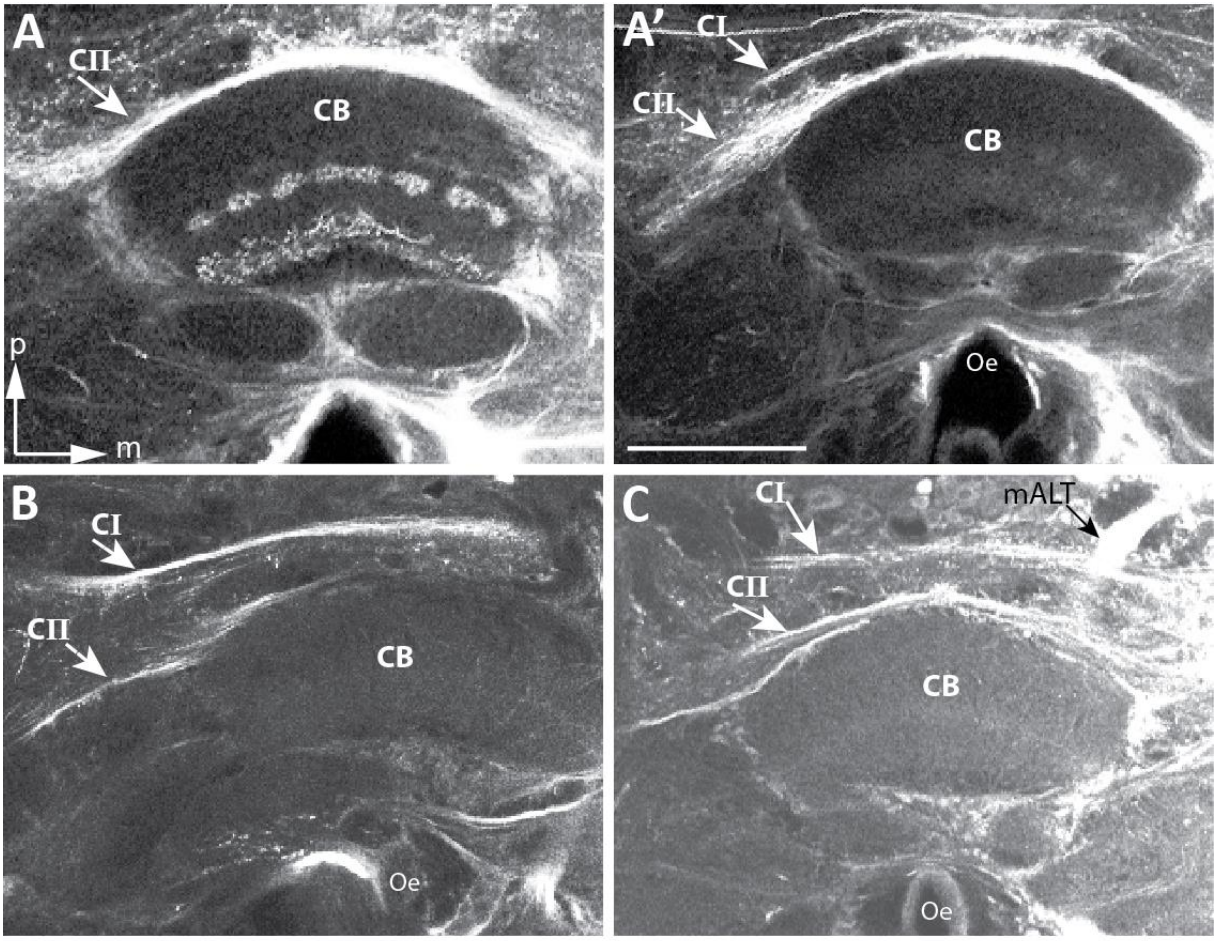

Figure 12. Confocal images of three different preparations, one from a male and two from females, displaying the projection patterns of commissure I (CI) and commissure II (CII) as they cross the brain midline (all dorsally oriented): **A-A':** Two sections of preparation No.111 (male) (from dorsal to ventral) demonstrating the positions of the two commissures: CII projects anterodorsally of commissure I (CI). **B-C**: Single slices of preparation No.133 and 190 (both females), showing CII projecting anteriorly of CI. Both commissures pass the central body (CB) posteriorly, with CII projecting along the posterior edge of CB. Esophagus (Oe), posterior (p), medial (m). 20X objective. Scale bar: 100 µm.

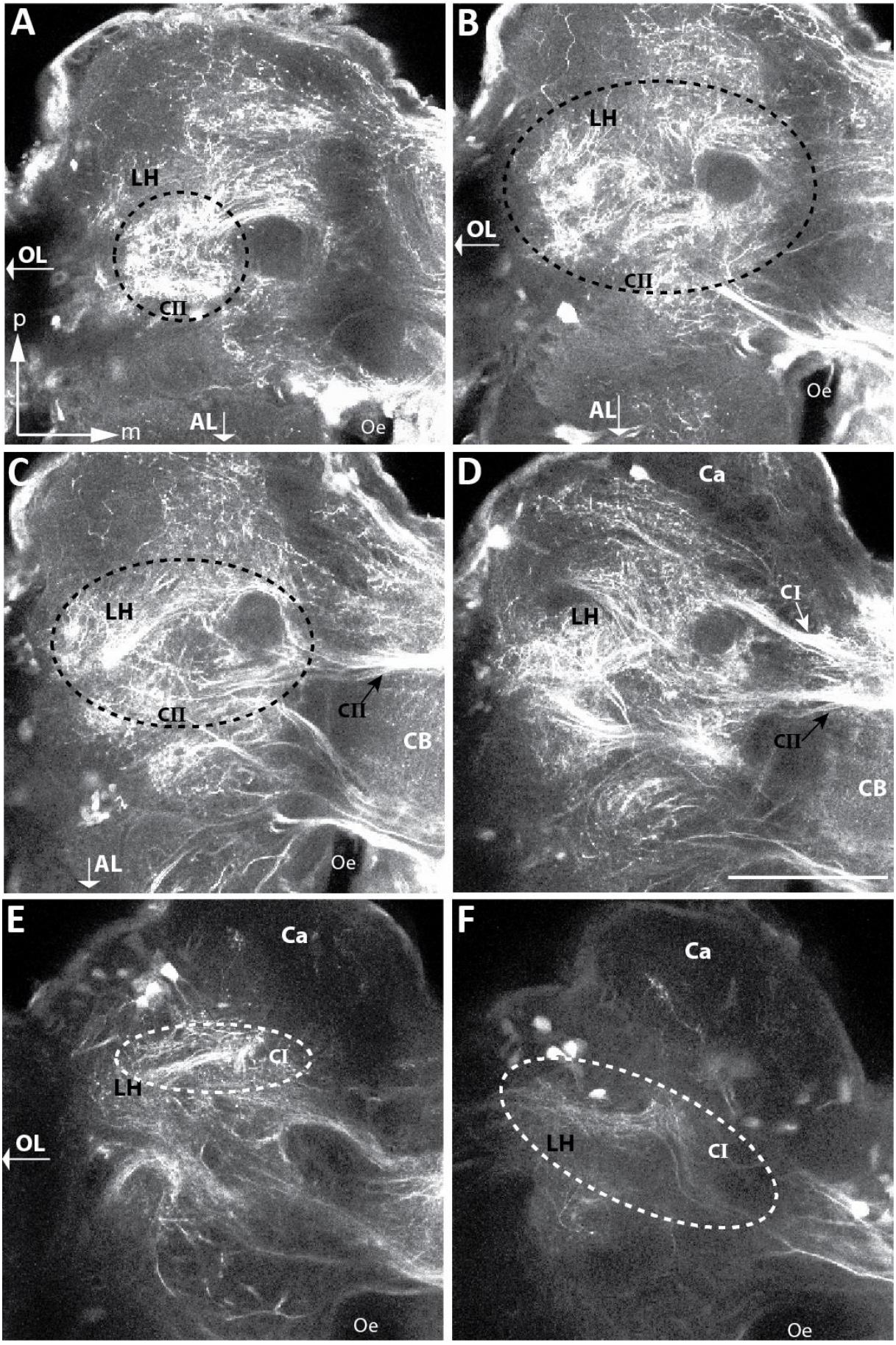
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Figure 13. Single-slice confocal images of preparation No. 7 (male), showing the projection pathways and innervation areas of the two commissures in the contralateral hemisphere. Close to their innervation areas, the two commissures turn into different directions; commissure II (CII) bends slightly dorsally whereas commissure I (CI) turns slightly ventrally. The slices from the confocal stack are presented from dorsal to ventral position and the final target regions of CII thus appear first in this series of confocal images. **A-D:** CII terminates in a relatively large region located dorsoanteriorly in the lateral horn (LH). The projection of CI, crossing the midline, becomes apparent in the ventrally located slice (D). **E-F**: As demonstrated from the two most ventrally located slices, CI projects ventrally and posteriorly of CII. On its course, CI divides and sends off branches to LH-regions located more posteriorly and ventrally as compared to those innervated by CII. Calyces of the mushroom bodies (Ca), optic lobe (OL), esophagus (Oe), antennal-lobe (AL), posterior (p), medial (m). 20X objective. Scale bar: 100 µm.

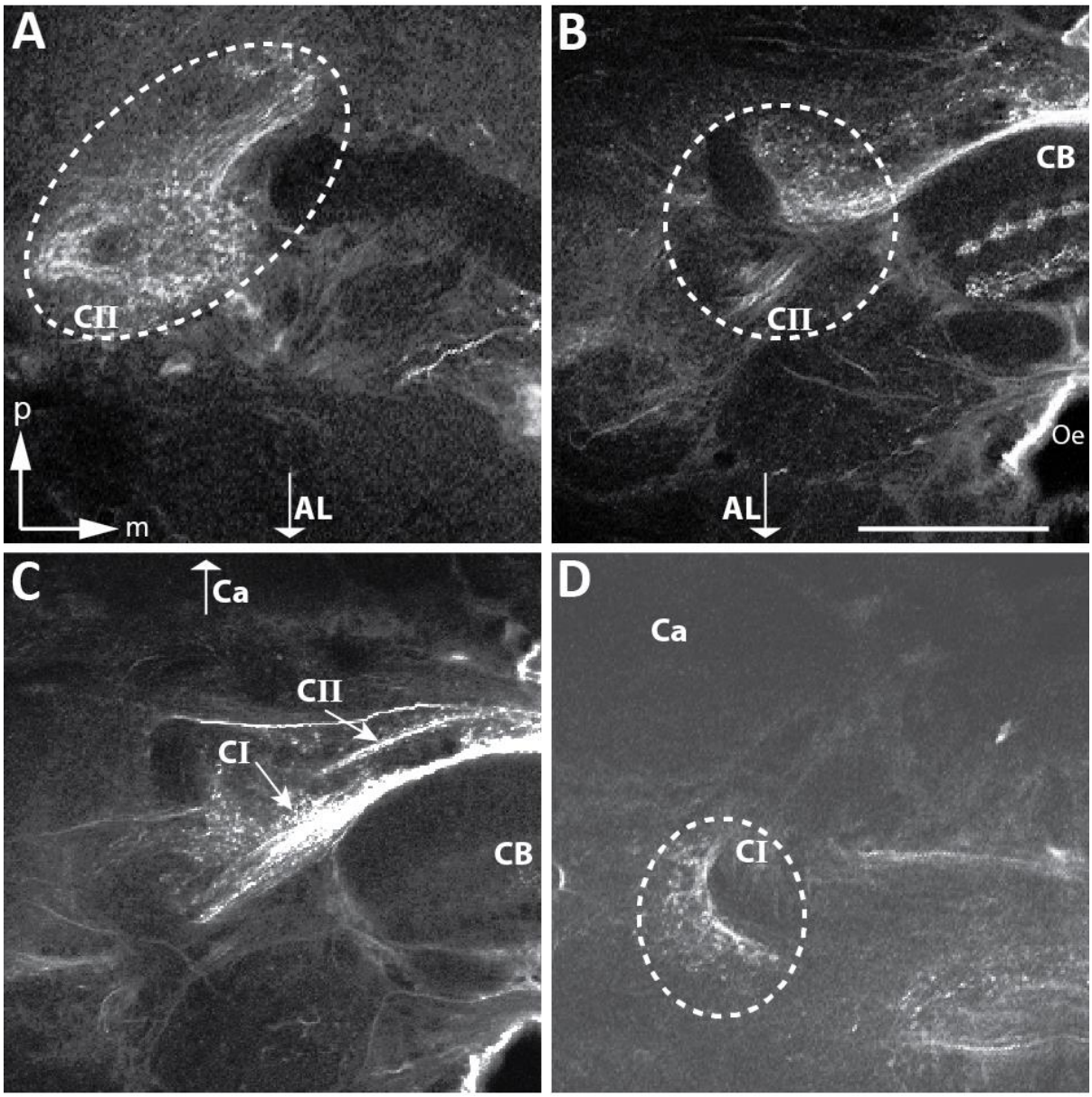

Figure 14. Single-slice confocal images of preparation No.111 (male) displaying the projection patterns and target regions of commissure II (CII) and commissure I (CI) (indicated by dashed circles) in the contralateral hemisphere (from dorsal to ventral). The staining patterns of the two commissures are in full agreement with those demonstrated in the previous figure, Fig. 13. **A:** CII coalesces as it innervates the target region in the dorsal and anterior part of the lateral horn (LH). **B:** CII disperses as it passes the lateral edge of the central body (CB). **C:** The projection of CI becomes apparent in more ventral sections. Both commissures project posteriorly to the CB, with CI passing posteriorly to CII, which projects along the border of the CB. **D:** CI innervates a more ventral and posterior part of the LH as compared to CII. Calyces of the mushroom bodies (Ca), esophagus (Oe), antennal-lobe (AL), posterior (p), medial (m). 20X objective. Scale bar: 100 µm.

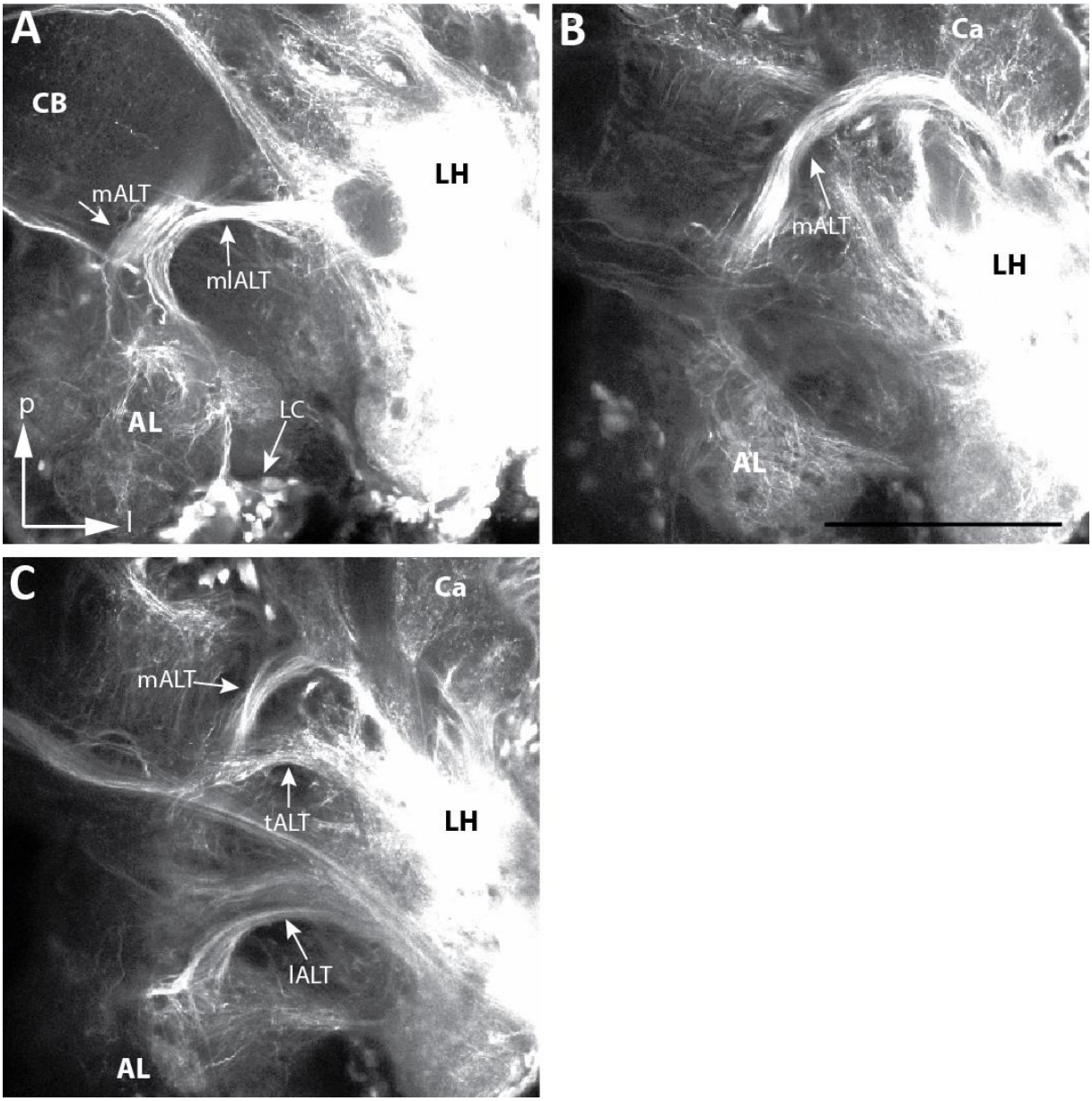

Figure 15. Single-slice confocal images of preparation No.7 at three different depths, displaying the labelled antennal-lobe tracts (ALTs) in the ipsilateral hemisphere (from dorsal to ventral). **A:** The medial ALT (mALT) exits the antennal-lobe (AL) together with the mediolateral ALT (mlALT). The mlALT projects directly to the lateral horn (LH) in the lateral protocerebrum. **B:** The mALT projects to the calyces of the mushroom bodies (Ca) before turning anteriorly and terminating the LH. **C:** The lateral ALT (lALT) exits the AL more ventrally and projects directly to the LH. The transverse tract (tALT) bifurcates from the mALT. Central body (CB), lateral cell cluster (LC), posterior (p), lateral (l). 20X objective. Scale bar: 100 µm.

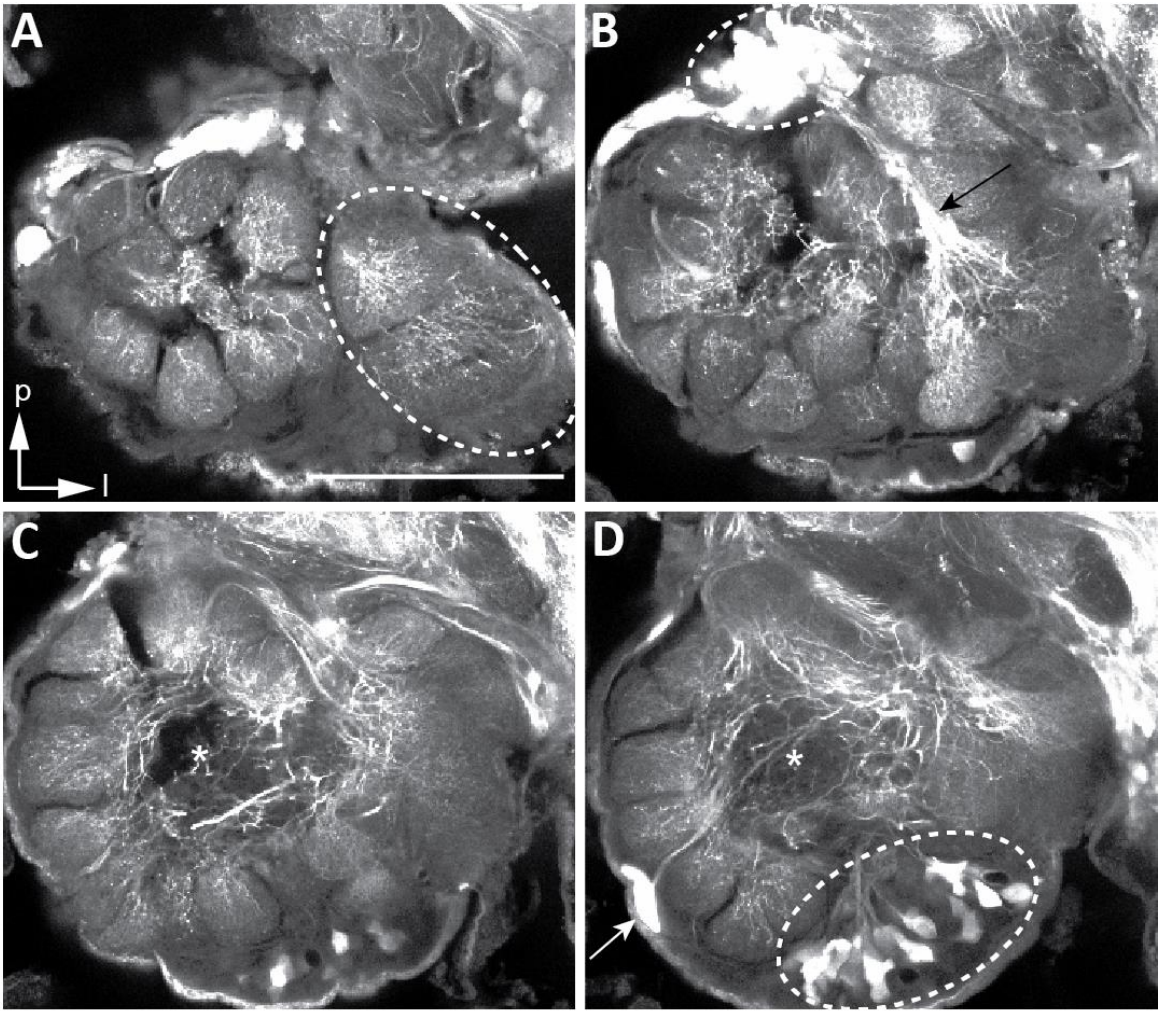

Figure 16. Single-slice confocal images of brain preparation No.7, showing detailed staining patterns in the ipsilateral antennal-lobe (AL) (from dorsal to ventral). **A:** Dorsal section showing stained processes innervating two enlarged glomeruli constituting a part of the macroglomerular complex (MGC) (indicated by dashed circle) in addition to numerous ordinary glomeruli. **B:** The medial cell cluster (MC), located dorso-medially in the AL (indicated by dashed circle), is strongly labeled. Processes of projection neurons connecting the MGC glomeruli with somata in the MC can be seen (arrow). **C-D:** The two more ventral slices demonstrate stained fibers arborizing in the non-glomerular core of the AL (indicated by an asterisk). In D, numerous stained somata in the lateral cell cluster are labeled (indicated by dashed circle). In addition, a few somata confined to the anterior cell cluster are visible (arrow). Posterior (p), lateral (l). 20X objective. Scale bar: 100 µm.

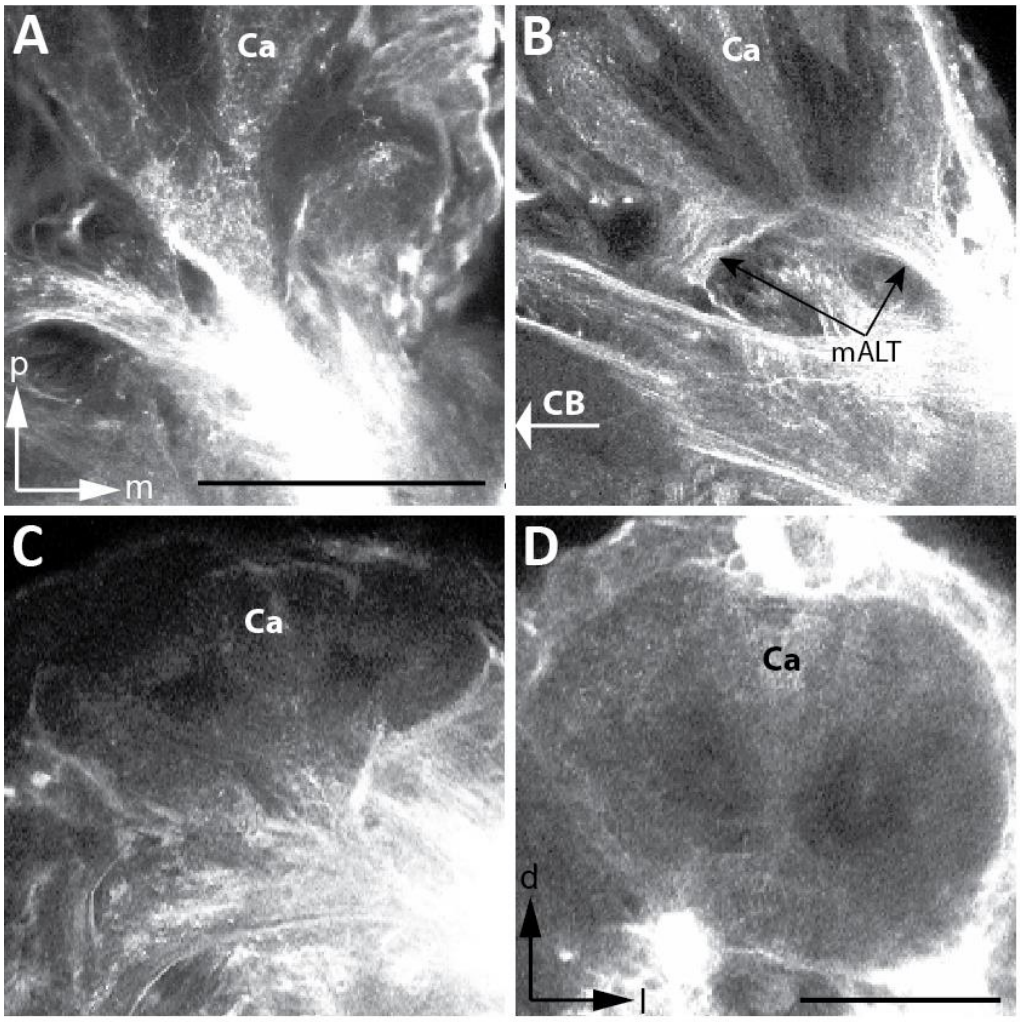

Figure 17. Confocal images from 4 different preparations, displaying labeling of the calyces (Ca) in the ipsilateral hemisphere. **A-C:** Preps No. 7 (A), 133 (B), 111 (C), all dorsal oriented. **D:** Prep. No.193, frontal oriented. Central body (CB), medial (m), posterior (p), dorsal (d), lateral (l). 20X objective. Scale bars: 100 µm.

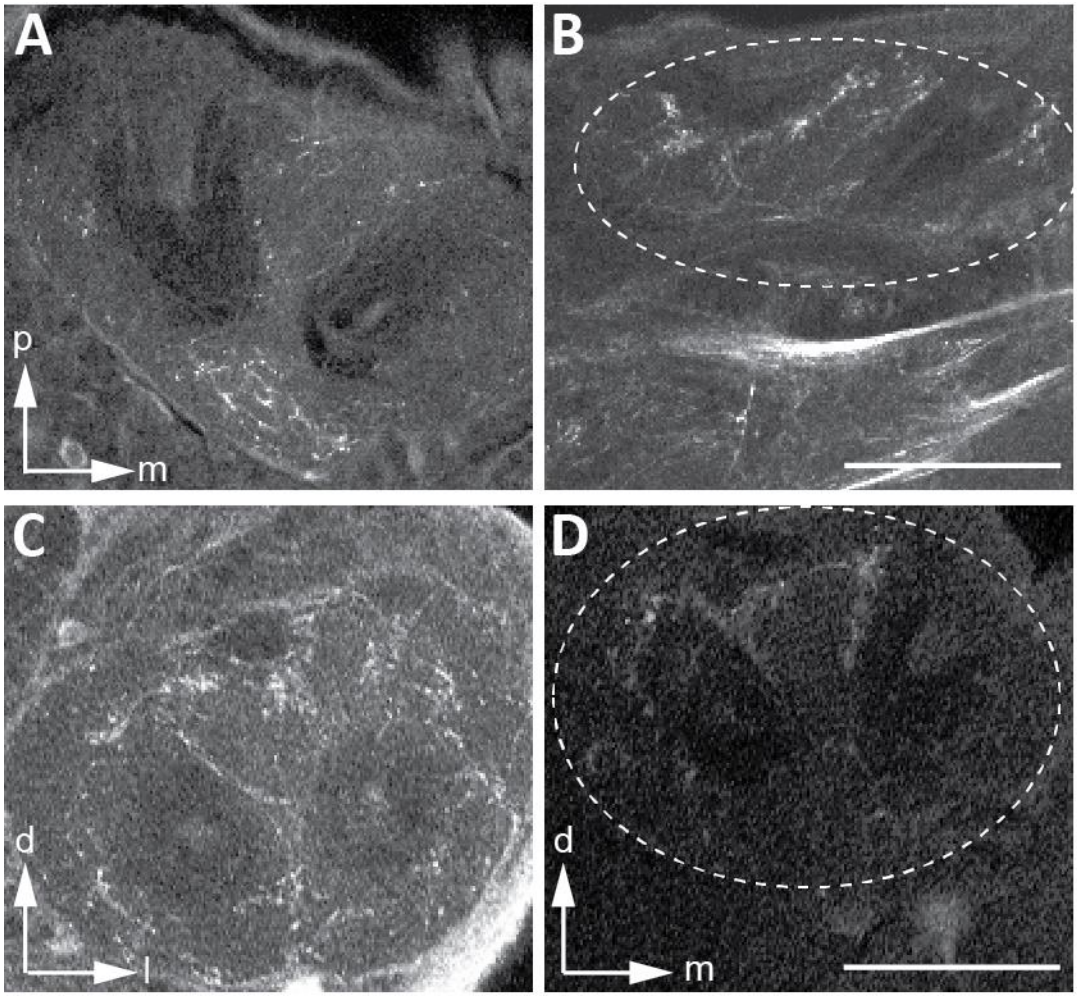

Figure 18. Confocal images from four different preparations displaying the labeling pattern in the contralateral calyces of the mushroom bodies (Ca). **A-B:** The images of preparation No.124 (A) and No.133 (B) show a dispersed labelling pattern in the calyces (indicated by a dashed circle in B). **C:** Preparation No.79, which is frontally oriented, displays a similar dispersed innervation pattern. **D:** The Ca of preparation No.193 (frontal oriented) is slightly less labelled (indicated by dashed circle), as compared to the other preparations, but displays the similar dispersed pattern. Posterior, (p), medial (m), dorsal (d), lateral (l). 20X objective. Scale bar: 100 µm.

Discussion

Characterizing all levels of the olfactory pathways is essential for understanding the mechanisms of smell. While the first and second order neurons of the olfactory pathway are well documented, knowledge concerning organization and morphology of third order olfactory neurons is particularly deficient; hence, the role of the LH in olfaction is still poorly understood. The present study is the first to map brain regions directly connecting with the LH by means of mass staining in heliothine moths. The results obtained are thus considered to be scientifically highly relevant. The findings include characterization of neuronal networks connected to the LH, both in the ipsi- and contralateral hemisphere. Exploring the organization of input to and output from the LH provides an important contribution to further understanding processing of olfactory information.

Brief summary of the results

The principal goal of this study was to explore neuronal networks linked to the LH. The specific goals were to 1) map the dendritic arborizations of ipsilateral and contralateral AL PNs targeting the LH, and 2) identify termination regions of third-order neurons projecting out from the LH. Indeed, successfully stained preparations, obtained by applying dye into the LH, revealed extensive neural connections in both hemispheres. In addition to complementing existing data describing the morphology of the ALTs and the calyces in the ipsilateral hemisphere, the outcome of this study revealed a number of networks not previously described: 1) Two prominent commissures connecting the LH in each hemisphere were characterized. 2) A prominent contralateral ALT not previously described was discovered. 3) A population of approximately 50 contralateral PNs forming this tract was identified. 4) A distinct connection between the LPOG and the contralateral LH was proven. 5) Extensive labeling in the non-glomerular core in the ipsilateral AL, demonstrates that some of the PNs arborize outside the glomeruli. 6) Finally, observation of a scattered staining pattern in the contralateral calyces, demonstrates a particular connection with the LH in the opposite hemisphere. In addition to these new data, the present study confirms a number of previous findings as well. However, the following discussion will focus on some selected issues concerning the new data. The results are therefore discussed according to the following topics:

1) Identification of two commissures connecting the LH in the two hemispheres
2) Connection between the contralateral LPOG and the LH
3) Identification of a new contralateral ALT

Identification of two commissures connecting the LHs in the two hemispheres

Two prominent commissures projecting from ipsilateral LH to contralateral LH were characterized. The presence of two stained commissures was observed in all preparations successfully labeled, including males and females. However, the data providing detailed information about the innervation patterns of the fiber bundles were obtained from two male preparations (No.7 and 111). The presence of two stained commissures displaying a clear and identical morphology across the two male preparations provides strong verification for the following interpretation of the obtained data.

The two commissures connect two different regions of the LH in a seemingly symmetrical manner. In the ipsilateral hemisphere, CΠ emerged from a dorsal and anterior position of the LH, and innervated a dorsal and anterior region of the contralateral LH (Fig. 11B and 13A-D). The other commissure, CI, emerged more ventrally and posteriorly in the ipsilateral hemisphere and terminated in a corresponding manner contralaterally (Fig. 11B and 13D-F).

In addition to displaying bilateral connections of the LH in the two hemispheres, the identification of the commissures also indicates a distinct division of the LH. More specifically, the two different LH regions in the male brain, as identified in the present study − one region located more dorsally and anteriorly than the other − resembles the separated areas in the LH of several moth species, linked to pheromone and plant odor signals, respectively (*M. sexta*: Homberg et al., 1988; *B. mori*: Namiki et al., 2014; *H. virescens/H. assulta*: Zhao et al., 2014). In general, the region innervated by pheromone neurons, named ΔILPC by Namiki et al. (2014), is described as being located slightly more dorsally, medially, and anteriorly than that innervated by plant odor neurons. The previous studies were based on intracellular recordings from individual PNs and mass staining of PNs originating from the MGC and ordinary glomeruli, respectively. The innervation areas of CI and CΠ in the LH, characterized in the present study, thus correspond to the regions for pheromone and plant odor signals, reported in previous studies of moths. Labelling of two commissures was also present in female preparations. However, the exact innervation areas of the fiber bundles were not possible to identify, as the staining in the relevant brain regions of these preparations was more diffuse.

Also, the data obtained here correspond to previous findings obtained from another kind of investigation. One mass staining study including dye application into the LH, similar to that performed here, has been performed previously, on *B. mori* (Namiki et al. 2014). In this study they managed to inject dye solely in the region of the LH that receives pheromone

information, due to the considerably larger size of the silk moth. This resulted in labelling of the pheromone region in the contralateral hemisphere, whereas the region which receives plant odor information was unlabeled. Dye injection into the plant odor region (posterior-lateral region of LH), on the other hand, resulted in labelling of the contralateral plant odor region, but not the pheromone region. These findings demonstrate that each of the subdivisions, representing behaviorally different odor stimuli, is selectively connected to a corresponding region in the contralateral hemisphere. Due to the small size of the brain of the heliothine moth, it is considerably more difficult to approach these regions separately in our preparation. In addition, mass staining of the pheromone region and plant odor region in the study by Namiki et al. (2014) was performed via an electrode attached to a micromanipulator, which allows for a more fixed and controlled insertion of the dye in the respective areas. The mass staining technique used in the present study was performed by manually inserting dye crystal attached to a needle into the LH.

The presence of lateral connections between the two hemispheres, such as those described here, indicates that the segregation of biologically relevant stimuli is maintained by third order neurons forming these commissures. It also complements previous reports commenting on the role of the LH in bilateral processing of sensory information. Segregating pheromones and general odorants at this level, is in line with the description of LH as an area particularly involved in innate behaviors. Bilateral integration mediated by third order neurons may allow for rapid comparison of particular aspects related to the stimuli, and thus contribute to odor- tracking behavior (Gupta & Stopfer, 2012).

Contralateral connection between the LPOG and the LH

The present study demonstrates a contralateral connection between the LPOG and the LH (Fig. 9). In addition, staining of 9 unusually small somata exclusively connected to the LPOG in the same preparation demonstrates the number of PNs forming this particular connection. The cell bodies formed a separate group located most ventrally in the lateral cell cluster.

Generally, the LPOG is a glomerulus specialized for processing of carbon dioxide (CO_2) information. CO_2 influences a variety of behaviors in different insects. Lepidoptera, for example, use CO_2 emitted from plants to detect and orient towards nutritious food sources, and selecting optimal plants for oviposition (Guerenstein & Hildebrand, 2008; Kent, Harrow, Quartararo & Hildebrand, 1986). Sensory neurons tuned to CO_2 in moths are located in sensilla situated on the tip of the labial palp, in a separate organ called the *labial palp organ.*

The axons of these sensory neurons project bilaterally to each AL via the *labial pit nerve*, and terminate in the LPOG (Kent et al., 1986; Zhao et al., 2013). This means that the CO_2 system is bilaterally connected already at the peripheral level, as opposed to the OSNs located on the antenna which only project ipsilaterally. What is obvious is that the CO_2 system is an arrangement separated from that mediating plant- and pheromone odorants; the LPOG receives no input from OSNs located on the antennae (Zhao et al., 2013). The present study demonstrates that the bilateral integration of CO_2 information is maintained at the subsequent level of the sensory pathway, confirming the conception of the CO_2 pathway as a distinct system, all the way from the periphery to the protocerebrum.

The stained somata demonstrate the presence of at least 9 uniglomerular PNs innervating the LPOG, all with terminal branches in the contralateral hemisphere. The complete axonal projections of these PNs could not be traced. However, a previous master's study has reported about LPOG PNs passing along the contralateral mlALT (Moe Dahl, 2013). In the former study, dye applied in the LPOG resulted in labeling of PNs mainly following the mlALT and lALT of the ipsilateral hemisphere (Moe Dahl, 2013). However, two stained fibers crossing the brain midline anteriorly of the CB and terminating in the contralateral LH were reported as well. Furthermore, these axons were described to target in a region of the LH symmetrical to that innervated by the mlALT PNs in the ipsilateral hemisphere. Findings from the present study provide evidence for additional fibers connecting the LPOG and the contralateral LH. Whether these are part of the mlALT, as found in *H. virescens* by Moe Dahl (2013), or the lALT, is impossible to say from the present data. As these originate from *H. armigera* and not *H. virescens* as in the former study, there may also be species-specific differences regarding the number of LPOG PNs following the different tracts.

The preference for CO_2 seems to be an innate ability, which may also explain why so many LPOG PNs project directly to the LH. Put together, the appearance of the CO_2 system as a separate arrangement, including as much as 9 uniglomerular LPOG PNs targeting the contralateral LH, provides evidence for its significant role for the behavior of the moth.

Identification of a new contralateral tract

A new contralateral tract (cALT) was found in one preparation. Due to the strong labeling of a fiber bundle originating in the AL and its projection to the contralateral hemisphere, the identification of this particular tract was unmistakable. Also, the relatively large number of fibers forming the cALT was indicated by a group of stained somata located

in the lateral cell cluster of the AL. The number was countable in another preparation and included 51 somata.

The cALT has not been described in any previous literature. However, Homberg et al. (1988) described 45 fibers connecting the AL and the contralateral protocerebrum in *M. sexta*, but declared these as a part of a dorsal tract. The stated number of fibers corresponds approximately to the assumed number of cALT PNs obtained here, i.e. 51. The study by Homberg et al. (1988) did neither visualize the glomerular innervation pattern nor the somata of these contralateral PNs.

The innervation of the contralateral PNs was restricted mainly to posterior glomeruli in the AL. As shown in figure 7C-D, the LPOG was among these glomeruli. The exact target of the cALT was not possible to determine as the tract projected towards an area being occluded by the mass staining. It was however clear that it extended laterally in the protocerebrum, possibly projecting to the same area as the mlALT (Fig. 8C). Taken together, the LH seems to receive odorant information, as well as CO_2 information, from a substantial number of PNs originating in the contralateral AL. The projection pattern of the cALT, which seems to target the LH specifically, without innervating the calyces, indicates that it is involved in experience-independent behavior. Behavioral studies on *Drosophila* larvae have demonstrated that bilateral integration of olfactory input enhances the accuracy of odor localization (Louis, Huber, Benton, Sakmar & Vosshall, 2007).

As previously mentioned, the finding of two commissures in the present study, demonstrates the bilateral representation of odor signals at the level of third order neurons in the moth brain. However, the identification of the contralateral ALT proves that an essential proportion of odor information is relayed to the other hemisphere already at the level of second order neurons.

Methodological considerations

The main aim of this project was to explore neural networks linked to the LH, using a mass staining technique. This implies that dye was to be injected in a very specific area, which is a complicated procedure in itself. Furthermore, the LH constitutes a diffuse area devoid of distinctive borders; it lacks a prominent and recognizable architecture, such as the calyces and the AL, making the LH specifically hard to target. Staining of the current brain region thus relied solely on a personal and visual assessment of the supposed location of the LH in the brain preparation. This was based on thoroughly studying anatomical schematics described in previous studies, which provided some guidance in localizing the structure.

Taking notes of the approximate point of insertion in each preparation, and replicating the approach from those successfully stained, was an essential part of this experimental study.

The mass staining technique has its implications on the results. Inserting an electrode manually, by means of a microscope, highly relies on visual, motoric, and eye-hand coordinating skills. Taken together; due to the combination of this particular method and the diffuse target area, it was therefore anticipated that each preparation would provide slightly different staining patterns. This was indeed evident, as all preparations displayed slightly different labelling. This does not however, indicate that the findings are invalid. It is well known that the anatomical organization of the nervous system is stereotyped across individuals/gender of a particular species. Thus, the different brains do not exhibit individual variations that can account for inconsistent results. The results indicate, however, that structures being labelled only occasionally may innervate a relatively restricted region in the site of dye application. Furthermore, due to the small size of the heliothine moth brain, it was not possible to stain distinct regions neither in the lateral nor in the medial protocerebrum in the ipsilateral hemisphere. The data presenting the commissures may therefor include protocerebral regions that are part of an ipsilateral olfactory pathway proceeding from the LH. This interval of the pathway is however occluded due to the mass staining technique. Finally, in addition to the two commissures connecting the LH, a number of other commissures were also labelled. These were assessed as not being related to olfaction, thus eliminated from the results.

The head of the moth was usually oriented such that the brain was exposed in a dorsal, slightly frontally tilted orientation. This proved to be the most suitable position for dye injection of the LH, as it was most accessible in this orientation. This may of course limit the results to some extent, but considering the reliable success in labelling the ipsilateral ALTs, this was found to be the optimal position.

Slightly different application techniques were tested intentionally in order to see if some were more fruitful than others: Insertion of only one crystal versus several were tested in different preparations, in which something in between turned out to provide most satisfactory results. During the experimental process, staining with and without Vaseline was also tested. Vaseline was used in order to make the crystals attach better to the electrode. However, this seemed to affect the quality of the labelling to some degree, resulting in some preparations displaying diffuse staining. This may be due to the osmotic properties of the brain, in which the fatty acids of the Vaseline create a barrier in the transportation of the dye molecules. Staining without Vaseline was therefore the safest method, and turned out to work very well.

Conclusions

- The method of applying dye into the LH of the moth brain resulted in successfully stained preparations revealing neuropil structures and distinct tracts being linked to this region in both hemispheres.
- The identification of two commissures in male preparations, each connecting symmetric regions of the LH in the two hemispheres, indicates bilateral processing of odor information by third order neurons.
- One commissure innervates the dorsal-anterior region of the LH, formerly shown to represent pheromone signals and the other innervates a more ventral-posterior region of the LH, formerly shown to represent plant odor signals.
- The LPOG is contralaterally connected to the LH by means of (at least) 9 PNs having their small-sized somata located in a distinct cell group located ventrally in the lateral cell cluster.
- In addition to the ipsilateral ALTs of the moth, there is a relatively thick tract projecting contralaterally.
- This tract, which is not previously described, and here named the cALT, comprises approximately 50 PNs originating from mainly posterior glomeruli and having their somata in a particular cell group located in the lateral cell cluster. The cALT, which projects along the course of the mALT in the ipsilateral hemisphere, crosses the brain midline anteriorly of the central body and seems to pass along the trajectory of the mlALT in the contralateral hemisphere.
- The cALT demonstrates that a substantial proportion of odor information is relayed to the contralateral hemisphere already the level of second order neurons.

Abbreviations

AL	Antennal-lobe
ALT	Antennal-lobe tract
cALT	Contralateral antennal-lobe tract
CB	Central body
CI	Commissure I
CΠ	Commissure Π
GABA	γ-Aminobutyric acid
KC	Kenyon cell
lALT	Lateral antennal-lobe tract
LH	Lateral horn
LN	Local interneuron
LPOG	Labial pit organ glomerulus
mALT	Medial antennal-lobe tract
MGC	Macroglomerular complex
mlALT	Mediolateral antennal-lobe tract
OBP	Odorant-binding protein
OG	Ordinary glomeruli
ODE	Odorant-degrading enzyme
OR	Olfactory receptor
OSN	Olfactory sensory neuron
PN	Projection neuron
ΔILPC	Delta area of the inferior lateral protocerebrum

References

Ache, B. W., & Young, J. M. (2005). Olfaction: Different species, conserved principles. *Neuron, 48*(3), 417-430. doi: 10.1016/j.neuron.2005.10.022

Aungst, J. L., Heyward, P. M., Puche, A. C., Karnup, S. V., Hayar, A., Szabo, G., & Shipley, M. T. (2003). Centre-surround inhibition among olfactory bulb glomeruli. *Nature, 426*, 623-629. doi: 10.1038/nature02187

Bear, M. F., Connors, B. W., & Paradisio, M. A. (2007). *Neuroscience: Exploring the brain.* Baltimore, MD: Lippincott Williams & Wilkins.

Belle, J., & Heisenberg, M. (1994). Associative odor learning in Drosophila abolished by chemical ablation of mushroom bodies. *Science, 263*(5147), 692-695. doi: 10.1126/science.8303280

Berg, B. G., Almaas, T. J., Bjaalie, J. G., & Mustaparta, H. (1998). The macroglomerular complex of the antennal lobe in the tobacco budworm moth *Heliothis virescens*: specified subdivision in four compartments according to information about biologically significant compounds. *Journal of Comparative Physiology A, 183*(6), 669-682. doi: 10.1007/s003590050290

Berg, B. G., Galizia, C. G., Brandt, R., & Mustaparta, H. (2002). Digital atlases of the Antennal Lobe in Two Species of Tobacco Budworm Moths, the Oriental *Helicoverpa assulta* (Male) and the American *Heliothis virescens* (Male and Female). *The Journal of Comparative Neurology, 446*(2), 123-134. doi: 10.1002/cne.10180

Berg, B. G., Schachtner, J., & Homberg, U. (2009). γ-Aminobutyric acid immunostaining in the antennal lobe of the moth *Heliothis virescens* and its colocalization with neuropeptides. *Cell and tissue research, 335*(3), 593-605. doi: 10.1007/s00441-008-0744-z

Berg, B. G., Zhao, X., & Wang, G. (2014). Processing of pheromone information in related species of heliothine moths. *Insects, 5*(4), 742-761. doi: 10.3390/insects5040742

Brodal, P. (2009). *Sentralnervesystemet.* Oslo, Norway: Universitetsforlaget.

Buck, L., & Axel, R. (1991). A novel multigene family may encode odorant receptors: A molecular basis for odor recognition. *Cell, 65*(1), 175-187. doi: http://dx.doi.org/10.1016/0092-8674(91)90418-X

Buck, L. B., & Bargmann, C. I. (2013). Smell and taste: The chemical senses. In E. R. Kandel, J. H. Schwartz, T. M. Jessell, S. A Siegelbaum & A. J. Hudspeth (Eds.), *Principles of Neural Science* (pp. 712-725). USA: McGraw-Hill companies.

Chang, H., Liu, Y., Yang, T., Pelosi, P., Dong, S., & Wang, G. (2015). Pheromone binding proteins enhance the sensitivity of olfactory receptors to sex pheromones *in Chilo suppressalis*. *Scientific Reports, 5,* 1-12. doi:10.1038/srep13093

Christensen, T. A., Waldrop, B. R., Harrow, I. D., & Hildebrand, J. G. (1993). Local interneurons and information processing in the olfactory glomeruli of the moth *Manduca sexta*. *Journal of Comparative Physiology, 173*(4), 385-399. doi: 10.1007/BF00193512

Dacks, A. M., Christensen, T. A., & Hildebrand, J. G. (2006). Phylogeny of a serotonin-immunoreactive neuron in the primary olfactory center of the insect brain. *The Journal of Comparative Neurology, 498*(6), 727-746. doi: 10.1002/cne.21076

Dahl, I. M. (2013). *Mapping of central pathways for CO_2 information in the brain of the moth Heliothis virescens.* (Master's thesis, Norwegian university of science and technology). Department of biology: Trondheim.

Fahrbach, S. E. (2006). Structure of the mushroom bodies of the insect brain. *Annual Review of Entomology, 51*, 209-232. doi: 10.1146/annurev.ento.51.110104.150954

Fitzpatrick, D., & Mooney, R. D. (2012). The chemical senses. In D. Purves, G. J. Augustine, D. Fitzpatrick, W. C. Hall, A. S. LaManita, & L. E. White (Eds.), *Neuroscience* (pp. 321-340). Sunderland, Massachusetts: Sinauer Associates.

Galizia, G.C. & Lledo, P. (2013). Olfaction. In G. C. Galizia & P. Lledo (Eds.), *Neurosciences: From molecule to behavior* (pp. 253-283). Springer spectrum.

Galizia, G. C., & Rössler, W. (2010). Parallel olfactory systems in insects: Anatomy and function. *Annual Review of Entomology, 55*, 399-420. doi: 10.1146/annurev-ento-122408-085422

Guerenstein, P. G., & Hildebrand, J. G. (2008). Roles and effects of environmental carbon dioxide in insect life. *Annual Review of Entomology, 53,* 161-178. doi: 10.1146/annurev.ento.53.103106.093402

Gupta, N., & Stopfer, M. (2012). Functional analysis of a higher olfactory center, the lateral horn. *The Journal of Neuroscience, 32*(24), 8138-8148. doi: 10.1523/JNEUROSCI.1066-12.2012

Hammer, M. (1997). The neural basis of associative reward learning in honeybees. *Trends in Neurosciences, 20*(6), 245-252. doi: 10.1016/S0166-2236(96)01019-3

Haupt, S. S., Sakurai, T., Namiki, S., Kazawa, T., & Kanzaki, R. (2010). Olfactory information processing in moths. In A. Menini (Ed.), *The neurobiology of olfaction* (pp.126-161). Boca Raton: CRC Press.

Heisenberg, M. (1998). What do the mushroom bodies do for the insect brain? An introduction. *Learning & Memory, 22*(5), 1-10. doi: 10.1101/lm.5.1.1

Homberg, U., Christensen, T. A., & Hildebrand, J. (1989). Structure and function of the deutocerebrum in insects. *Annual Review of Entomology, 34*(1), 447-501. doi: 0066-4170/8910101-0477$02.00

Homberg, U., Montague, R. A., & Hildebrand, J. G. (1988). Anatomy of antenna-cerebral pathways in the brain of sphinx moth *Manduca sexta*. *Cell and Tissue, 254*(2), 255-281.doi: 10.1007/BF00225800

Ian, E., Berg, A., Lillevoll, S., & Berg, B. (In revision). Antennal-lobe tracts in the noctuid moth, Heliothis virescens: new anatomical findings. *Under revision in Cell and Tissue Research.*

Ito, K., Shinomiya, K., Masayoshi, I., Armstrong, J. D., Boyan, G., Hartenstein, V.,… Vosshall, L. B. (2014). A systematic nomenclature for the insect brain. *Neuron, 81*(4), 755-765. doi: 10.1016/j.neuron.2013.12.017

Jefferis, G. S. X. E., Potter, C. J., Chan, A. M., Marin, E. C., Rohlfing, T., Maurer, C. R., & Luo, L. (2007). Comprehensive maps of *Drosophila* higher olfactory centers: Spatially segregated fruit and pheromone representation. *Cell, 128*(6), 1187-1203. doi: doi:10.1016/j.cell.2007.01.040

Jortner, R. A. (2016). Neural coding in the olfactory system. In R. Q. Quiroga & S. Panzeri (Eds.), *Principles of Neural Coding* (pp. 225-262). Retrieved from http://www.crcnetbase.com/isbn/9781439853313

Kent, K. S., Harrow, I. D., Quartararo, P., & Hildebrand, J. G. (1986). An accessory olfactory pathway in Lepidoptera: the labial pit organ and its central projections in *Manduca sexta* and certain other sphinx moths and silk moths. *Cell and Tissue Research, 245*(2), 273-245. doi: 10.1007/BF00213927

Louis, M., Huber, T., Benton, R., Sakmar, T. P., & Vosshall, L. B. (2007). Bilateral olfactory sensory input enhances chemotaxis behavior. *Nature Neuroscience, 11,* 187-199. doi: 10.1038/nn2031

Martin, J. P., Beyerlein, A., Dacks, A. M., Reisenman, C. E., Riffell, J. A., Lei, H., & Hildebrand, J. G. (2011). The neurobiology of insect olfaction: sensory processing in a comparative context. *Progress in Neurobiology, 95*(3), 427-447. doi: 10.1016/j.pneurobio.2011.09.007

Menzel, R. (2013). Learning, memory, and cognition: Animal prespectives. In G. C. Galizia & P. Lledo (Eds.), *Neurosciences: From molecule to Behavior* (pp. 629-653). Springer spectrum.

Menzel, R., Leboulle, G., & Eisenhardt, D. (2006). Small brains, bright minds. *Cell, 124*(2), 237-239. doi: 10.1016/j.cell.2006.01.011

Namiki, S., Iwabuchi, S., Kono, P. P., & Kanzaki, R. (2014). Information flow through neural circuits for pheromone orientation. *Nature Communications, 5,* 1-11. doi: 10.1038/ncomms6919

Pelosi, P., Zhou, J. J., Ban, L. P. & Calvello, M. (2006). Soluble proteins in insect chemical communication. *Cellular and Molecular Life Sciences, 63*(14), 1658-1676.doi: 10.1007/s00018-005-5607-0

Pophof, B. (2004). Pheromone-binding proteins contribute to the activation of olfactory receptor neurons in the silkmoths *Antheraea polyphemus* and *Bombyx mori*. *Chemical Senses, 29*(2), 117-125. doi: 10.1093/chemse/bjh012

Roth, G. (2013). *The long evolution of brains and minds.*
Retrieved from: http://download.springer.com/static/pdf/481/bok%253A978-94-007-6259-6.pdf?originUrl=http%3A%2F%2Flink.springer.com%2Fbook%2F10.1007%2F978-94-007-6259-6&token2=exp=1461160219~acl=%2Fstatic%2Fpdf%2F481%2Fbok%25253A978-94-007-6259-6.pdf%3ForiginUrl%3Dhttp%253A%252F%252Flink.springer.com%252Fbook%252F10.1007%252F978-94-007-6259-6*~hmac=b0f7d958dec71ced2c90b198f492e649770ba291db8416db55ff1f9e013f12b4

Ruta, V., Datta, S. R., Vasconcelos, M. L., Fræland, J., Looger, L. L., & Axel, R. (2010). A dimorphic pheromone circuit in *Drosophila* from sensory input to descending output. *Nature, 468,* 686-690. doi: 10.1038/nature09554

Rø, H., Müller, D., & Mustaparta, H. (2007). Anatomical organization of antennal lobe projection neurons in the moth *Heliothis virescens. The Journal of Comparative Neuorology, 4*(500), 658-675. doi: 10.1002/cne.21194

Rössler, W., Tolbert, L. P., & Hildebrand, J. G. (1998). Early formation of sexually dimorphic glomeruli in the developing olfactory lobe of the brain moth *Manduca sexta*. *The Journal of Comparative Neurology, 396*(4), 415-428. doi: 10.1002/(SICI)1096-9861(19980713)396:4<415::AID-CNE1>3.0.CO;2-4

Røstelien, T., Stranden, M., Borg-Karlson, A. K., & Mustaparta, H. (2005). Olfactory receptor neurons in two heliothine moth species responding selectively to aliphatic green leaf volatiles, aromatic compounds, monoterpenes and sesquiterpenes of plant origin. *Chemical Senses, 30*(5), 443-461.doi: 10.1093/chemse/bji039

Shepherd, G. M. (2006). Smell images and the flavor system in the human brain. *Nature, 444*, 316-321. doi: 10.1038/nature05405

Stranden, M., Røstelien, T., Liblikas, I., Almaas, T. J., Borg-Karlson, A. K., & Mustaparta, H. (2003). Receptor neurons in the three heliothine moths responding to floral and inducible plant volatiles. *Chemology, 13*(3), 143-154. doi: 10.1007/s00049-003-0242-4

Strausfeld, N. J., Hansen, L., Yongsheng, L., Gomez, R. S., & Ito, K. (1998). Evolution, discovery, and interpretations of arthropod mushroom bodies. *Learning & Memory, 22*(5), 11-37. doi: 10.1101/lm.5.11

Tegoni, M., Pelosi, P., Vincent, F., Spinelli, S., Campanacci, V., Grolli, S., Ramoni, R., & Cambillau, C. (2000). Mammalian odor binding proteins. *Biochimica et Biophysica Acta, 1482*(1-2), 229-240. doi: 10.1016/S0167-4838(00)00167-9

Zhao, X. C., & Berg, B. G. (2009). Morphological and physiological characteristics of the serotonin-immunoreactive neuron in the antennal lobe of the male oriental tobacco budworm, *Helicoverpa assulta*. *Chemical Senses, 34*(5), 363-372. doi: 10.1093/chemse/bjp013

Zhao, X. C., Chen, Q. Y., Guo, P., Xie, G. Y., Tang, Q. B. Guo, X. R., & Berg, B. G. (2016). Glomerular identification in the antennal lobe of the male moth *Helicoverpa armigera. The Journal of Comparative Neurology,* accepted article. doi: 10.1002/cne.24003

Zhao, X. C., Kvello, P., Løfaldli, B. B., Lillevoll, S. C., Mustaparta, H., & Berg, B. G. (2014). Representation of pheromones, interspecific signals, and plant odors in higher olfactory centers; mapping physiologically identified antennal-lobe projection neurons in the male heliothine moth. *Frontiers in System Neuroscience, 8*, 1-14. doi: 10.3389/fnsys.2014.00186

Zhao, X. C., Tang, Q. B., Berg, B. G., Liu, Y., Wang, Y. R., Yan, F. M., & Wang, G. R. (2013). Fine structure and primary sensory projections of sensilla located in the labial-pit organ of *Helicoverpa armigera* (Insecta). *Cell and Tissue Research, 353*(3), 399-408. doi: 10.1007/s00441-013-1657-z

Appendix

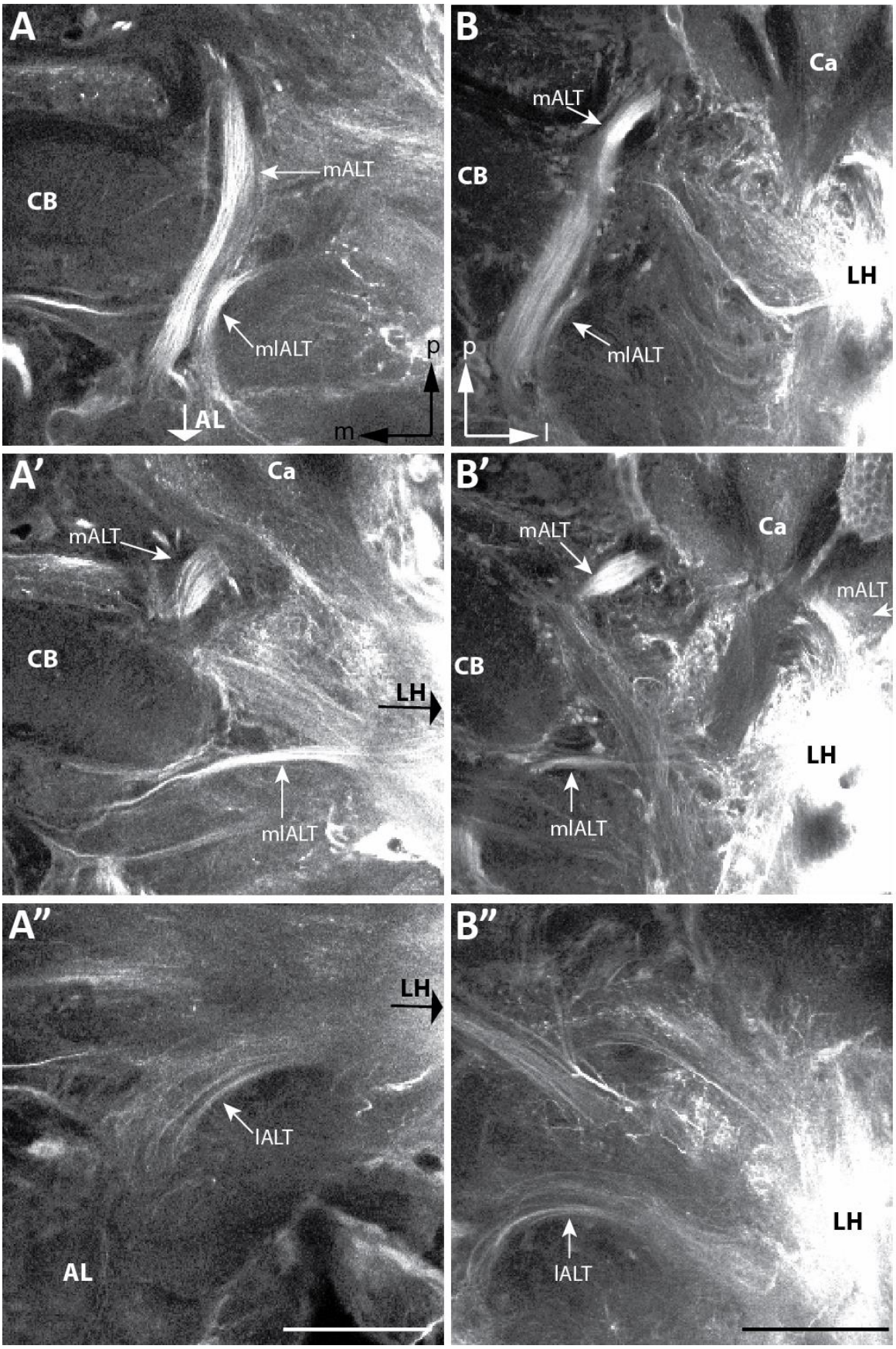
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Figure A1. Single-slice confocal images of two different preparations (from dorsal to ventral), showing the three main ipsilateral antennal-lobe tracts (ALTs). The overall staining pattern is in full accordance with that demonstrated in Fig. 16. **A-A'':** Preparation No.133. **B-B'':** Preparation No.190. As demonstrated, the medial ALT (mALT) leaves the antennal-lobe (AL) together with the mediolateral ALT (mlALT) and bypasses the central body (CB). The mALT targets the calyces of the mushroom bodies (Ca) before it continues to the lateral horn (LH). The mlALT projects directly to the LH. The lateral ALT (lALT) leaves the AL most ventrally and projects directly to the LH. Posterior (p), medial (m). 20X objective. Scale bars: 100 μm.

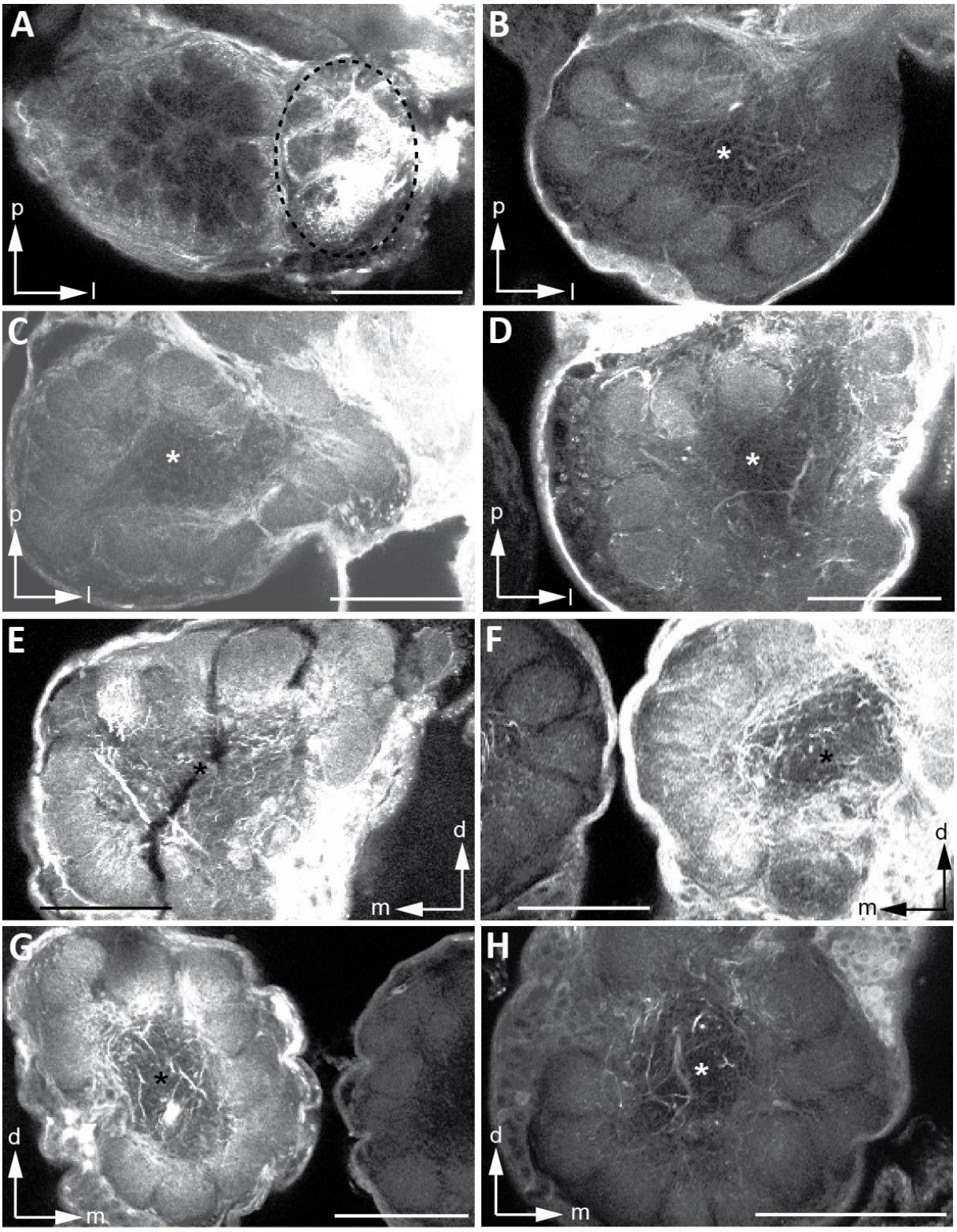
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Figure A2. Single-slice confocal images showing staining patterns in the ipsilateral antennal-lobe (AL) of eight different brain preparations in a dorsal (A-D) and frontal (E-H) orientation. Similarly to the staining presented in Fig. 17, the preparations display extensive staining in all glomeruli as well as in the non-glomerular central core (indicated by an asterisk). **A:** The AL of preparation No.111 shows four enlarged glomeruli constituting the macroglomerular complex (MGC) located dorso-laterally in the AL (indicated by dashed circle). **B:** Prep. 133. **-C:** Prep. 190. **D:** Prep. 157**. E:** Prep. 193**. F:** Prep. 124. **G:** Prep. 24. **H:** Prep. 18. Posterior (p), medial (m), lateral (l), dorsal (d). 20X objective. Scale bar: 100 µm.

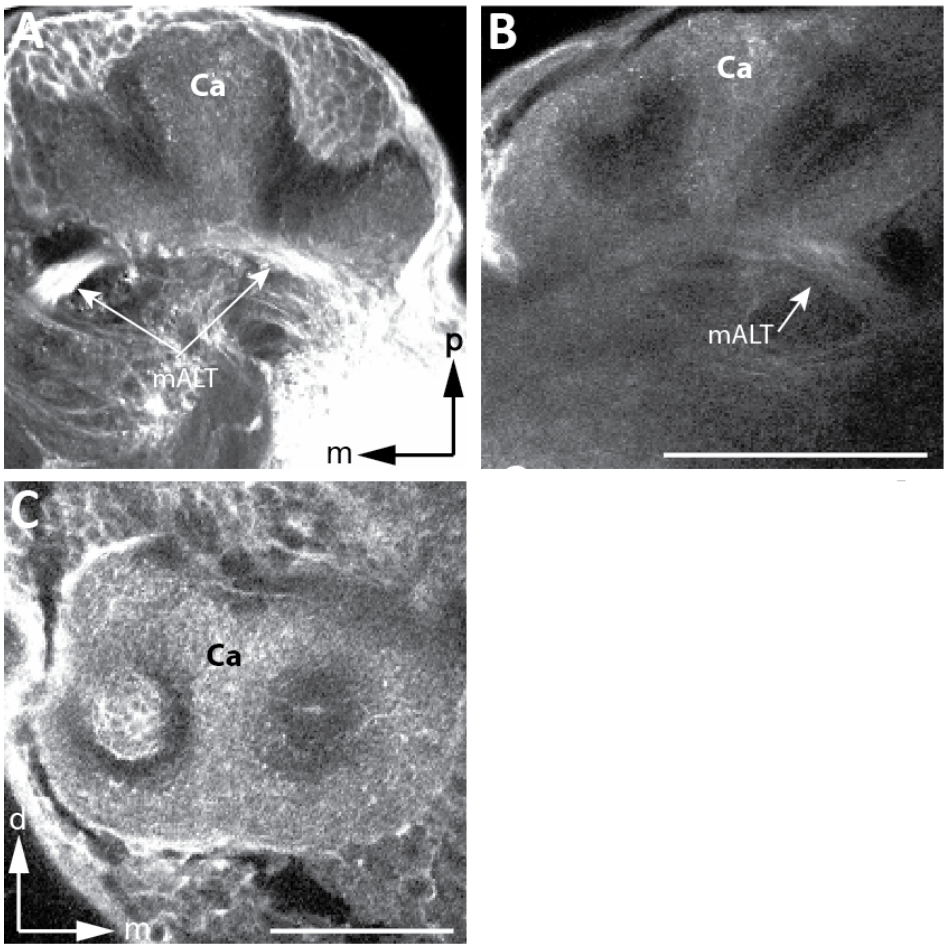

Figure. A3. Confocal images from 3 different preparations, showing labelling of the calyces (Ca) in the ipsilateral hemisphere. **A-B:** Preps. No.24 (A), and 150 (B), dorsal oriented. **C:** Prep. nr.190, frontal oriented. Medial (m), posterior (p), dorsal (d). 20X objective. Scale bars: 100 µm.