NTNU – Trondheim
Norwegian University of
Science and Technology

Mapping of Central Pathways for CO_2 Information in the Brain of the Moth *Heliothis virescens*

Ingrid Moe Dahl

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Supervisor: Bente Gunnveig Berg, IBI
Co-supervisor: Tor Jørgen Almaas, IBI

Norwegian University of Science and Technology
Department of Biology

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It's been a blast!

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Ingrid Moe Dahl

Sammendrag

Luktesystemet hos insekter er essensielt for deres evne til å søke matkilder og eggleggingssteder, samt for reproduksjon. Informasjon om ulike dufter produsert av planter og av insektene selv, fremkaller responsbestemt atferd. Kunnskap om hvordan ulike kjemiske substanser endrer insektets atferd kan benyttes i utvikling av spesifikke og biologisk ufarlige pesticider. En viktig kjemisk signal substans hos insekter er karbondioksid (CO_2). Dette masterprosjektet benyttet nattsvermeren *Heliothis virescens* som forsøksdyr for å kartlegge de sentrale nervebanene som er involvert i prosessering av CO_2 informasjon. Ved injisering av fargestoff i det sensoriske organet for CO_2 på de labiale palpene, var det mulig å visualisere det spesifikke området for primær prosessering av CO_2 i antenneloben, kalt labial pit organ glomerulus (LPOG), i levende individer. Ved påfølgende retrograd farging av andreordens nevroner fra LPOG, ble de distinkte projeksjonsbaner til høyere ordens hjernesentre visualisert. Projeksjonsnevronene viste seg å følge to av de antenno-protocerebrale traktene; henholdsvis den laterale- og medio-laterale trakten. Begge nervebuntene viste omfattende forgreininger i protocerebrum. To terminalområder mottok informasjonen fra LPOG; det laterale protocerebrum og superior protocerebrum. Ingen av de fargede projeksjonsnevronene fulgte ruten via den mediale antenno-protocerebrale trakten til mushroom body calyces. Dette indikerer at informasjon om CO_2 kun prosesseres i en lærings-uavhengig bane. Alle fargede projeksjonsnevroner fra LPOG var uniglomerulære, også de som fulgte den medio-laterale trakten. Dette skiller seg fra det generelle luktesystemet der sistnevnte trakt inneholder fibre fra multiglomerulære projeksjonsnevroner. Ingen kjønnsbestemt dimorfisme ble påvist ved sammenlikning av CO_2-banene hos hunner og hanner. Totalt viser resultatene i dette masterprosjektet at det er vesentlige forskjeller mellom det generelle luktesystemet og CO_2-systemet hos *H.virescens*.

Abstract

The olfactory system is vital for insects in their ability to seek food, reproduce, and orientate. Information about different odors, produced by plants or conspecifics, elicits behavioral responses in the insects. Knowledge about how chemical substances change the insects' behavior may be used in development of pest-specific and biologically harmless insecticides. One biological relevant odor cue is carbon dioxide (CO_2). This Master's project used the noctuid moth *Heliothis virescens* as model organism for mapping the central neural pathways involved in processing of CO_2 information. By injecting dye into the CO_2 sensory organ on the labial palps, it was possible to visualize the labial pit organ glomerulus (LPOG) in the antennal lobes of living individuals. Subsequent retrograde stainings of the LPOG revealed distinct projection pathways to higher brain centers. The stained projection neurons were demonstrated to follow two of the main antenno-protocerebral tracts; the lateral- and the medio-lateral tract. Both of the neural bundles showed extensive branching in the protocerebrum. Two main termination areas were found to receive information from the LPOG; the lateral- and superior protocerebrum. No projection neurons followed the route via the medial antenno-protocerebral tract to the mushroom body calyces. This indicates that information about CO_2 is processed only by an experience-independent pathway. All projection neurons originating from the LPOG were found to be uniglomerular, also those passing in the medio-lateral tract. This is in contrast to findings related to the general olfactory system where the last mentioned tract includes fibers of multiglomerular projection neurons. Comparison of the CO_2 pathways in males and females showed no indications of sexual dimorphism. In total, the results from this study show significant differences between the general olfactory system and the CO_2 system of *H. virescens*.

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1 Introduction

Olfaction is one of the most ancient of all senses, and the neural mechanisms underlying olfactory information processing are well preserved through evolution. This becomes apparent in the many striking similarities between the olfactory pathways in vertebrates and insects. The olfactory system of insects therefore makes up a good experimental model for studying the principles connected to this arrangement in general, including that of both invertebrates and vertebrates. Olfaction is vital to insects in their ability to seek food, reproduce, and orientate. These organisms also have many direct and indirect impacts upon our environment, both negative as agricultural pests, and positive as honey producers and pollinators. Consequently, insect olfaction has become a well-studied field of science. By investigating the insect sensory mechanisms in general, and olfaction in particular, we can use this information in monitoring and managing pest insects, as well as improve our understanding of how to better utilize the insects we benefit from. Additionally, one should not underestimate the importance of gaining knowledge about the nature surrounding us, and how different organisms are adapted to their specific habitats.

Many different odor cues are involved in stimulating insect behavior, one of them being CO_2. This project aims to study the central pathways for processing of CO_2 information, using the moth *H. virescens* as model organism. The study is conducted as a part of the basic scientific neurobiology research that considers coding of chemosensory signal information. Knowledge about the processing of CO_2 in biological systems, particularly on the neurological level, will undoubtedly be advantageous, not only to expand our knowledge about the sensory systems, but also considering that we live in a time where we observe an ongoing increase in the atmospheric CO_2 concentration.

1.1 The insect as a model organism

Lepidoptera is one of the world's largest orders of organisms and makes up about 10 % of all known species, beaten only by the beetles (Coleoptera). The approximately 200,000 different moth species inhabit almost all terrestrial environments on earth, including polar regions as well as tropical areas (Majerus, 2002). The noctuid moth *H. virescens* is considered to be an agricultural pest as it feeds on crops. The larvae eat off the buds of the cotton plant, hence causing damages on the crops and huge economic losses for the industry (Matthews, 1999). After the Second World War, the use of insecticides increased significantly, and is still the most common method for controlling pest insects. The technique is efficient for decreasing the number of pest insects in an area, but it is both unspecific and very poisonous, often affecting the other living organisms present (Gullan, 2010). As part of an alternative strategy it should therefore be of great importance and interest to increase the knowledge about how these insects respond to and process chemical signals, both in selection of host plants and mates. This information can be used in development of biologically harmless methods to monitor and control the specific pest insects of concern, while avoiding possible harmful effects on other species.

Among other heliothine species, *H. virescens* has been used in research on brain anatomy and physiology by the Neuroscience Unit at Dept. of Biology and Dept. of Psychology at the Norwegian University of Science and Technology (NTNU). Their easy accessible neural system and unique adaptability have made it possible to study which odor substances they detect, how chemosensory information is being processed in the brain, and how this information is linked to behavior. The thorough knowledge already obtained from heliothine moths, and also other moth species, constitutes a strong basis for the current work involving mapping of the central pathways mediating CO_2 information in *H. virescens*.

1.2 The organization of the olfactory pathway in insects

Odors have a very important role in the life of insects in detecting attractive or unattractive sources. The nocturnal moths are dependent upon the olfactory system in food search, communication, reproduction and orientation (Hansson, 1995, Stranden et al., 2003). Both plant-produced odors, and odors produced by the insects themselves, are important signal inputs in the Lepidoptera. (Hansson, 1995).

1.2.1 Primary processing of olfactory information

In Lepidopterans, the main olfactory organ is the antennae with up to 100,000 olfactory sensilla. In addition, many species are found to have an organ specialized for detection of CO_2, called the labial pit organ. This will be further introduced in later sections. The lumen of the antennal sensilla houses the dendrites of the olfactory receptor neurons (ORNs) where they are surrounded by sensillum lymph. The wall pores of the sensilla allow odor molecules to be transported through the cuticula and into the lymph. Odor binding proteins (OBPs) ensure the transportation of the lipophilic odor molecules to the olfactory receptors (ORs) on the ORN dendrite surface (reviewed by Hansson, 1995). The insect ORs constitute a heteromeric complex, consisting of one variable odorant-binding subunit, and one Or83b co-receptor. This complex is shown to make up a ligand-gated ion channel, hence also functioning as ionotropic receptors (Kaupp, 2010, Sato et al., 2008, Wicher et al., 2008) The receptor complex is made up by 7-transmembrane G-protein-coupled receptors. However, in insects the receptors are structurally and genetically different from those first described in mammals (Buck and Axel, 1991). The membrane topology is inversed with the N-terminal facing towards the cytoplasm (Benton et al., 2006, Kaupp, 2010, Lundin et al., 2007). In moths, the ORNs seem to be narrowly tuned, being most sensitive to one key odorant (Almaas et al., 1991, Berg et al., 1995, Røstelien et al., 2000, Røstelien et al., 2005, Stranden et al., 2002). The binding of the stimulus molecule activates a signal transduction mechanism which produces a transformed signal, called action potentials. These signals are then mediated via the ORN axons forming the antennal nerve (AN). The olfactory signals are sent directly to the antennal lobe (AL), the primary olfactory center of the insect brain. The AL is made up of a collection of spherical neuropils called glomeruli (reviewed by Boeckh and Tolbert, 1993, Hildebrand, 1996). Studies on *Drosophila melanogaster* have revealed that each ORN expresses one OR type, and each glomerulus receives input from one specific type of ORN (Couto et al., 2005, Gao et al., 2000, Vosshall et al., 2000, reviewed by Keene and Waddell, 2007). This seems to be valid also for moths, as staining of physiologically characterized ORNs show corresponding projection patterns (Berg et al., 1998, Berg et al., 2005, reviewed by Hansson et al., 1995). The AL is thus functioning as a topographic map of receptor activity (Wong et al., 2002). This has been referred to as "the molecular logic of smell" (Axel, 1995). Figure 1.1 shows a three-dimensional reconstruction of the heliothine moth brain, with its prominent antennal lobes.

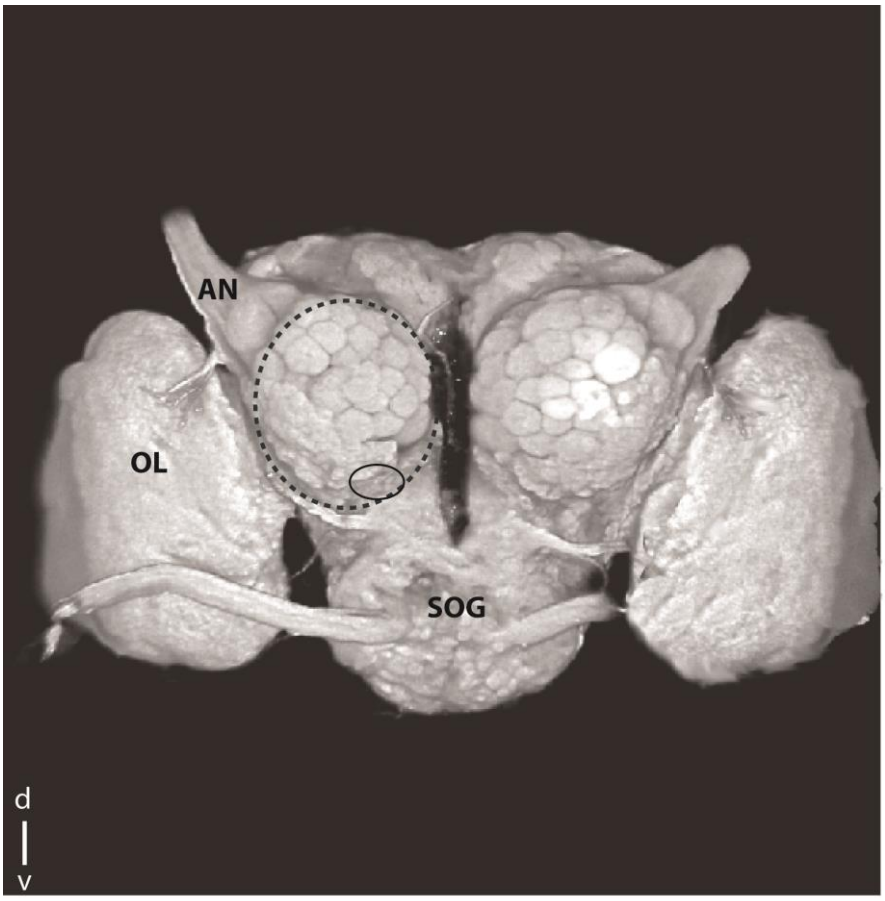

Figure 1.1: Three-dimensional confocal reconstruction of the brain of a heliothine moth. The dashed circle indicates the antennal lobe and the whole-lined circle indicate the approximate position of the labial pit organ glomerulus. **AN:** antennal nerve, **OL:** optic lobe, **SOG:** sub-oesophageal ganglion. Frontal view. (Berg et al., 2002).

The total assembly of AL glomeruli in the male moth includes two subdivisions; the ordinary glomeruli and the glomeruli processing pheromone information. The number of ordinary glomeruli in the AL varies between insect species; 43 in *D. melanogaster* (Wong et al., 2002), 62 in *H. virescens* (Berg et al., 2002, Løfaldli et al., 2010), 63 in *Manduca sexta* (Rospars and Hildebrand, 2000), and about 160 in *Apis mellifera* (Arnold et al., 1985). In male moths detecting long-range sex pheromones from the females, a part of the glomerular structure has been transformed into a characteristic array of 2-6 glomeruli making up the macroglomerular complex (MGC). In *H. virescens*, for example, the MGC includes four enlarged glomeruli located at the entrance of the AN in the AL (Berg et al., 1998, Vickers et al., 1998). In addition to the sensory projections, the glomeruli receive information from two main types of AL-neurons; local interneurons (LNs) and projection neurons (PNs). The LNs are mainly GABA-ergic inhibitory neurons and are restricted locally in the AL, mediating information laterally between glomeruli (Berg et al., 2009, Waldrop et al., 1987, reviewed by Mustaparta,

2002). The PNs receive information from both ORNs and LNs, and mediate this information from the AL via several antenno-protocerebral tracts to higher-order brain centers in the protocerebrum (Homberg et al., 1988, Rø et al., 2007). Additionally, the AL receives modulatory input from centrifugal neurons.

1.2.2 The antenno-protocerebral tracts

To understand how sensory information is coded in the nervous system, it is inevitable to gain knowledge about the morphology of central cell types and how they are organized relative to each other in the brain. As concerns the moth brain, this encompasses the projection pathways of PNs from the AL to higher brain centers. By use of intracellular recording and staining, it has been possible to investigate the physiological properties of olfactory PNs in several moth species, heliothine moths included. Visualization by confocal laser-scanning microscopy makes the brain available for studies on neuron morphology and projection patterns (Rø et al., 2007). Three-dimensional reconstructions of the insect brain have been constructed for several insect species, and are used to create standard brain atlases (SBAs) which make excellent tools for studying the distribution and spatial relationship of brain features and neurons (Brandt et al., 2005, Kurylas et al., 2008, Kvello et al., 2009, Rein et al., 2002). In *H. virescens*, the glomerular layer of the AL was added to the SBA, making it possible to integrate a model of the individual glomeruli, thus constituting a remedy to study the PN innervation of the different glomeruli separately, in addition to the projection patterns in the protocerebrum (Kvello et al., 2009, Løfaldli et al., 2010).

Olfactory information is transported by the PNs to higher protocerebral centers through three main antenno-protocerebral tracts; the medial antenno-protocerebral tract (m-APT), the medio-lateral antenno-protocerebral tract (ml-APT) and the lateral antenno-protocerebral tract (l-APT). These are presented in figure 1.2. The nomenclature of these tracts has differed widely among species. In this study it is used as suggested by Galizia and Rösler (2010) and indicate the position of the tracts relative to each other without necessarily implying a functional homology between species (Galizia and Rössler, 2010).

The PNs can originate from only one glomerulus (uniglomerular) or have dendritic arborizations in several glomeruli (multiglomerular) (Mustaparta, 2002).

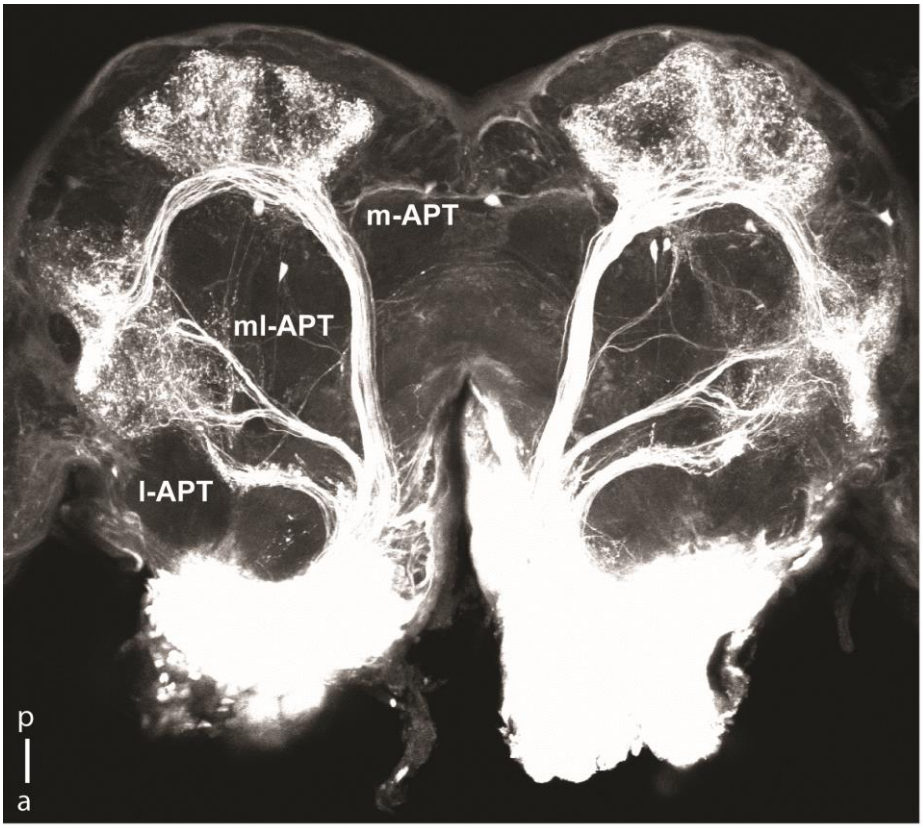

Figure 1.2: The three main antenno-protocerebral tracts in a moth brain. **m-APT:** medial antennoprotocerebral tract, **ml-APT:** medio-lateral antennoprotocerebral tract, **l-APT: l**ateral antenno-protocerebral tract. Dorsal view. Shown with permission from Xin-Cheng Zhao (unpublished data).

The most prominent tract is found to be the m-APT. It leaves the AL dorso-medially, projects posteriorly, before turning laterally at the posterior, ventral-lateral edge of the central body (CB). Towards its final target area in the ipsilateral protocerebrum, it gives off terminal projections to the mushroom body calyces. The PNs running in the m-APT are mainly uniglomerular with dense arborizations in one single glomerulus. Some sexual dimorphism is evident in the number of fibers in this tract due to male-specific PNs arborizing in the MGC (Homberg et al., 1988). The considerably thinner ml-APT leaves the AL together with the m-APT and joins this tract for a short distance before it turns laterally at the anterior part of the CB, and projects directly towards the optic lobe. Most of the axons making up this tract terminate in the lateral protocerebrum (Homberg et al., 1988, Rø et al., 2007). So far, all PNs in the ml-APT are found to be multiglomerular. The l-APT contains both uni- and multiglomerular PNs, and constitutes a loose bundle of small diameter fibers leaving the AL medio-ventrally, projecting laterally with some branching, and targets the lateral protocerebrum. Some of the fibers continue dorsally from the lateral protocerebrum and have

been seen to innervate the ipsilateral mushroom body calyces (Homberg et al., 1988, Rø et al., 2007). Homberg et al (1988) described and named the PNs of the three main tracts (m-APT, ml-APT and l-APT) PI, PM and PO, respectively (based upon old nomenclature of the tracts). Mass staining of AL PNs in *H. virescens* revealed neurons of the same morphological type and they were classified according to the study on *M. sexta* (Rø et al., 2007).

The cell somata of all AL neurons, including PNs and LNs, are located in three different clusters enveloping the glomeruli. The lateral cluster, which is situated along the lateral side of AL, adjacent to the optic lobe, constitute the most numerous group of AL-somata and is subdivided in a large dorsal cluster (LCI) and a smaller postero-ventral cluster (LCII) (Homberg et al., 1988). The medial cell cluster is positioned dorsally in the AL facing the midline of the brain, and the tiny anterior cell cluster is positioned at the most anterior site of the AL. Only the medial cluster shows sexual dimorphism in regards to the number of somata in the cell cluster (Homberg et al., 1988, Homberg et al., 1989, reviewed by Hansson, 1995). The lateral cell cluster consists of somata of both PNs and LNs, while the medial- and the anterior cluster only holds somata of PNs. Results from an ongoing Master's study describing the nervous branching patterns of the AL, indicates that PNs following APTs apart from the m-APT, all seem to have their somata in the lateral cell cluster (Berg, A., 2013, personal communication, unpublished data).

1.2.3 Odor processing in higher-order brain areas

In adult moths, the main protocerebral target areas of the PNs are the calyces of the mushroom bodies and the lateral protocerebrum, in which the PNs relay the olfactory information onto third order neurons. This is also reported for *H. virescens* (Berg et al., 1998, Christensen et al., 1995, reviewed by Hansson, 1995). The mushroom bodies is located in the posterior, dorsal area of the protocerebrum, and is made up of three main parts; lobes, calyces and pedunculus (Strausfeld et al., 1998, reviewed by Heisenberg, 2003). The calyces receive olfactory information from PNs running in the m-APT and l-APT, in opposite directions. The terminals of AL PNs synapse with intrinsic neurons of the mushroom body calyces, called Kenyon cells (KC) (Mauelshagen, 1993, Menzel and Muller, 1996, reviewed by Heisenberg, 2003, Keene and Waddell, 2007). The dendritic branches of the KCs make up the calyces, while their parallel axons form the pedunculus before they branch to form the output area of

the mushroom bodies, the mushroom body lobes (MBLs). In the MBLs the KCs synapse on extrinsic neurons which connect the mushroom body system to other higher order brain areas, such as the superior- and lateral protocerebrum (Ito et al., 1998, Mauelshagen, 1993, Rybak and Menzel, 1998, Tanaka et al., 2008, reviewed by Strausfeld et al., 1998). The mushroom bodies are known as the primary center for olfactory learning and memory, and also has an important role in motor control (Menzel, 2001, Menzel and Giurfa, 2001, reviewed by Heisenberg, 2003, Keene and Waddell, 2007).

The lateral protocerebrum is often designated as a premotoric region in the insect brain. Concerning olfactory information little is known about the efferent neurons mediating information from the protocerebrum. One descending neuron with dendrites in the lateral protocerebrum, and an axon projecting out of the brain via the ipsi-lateral connective ventrally in the suboesophageal ganglion has been described for female *H. virescens* (Løfaldli et al., 2012). Descending neurons from the lateral triangle in the lateral accessory lobe have been reported from a study on a dimorphic pheromone circuit in *D. melanogaster*. These neurons were found be part of a circuit consisting of a minimum of 4 neurons and 3 synapses (Ruta et al., 2010). The lateral protocerebrum receives information from the AL conveyed via all the APTs mentioned above. As compared to the m-APT, which makes up an indirect route to this region via the calyces, the lateral- and the medio-lateral tracts constitute a more direct pathway, considered to represent an experience-independent route (Menzel, 2001, Tanaka et al., 2004, reviewed by Heisenberg, 2003).

1.3 Carbon dioxide and insects

CO_2 is an important component in the chemical environment. The atmospheric concentration of CO_2 is increasing, partly due to human activities. The average level on a global scale increased from 280 parts per million (ppm) before the industrial revolution, to a daily average of about 380 ppm measured in 2005, and is still increasing about 2 ppm each year. Such an increment is expected to have major influences on the biology of a great number of living organisms, and studies on CO_2 processing and adaptations are important to reveal the possible consequences of the increased concentration on insect ecosystems (Guerenstein and Hildebrand, 2008).

As the biosphere contains numerous sources and sinks for CO_2, many terrestrial arthropods have evolved organs that are sensitive to the resulting CO_2 concentration gradients. The different CO_2 levels carry a lot of information about the surrounding environment, and have proven to be important sensory signals in insects. It has thus been considerable improvement in the understanding of the role of CO_2 in insect olfaction, as in interactions between insects and host plants in food search, orientation, climate control (in bee colonies), oviposition behavior, and more (Guerenstein et al., 2004b, Guerenstein and Hildebrand, 2008, Stange et al., 1995, Stange, 1997). Electrophysiological recordings from the CO_2 receptors in the LPO of *Helicoverpa armigera,* a species closely related to *H. virescens*, revealed the fluctuations of ambient CO_2 concentrations which can be detected by the receptor neurons. It was reported that several sources in the environment can be capable of modulating the CO_2 level within a particular range (Stange, 1992). Behavioral studies on *H. armigera* larvae demonstrated strong attractiveness towards CO_2, using it as a cue together with host-specific signals in host preference behavior (Rasch and Rembold, 1994). Orientation was shown to be clearly influenced by different quantities of CO_2, and the *H. armigera* larvae used the CO_2 gradients to choose between photosynthetically active or inactive plant tissues, as the plant organs with high respiration have a higher nutritional value for the insects (Rasch and Rembold, 1994). Such utilization of CO_2 gradients in orientation behavior, has also been shown in other moth species as *Cactoblastis cactorum* (Stange et al., 1995). It has thus become evident that several insect species use information about CO_2 in search for resources, and knowledge about this specific sensory system may be used in the development of new strategies for surveillance and control of pest insects (Guerenstein and Hildebrand, 2008).

1.4 Processing of CO_2 information

1.4.1 Primary processing of CO_2 information

Insects have specialized receptor cells that can detect CO_2 in the surroundings. These cells are located in sensilla, either on the antennae or the mouth parts, but never on both in the same species. In adult Lepidoptera, wall-pored sensilla are gathered on the terminal segment of the labial palps, constituting a specialized organ for detection of CO_2, named the labial pit organ (LPO). Electrophysiological as well as morphological studies on different species have collected evidence that the LPO with their underlying sensilla have a chemoreceptive role and show structural properties similar to antennal olfactory sensilla (Bogner et al., 1986, Kent et al., 1986, Lee et al., 1985, Rasch and Rembold, 1994, Stange, 1992, reviewed by Hansson, 1995). The number of sensilla differs among species and orders. The LPO contains about 1750 sensilla in *M. sexta*, 40 in *Antheraea* (Kent et al., 1986), 200 in *C. cactorum* (Stange et al., 1995), 200 in *Pieris* (Lee et al., 1985) and 1200 in *H. armigera* (Zhao et al., 2013). In *H. armigera*, a recent study has reported two morphologically different sensillum types in the LPO (Zhao et al., 2013). The properties of the CO_2 receptor cells have been studied in detail for several insect species, and show some disparities compared to the typical olfactory receptor cells. Sensilla detecting CO_2 seem to register information about air velocity and odor concentration in another manner than common olfactory sensilla. The CO_2 sensory organ is highly sensitive and selective, detecting several concentration levels, as well as swift bi-directional changes in CO_2 concentration. It is also capable of detecting rapid signal fluctuations and variations in background levels of CO_2 simultaneously (Guerenstein and Hildebrand, 2008). The LPO receptor cells of *M. sexta* are suggested to be specialized for detecting changes in the CO_2 concentration, without being affected by natural concentrations of other organic volatile compounds (Guerenstein et al., 2004a). A study on *Rhodogastria* (Arctiidae) moths tested the functional properties of the LPO by stimulating with several different olfactory compounds. The investigation demonstrated that the receptors cells respond uniformly, being most excitable by stimulation with CO_2, with only secondary sensitivity to other odorants (Bogner et al., 1986). This confirms that the LPO is primarily responsive to CO_2.

The moth *C. cactorum* shows sexual dimorphism in regard to the development of the LPO organ, being rudimentary in males and prominent in females. This might imply that the LPO could be involved in oviparous behavior. The hawkmoth *M. sexta* does not display a distinct sexual dimorphism of the LPO, but still uses CO_2 as an oviparous signal in search for food (Guerenstein and Hildebrand, 2008). However, a behavioral study on *M. sexta* by Kent et al. (1986) did not show any effects upon the female's choice of oviposition site after ablation of the LPO, indicating they are not dependent upon this organ for oviparous behavior.

The axons of the CO_2 receptor cells of Lepidoptera species project via the labial-palp pit nerve (LPN) to the suboesophageal ganglion in the central nervous system. Then they project bilaterally to each of the ALs where they terminate in a specific glomerulus called the labial pit organ glomerulus (LPOG). The LPOG does not receive any input from the antennae and thereby seems to be a specialized target area for CO_2 information (Guerenstein et al., 2004a, Guerenstein and Hildebrand, 2008, Kent et al., 1999). Since this information originating from receptors on the palps terminate in the AL, and not in the suboesophageal ganglion, it could imply that the information about CO_2 is integrated with sensory input from the antennae. This idea is supported by findings of multiglomerular LNs in the AL of *M. sexta* (Christensen et al., 1993). It is suggested that the LPO might provide the only non-directional olfactory information to the antennal lobes as the input from the LPO is bidirectional and not unilateral as the antennal input. This kind of bidirectional input to the AL have shown to be valid for several species of *Manduca*, as well as *Bombyx* and *Antheraea* (Kent et al., 1986), plus heliothine species as *H .armigera* and *H. virescens* (Kvello, 2003, Zhao et al., 2013).

1.4.2 Higher-order processing of CO_2 information in the insect brain

A study by Guerenstein et al. (2004a) on sensory processing of ambient CO_2 information in the brain of *M. sexta* was the first to describe the AL arborizations and projection patterns of CO_2 responsive PNs. Before this, there was no knowledge on the physiological properties of the LPOG and how the second order neurons carrying CO_2 information project in the brain. The antenno-protocerebral pathways from the LPOG to higher processing areas of the brain are still scarcely studied, and what we know is based on a few reports only.

Guerenstein et al. (2004a) reported that all CO_2 PNs arborizing from the LPOG were uniglomerular. This also supports the idea of the AL glomeruli constituting a spatial olfactory map based on the stimulus quality. In this study on *M. sexta*, they successfully stained 6 PNs

which could be subdivided in two groups based on their responses to decreasing CO_2 concentrations. All 6 PNs were classified as PIa neurons according to Homberg et al (1988), meaning that they all projected through the m-APT to the mushroom body calyces before most, if not all, projected further to the lateral protocerebrum. This study revealed that the CO_2 PNs of *M. sexta* seem to be heterogenous in regards to their physiological properties, but appear to be of the same morphological type (PIa neurons). The PN somata were shown to be located in the LCI group of the lateral cell cluster (Guerenstein et al., 2004a, Guerenstein and Hildebrand, 2008).

1.5 Aim of the thesis

The main objective of this study was to acquire new knowledge about sensory encoding mechanisms by mapping the central pathways for CO_2 information in the brain of the moth *H. virescens*. This study is the first attempt to identify the projection neurons and higher-order processing pathways of the CO_2 sensory system in this species.

Specific study aims:

i. Staining of the labial pit organ, the labial-palp pit nerve and the labial pit organ glomerulus
ii. Establishing a method for (retrograde) staining of the CO_2 projection neurons originating from the labial pit organ glomerulus (LPOG) in the moth *H. virescens*
iii. Visualizing the central pathways for CO_2 information using confocal microscopy and comparing their target areas with corresponding regions of the olfactory pathways
iv. Comparing the CO_2 pathways in brain preparations from males and females

2 Materials and Methods

2.1 Study location

The lab work was carried out at the Neuroscience unit, Department of Biology, NTNU, Norway. Confocal laser-scanning microscopy was performed at Department of Physics, NTNU, Norway.

2.2 The insects

The lab acquires pupae of the moth *H. virescens* (Heliothinae; Lepidoptera; Noctuidae) from the Syngenta laboratory culture (Syngenta AS, Basel, Switzerland). Upon arrival, the pupae are sorted by sex. They are separated in different incubators (Refritherm 200, Struers-Kebolab, Albertslund, Denmark) at 22-23°C, 70% air humidity, and a phase-shifted photoperiod (14 h light:10 h dark). After hatching, the adult moths are moved to plexiglass cylinder containers (height 20 cm, diameter 10 cm). The containers are covered with a perforated lid to ensure air supply, and marked with information about sex, species, and date of hatching. The moths have continuous access to fresh 0,15M sucrose solution upon which they feed. A maximum of 8 moths are kept together in each container. Handling of the insects is performed in fume hoods to reduce the risk of allergic reactions. Moths of both sexes and of seemingly good health, aged 2-5 days following eclosion, were used in experiments. A total number of about 140 moths were used in this study.

2.3 Preparation and staining

Before experiments, the moths were cooled at 4°C for 15 min in order to make them calm and easier to handle. The moths were then arranged in small plastic tubes (cut pipette tips) and immobilized using dental wax (Kerr Corporation Romulus, MI, USA). Only the head with antennae, proboscis, and other mouthparts was to protrude from the tube.

The staining of the sensory neurons and projection neurons of the moth brain was carried out in two steps. In order to display the LPOGs in a fluorescent light microscope, the glomeruli were pre-marked by applying fluorescent dye onto the labial palps. In the second step, the CO_2 PNs innervating the LPOG in one AL was stained by injecting dye into the glomerulus. To visualize the PNs and compare them to the corresponding areas in the olfactory pathways, the preparations were scanned by the means of a confocal laser-scanning microscope.

2.3.1 Staining of the labial palps

The labial palps were cut in the outer third section (figure 2.1), using a micro scissor and hence exposing the labial pit organ (LPO). Scales were removed in the area around the cut with a forceps to avoid their hydrophobic properties during staining. The LPO was stained with crystals of micro-Ruby (dextran, tetramethylrhodamine and biotin; 3000 MW, Lysine Fixable, D-7162, Invitrogen, Germany) using a micro needle. Droplets of tap water were applied in between appliance of crystals to make them dissolve and thereby ease the uptake by the sensory neurons of the LPO. To assure that enough amount of micro-Ruby had been applied, this procedure was repeated until the palp tissue around the cut was visibly colored pink (figure 2.2B). The animal was fed with sucrose water (0,15M) and placed in a glass container with paper soaked in water to ensure high air humidity at 4°C overnight, letting the dye be taken up and transported by the neurons innervating the AL. A total of 124 moths were stained from the labial palps.

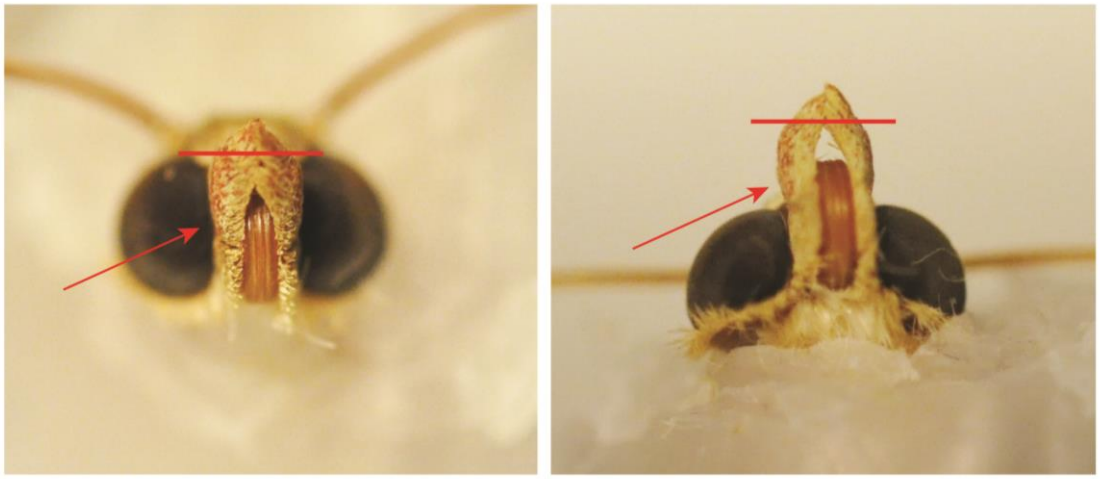

Figure 2.1: Images showing the labial palps of the moth *Heliothis virescens*, indicated with arrows. The lines indicate where the cut is made for staining of the labial pit organ in the terminal (third) segment of the palps. **A:** Frontally oriented. **B:** Ventrally oriented.

2.3.2 Staining of the labial pit organ glomerulus under visual control

Cephalic scales and hair on the head, and around the eyes and mouthparts, were removed with forceps and q-tip. Using light microscope and micro scissors, the proboscis and palps were removed. By using a micro knife, the cuticula was cut open; between the antennae, and alongside the inside of the compound eyes. The cuticula was then removed using a forceps, hence opening a window into the head cavity of the moth. Ringer's solution (In mM: 150

NaCl, 3 KCl, 10 TES buffer, 25 sucrose; pH 6.9) was applied to keep the brain tissue moist. All intracranial muscle tissue and trachea surrounding the brain were removed with forceps to expose the brain in a frontal direction (figure 2.2C). The AL was desheated using a sharp needle or forceps, making it accessible for perforation.

The moths, still in plastic tubes, were fastened onto a movable platform with dental wax and placed inside a faraday cage. The LPOGs were visualized under a stereo microscope (Zeiss, Discovery V12, Stereo, Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with long-working-distance objectives and a green fluorescence filter. This system allows a clear visualization of the target glomeruli, thereby facilitating the insertion of a microelectrode in the relevant region (figure 2.2D). All further staining was done using borosilicate glass microelectrodes. They were pulled using a flaming brown micropipette puller (P-97, Sutter Instrument Co, Novato, CA, USA).

Two different methods were used for retrograde staining of the LPOGs:

I. One method implied staining with micro-Ruby crystals added on the outer tip of a glass electrode. During dye application, the electrode was arranged in two different manners: either attached to a manually maneuvered custom-made manipulator or to a micro manipulator. Further procedure description was identical for the two arrangements.

   The glass electrode was moved into the cuticula window anterior to the brain. The dye crystal was visible through the microscope, making it easier to maneuver it towards the LPOG. Any hemolymph on the outside of the brain was dried off to avoid color spill. The crystal was inserted just underneath the surface in the ventral area of the LPOG. The electrode was left in the tissue for a few seconds to ensure the dye could be taken up by neurons innervating the glomerulus. Ringer's solution was used to wash off any redundant dye on the outside of the brain. The moth brain was kept moist by covering it with medical wipes (Kimberley-Clark® Professional) perfused with Ringer's solution. The moth was then kept at 4°C in a glass container in dark and humid conditions overnight, or at room temperature for 4-5 h. 36 moths were stained using this procedure.

II. The other method implied iontophoretic staining of the LPOG. The tip of the glass electrode was filled with a solution of potassium acetate (0,2M) containing 4% micro-Ruby. The glass electrode was then back-filled with potassium acetate, functioning as an electrolyte solution, before being positioned in the micro manipulator. The electrode was connected to an preamplifier (Axon CNS, Axoclamp 900A, computer-controlled microelectrode amplifier, Molecular Devices, CA, USA) through a silver chloride coated wire at the inside of the microelectrode.

A reference electrode was inserted into the extracellular fluid in the right eye. The LPOG to be stained was chosen regarding to the strength of the visual fluorescence. After drying off hemoplymph on the brain surface using medical wipes, the tip of the glass electrode was inserted underneath the surface of the ventral area of the LPOG with the aid of the micro manipulator. After insertion, the brain was superfused with Ringer's solution to keep it hydrated. The neuronal activity in the glomerulus could be seen on an oscilloscope screen. The AxoClamp 900A software was used to administer strength of current and duration of staining. The LPOG was stained by passing a 7-10 nA depolarizing current (pulses of 2Hz) through the glass electrode (iontophoretic injection). The staining persisted for 25-30 minutes. These settings were considered to be the most suitable as they gave the best results after test runs. Some brain preparations were stained by applying dye into one single LPOG others were stained by applying dye into both LPOGs. This was dependent upon the success of the first staining procedure. In order to allow the dye to diffuse into the PNs and travel to the terminal areas by retrograde transport, the moth was kept at 4°C overnight, or at room temperature for 3-4 h. 56 moths were stained using this procedure.

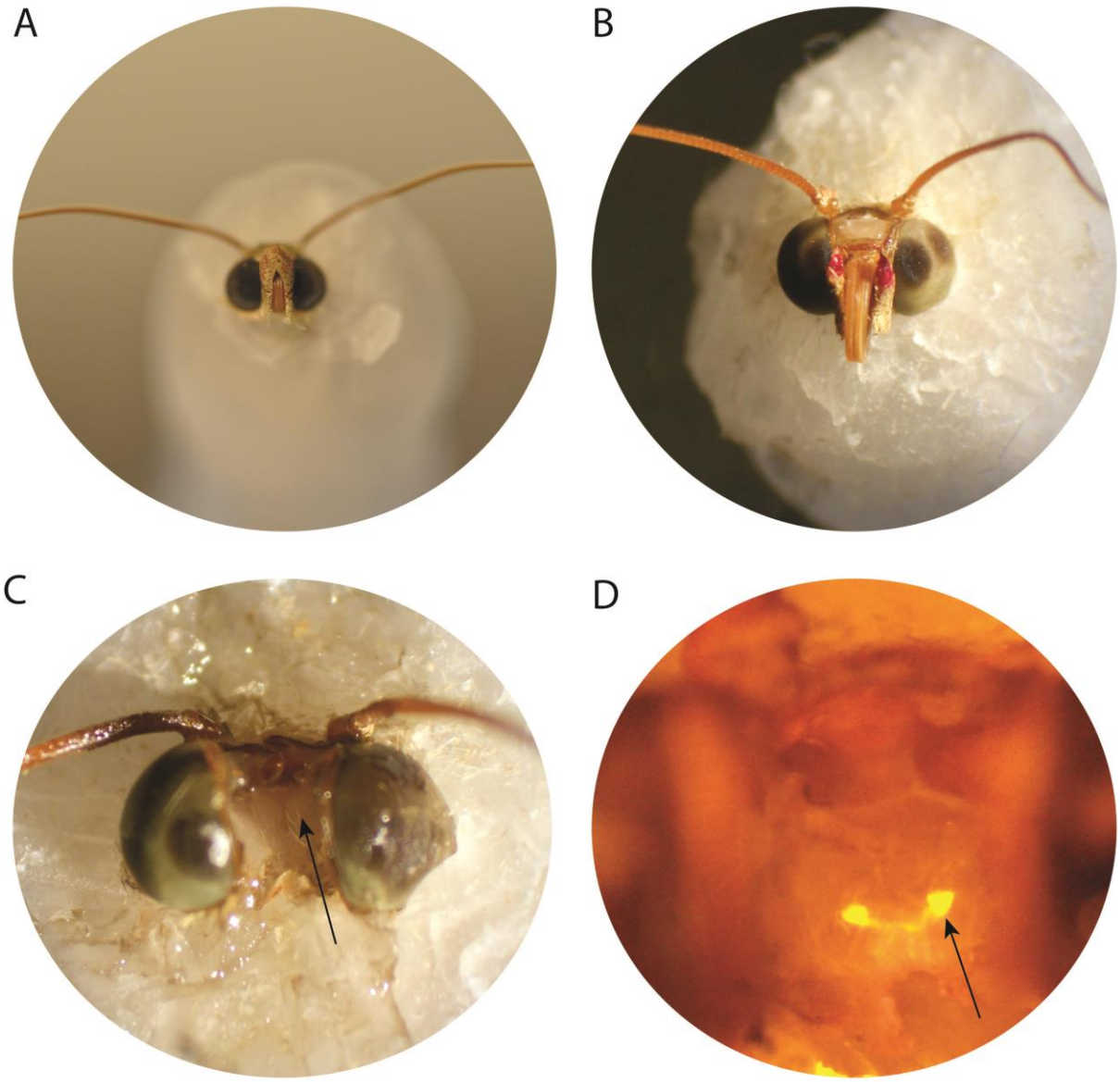

Figure 2.2: Images demonstrating immobilizing, staining, and dissection of the moth. **A:** The moth is fastened in a cut pipette tip and immobilized using dental wax so only the head is protruding. **B:** The third segment of the labial palps are cut, and the labial pit organ (LPO) is stained using micro-Ruby dye until the shaved palp tip turn pink. The animal is kept cooled overnight before dissection. **C:** All cuticula between the antennae and eye lobes is dissected, including the proboscis and mouth parts, and all intracranial muscle tissue and trachea are removed, leaving the brain exposed. The arrow indicates the position of the left antennal lobe. **D:** Pre-staining of the labial pit organ glomerulus (LPOG) from the LPO, is visualized using a stereo microscope with green fluorescence filter. The arrow indicates the position of the LPOG. This visualization allows navigation of the glass microelectrode for retrograde staining of the LPOG. **A** is slightly ventrally oriented, **B-D** are frontally oriented.

2.4 Dissection and protocol

The moth was decapitated and the brain dissected out in cold Ringer's solution. Dissection time never exceeded 25 minutes. The brain was fixated (Roti Histofix© , Carl Roth GmbH + Co. KG, Germany) overnight at 4°C, or for 1-2 h at room temperature to prevent degradation of the neural tissue. Following fixation, the brains were washed using a phosphate buffered solution (PBS; in mM: 684 NaCl, 13 KCl, 50.7 Na_2HPO_4, 5 KH_2PO_4; PH 7.2; 4 x 10 min).

Then the brain preparations were gradually dehydrated in an increasing alcohol sequence (50%, 70%, 90%, 96%, 100%, 100%; each 10 min), and finally treated with the hydrophobic compound methyl 2-hydroxybenzoate (methyl salicylate). Methyl salicylate is used as clearing agent, and makes the brain preparations transparent so they can be studied with a laser-scanning microscope. The brains were arranged inside holes of custom-made aluminum plates, enclosed by a cover glass on each side. Embedding medium in the holes was methyl salicylate. The brains were preferably mounted in a frontal position.

Brains that showed weak neuronal staining after confocal imaging were treated according to an intensification protocol. The preparations were first rehydrated with a decreasing ethanol sequence (100%, 100%, 96%, 90%, 70%, 50%; 10 min each). Then the staining was amplified by immersing the brains in Streptavidin-CY3 (Jackson Immunoresearch, West Grove, PA, USA) diluted in PBS (1:200) at 4°C for 24 h. Following intensification, the brains were again washed with PBS (4 x 10 min), dehydrated, and treated with methyl salicylate.

2.5 Visualization of stained neurons

2.5.1 Confocal laser-scanning microscopy

The CO_2 PNs were studied using a confocal laser-scanning microscope (Zeiss, LSM 510 META, Carl Zeiss Microscopy, GmbH, Jena, Germany).

The PNs stained with micro-Ruby (Ex/em: 555/580) were examined at an excitation wavelength of 543nm, using a green laser pointer (Helium Neon laser, transmission 49.5%, 1mV max), and filtered through a band-pass filter (Rhodamine BP565-615) in whole

preparation mounts. All brain preparations were studied using these laser settings. Brains with no visible staining at this experimental stage, were not further examined.

Successfully stained brain preparations were scanned in the z-axis in an anterior-posterior, or dorsal-ventral direction to retrieve a stack of images. These stacks were used to create three-dimensional images of the stained neurons in the moth brain. All brain preparations were scanned with a Plan-Neofluar 10x 0.4 dry objective to retrieve an overview of both hemispheres, and with a Plan-Neofluar 20x 0.7 dry objective to retrieve more detailed images of each hemisphere. A few areas of special interest were also scanned with a C-Achroplan 40x 0.8 water immersion objective (refractive correction: 1.15). The resolution frame size of the XY-plane was set to 1024 x 1024 pixels, and the scan speed to 6. In order to remove noise, the scan average per line was increased to 4 times, using the mean method. Optimal pin-hole diameter (1 Airy Unit) and z-slice interval distance were calculated by an optimization formula in the Zeiss software. Detector gain and amplifier offset were adjusted for each preparation. The resulting stack of images was saved as Zeiss image files (.lsm). A total of 29 brains preparations were scanned.

The picture editing systems used to prepare the images, were LSM 510 Image browser (Carl Zeiss Microscopy, Jena, Germany, Version 4.2), Adobe Photoshop CS6 and Adobe Illustrator CS6 (Adobe systems, San Jose, CA). Contrast and brightness of images have been modified to emphasize the areas of interest. The confocal images are labeled with directional indications according to Homberg et al. (1988).

All experimental procedures used in this study were exposed to several test runs for ensuring optimization of the procedures and protocols. Procedures that were excluded after test trials are presented in Appendix II.

2.6 Evaluation of the preparations

After studying the confocal data, a representative collection of images were chosen to be presented in the result section of this thesis. As the main focus was to map PNs arborizing in the LPOG, these confocal images were selected by a subjective comprehension about which gave the best visualization of the current neural pathways.

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3 Results

Injection of dye into the LPO on the labial palps of the moth *H. virescens* resulted in staining of the axons of the LPN and their termination area in the LPOG. Subsequent dye application into the pre-stained LPOG, revealed the ascending PNs and their targets in higher integration areas in the brain. The stained PNs were found to mainly follow the l-APT and the ml-APT. No PNs were found to follow the m-APT. Schematic presentations of these findings are presented in figures 3.1-3.3. The staining patterns in males and females were similar, suggesting no sexual dimorphism in regard to higher-order processing of CO_2 information. The majority of the PNs originating from the LPOG seem to terminate in particular regions of the ipsilateral protocerebrum with slightly different target areas, regarding to which tract the PNs follow.

The results are presented in 3 parts:

3.1: Staining of the sensory neurons located in the LPO

3.2: Staining of projection neurons originating from the LPOGs

3.3: Comparison between males and females

3.1 Staining of the sensory neurons located in the LPO

The LPO of 124 moths was injected with micro-Ruby dye, followed by the procedure described in the materials and methods section. Confocal images of 14 brain preparations constitute the basis for the data presented in the main result section and are discussed in the next chapter. Figures A1-A7 in Appendix I support the results presented in this section.

All the brain preparations that were successfully stained show the same projection pattern. Staining of both palps demonstrates that each of the two LPNs enter the ipsilateral suboesophageal ganglion, and send off some branches on its way towards the oesophagus. Ventrally to the oesophagus, the LPN from each palp divides. One branch projects to the ipsilateral LPOG and the other to the contralateral LPOG (figure 3.4A).

In preparations where only one palp was stained, it appears clear that still both the ipsi- and contralateral LPOGs are innervated by the sensory nerve fibers of the LPN, but the number of stained axons is fewer in the contralateral pathway (figure 3.4B).

As shown in figure 3.4, the LPOG is located most ventrally and posterior in the AL. The axons of the sensory nerves from the LPO enter the ventral area of the glomerulus (figure 3.5) and make up a thick layer of neural innervation in the outer part of the structure, leaving the inner part empty (figure 3.6).

3.2 Staining of projection neurons originating from the LPOGs

Of 29 scanned brain preparations, a total of 16 brain preparations showed successful staining of the LPOG PNs. The confocal images of 14 preparations are included in the main result section. Of these, 6 preparations are presented in different angles or z-sections in the Appendix I, together with 2 preparations not presented here, in the means of supporting the results. Thus the general staining pattern of the PNs presented in the Appendix I, is similar to those presented in the result section.

3.2.1 Antenno-protocerebral pathways formed by the output neurons originating from the LPOG

The successful stainings show labeling along two main antenno-protocerebral pathways, one projecting in the lateral APT and the other in a more medially positioned track, likely the medio-lateral APT (figures 3.7, 3.9, 3.10). Figure 3.8 describes these pathways' relative position to each other in the anterior-posterior direction. The PNs arborizing in the LPOG target distinct areas of the protocerebrum, including the (ipsi- and contra-) lateral protocerebrum, and the superior (medio-lateral) protocerebrum. No clear innervation of the mushroom body calyces is evident. Most of the stained PNs innervate the ipsi-lateral protocerebrum. Figure 3.1 demonstrates a schematic overview of the overall results. None of the stained PNs show distinct arborizations in other AL glomeruli, indicating that all stained PNs stained are uniglomerular.

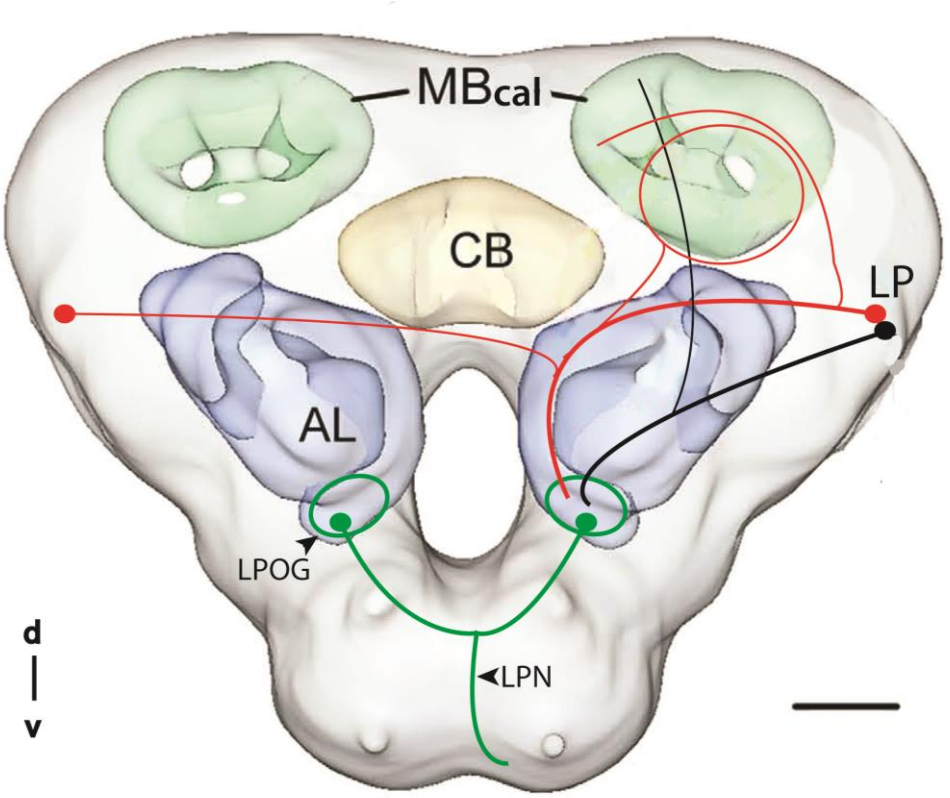

Figure 3.1: Schematic presentation of the primary and secondary projection pathways of the CO_2 system in the brain of the moth *H. virescens*. **Green:** The primary sensory pathway. The labial pit nerve (LPN) projects bilaterally to the labial pit organ glomerulus (LPOG) of both hemispheres. **Black:** Projection neurons (PNs) following the lateral antenno-protocerebral tract. The main termination area is in the lateral protocerebrum (LP). In addition, one sub-bundle projects adjacent to the posterior portion of the antennal lobe (AL) and terminate in an area of the superior protocerebrum (SP). **Red:** Projection neurons following the medio-lateral antenno-protocerebral tract. The main termination area is in the ipsi-lateral protocerebrum. Three additional sub-bundles branch off from the common tract. One projects to the LP of the contralateral hemisphere. The two other terminate in the SP, just anterior to the mushroom body calyces (MBcal). The central body (CB) is indicated for orientation. Scale bar: 100 µm.

3.2.1.1 PNs following the lateral antenno-protocerebral tract

The stained fibers projecting in the l-APT bifurcate in two sub-bundles shortly after leaving the LPOG; one is terminating in the lateral protocerebrum, and the other in a more medially located region also covering the superior protocerebrum. The l-APT leaves the AL more ventrally as compared to the medio-lateral route described in the next section. As shown in figure 3.9 and 3.10, it continues laterally as a relatively loosely defined bundle of at least 4 thin fibers, projecting towards the optic lobe. A significant portion of these fibers makes up the sub-bundle terminating in the ipsilateral protocerebrum (e.g. figure 3.10-3.13). The other sub-bundle, being a distinct, but loosely defined side-branch, bends off from the lateral path and projects dorsally. Its termination area covers a region of the protocerebrum located relatively medial and anterior in the ipsilateral hemisphere. This bundle forms a characteristic columnar structure, stretching in the dorsal-ventral direction, which causes it to cover also an area of the superior protocerebrum (figure 3.14). The fibers following the last mentioned sub-bundle, were often weaker stained than those projecting in the sub-bundle targeting the lateral protocerebrum, and appear only in well-stained preparations. Figure 3.15 shows a close-up confocal image of the terminal area of this dorsal branch scanned with a 40x objective. It clearly demonstrates that the axon terminals of this branch target a very narrow region. In an ongoing master's study performing mass staining of antennal-lobe PNs passing in the various antenno-protocerebral tracts, a similar columnar structure forms a part of the l-APT. This supports the observation that the current bundle of PNs originating from the LPOG, passes in this particular tract. (Lillevoll, S. 2013, personal communication, unpublished data). Figure 3.2 present a schematic demonstration of the PNs running in the l-APT.

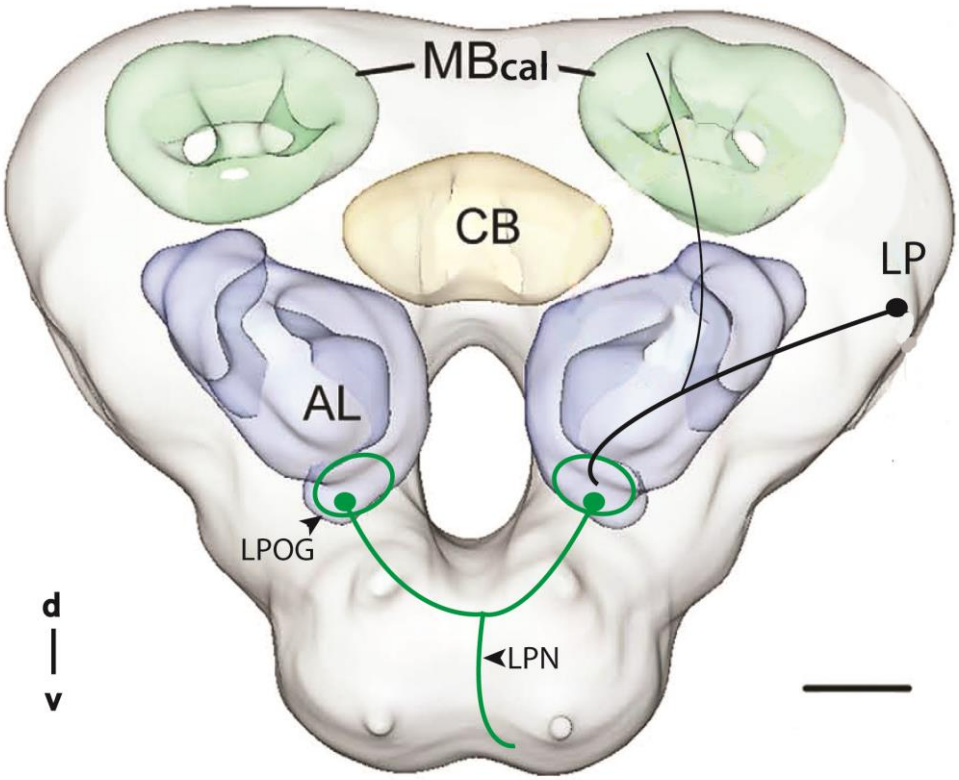

Figure 3.2: Schematic presentation of primary and secondary projection pathways of the CO_2 system in the brain of the moth *H.virescens*.
Green: The primary sensory pathway. The labial pit nerve (LPN) projects bilaterally to the labial pit organ glomerulus (LPOG) in the antennal lobe (AL) of both hemispheres. **Black:** Projection neurons (PNs) following the lateral antenno-protocerebral tract. The main termination area is in the lateral protocerebrum (LP). In addtition, one sub-bundle projects adjacent to the posterior portion of the AL and terminate in an area of the superior protocerebrum. The central body (CB) and mushroom body calyces (MBcal) are indicated for orientation. Scale bar: 100 µm.

3.2.1.2 PNs following the medio-lateral antenno-protocerebral tract

The PNs projecting via the more medial-lateral route as compared to the l-APT are assumed to follow the ml-APT, based upon morphological comparisons with previously stained preparations displaying the three main APTs. The PN bundle following the ml-APT separates in four branches, which terminates in three different brain areas; the lateral protocerebrum in the ipsi- and contralateral hemisphere, plus the superior protocerebrum, anterior to the mushroom body calyces. A schematic overview of these projections is presented in figure 3.3. At least 3 axonal fibers are stained in this tract, and they are relatively thick as compared to those running in the l-APT.

As shown in figure 3.9 and 3.16, the stained axons in the ml-APT leave the AL posterior-medially and project posteriorly along the oesophageus, before turning laterally and terminating in a distinct area of the lateral protocerebrum (figure 3.17). One or more of the PNs following this pathway do not end up in the lateral protocerebrum, but continues dorsally, and then medially, constituting a new route that terminates just anterior to the mushroom body calyces (figure 3.18).

Two distinct assemblies of nerve fibers branch off from the common medio-lateral route. One branch, bending off at the edge of the central body, sends one or more axons to the contralateral hemisphere. It terminates in a region of the lateral protocerebrum symmetrical to that innervated in the ipsilateral hemisphere (figure 3.19 and 3.20). The other branch turns laterally, also at the level of the central body, and then continues dorsally after a short distance, before finally terminating in a region located anterior to the mushroom body calyces. These PNs form a characteristic circular pattern (figure 3.21). The area in the lateral protocerebrum innervated by PNs following the ml-APT shows a minimum of overlap with that innervated by axonal fibers from the l-APT, by being located slightly more dorsally and posteriorly.

No brain preparations of this study show staining of PNs in the m-APT.

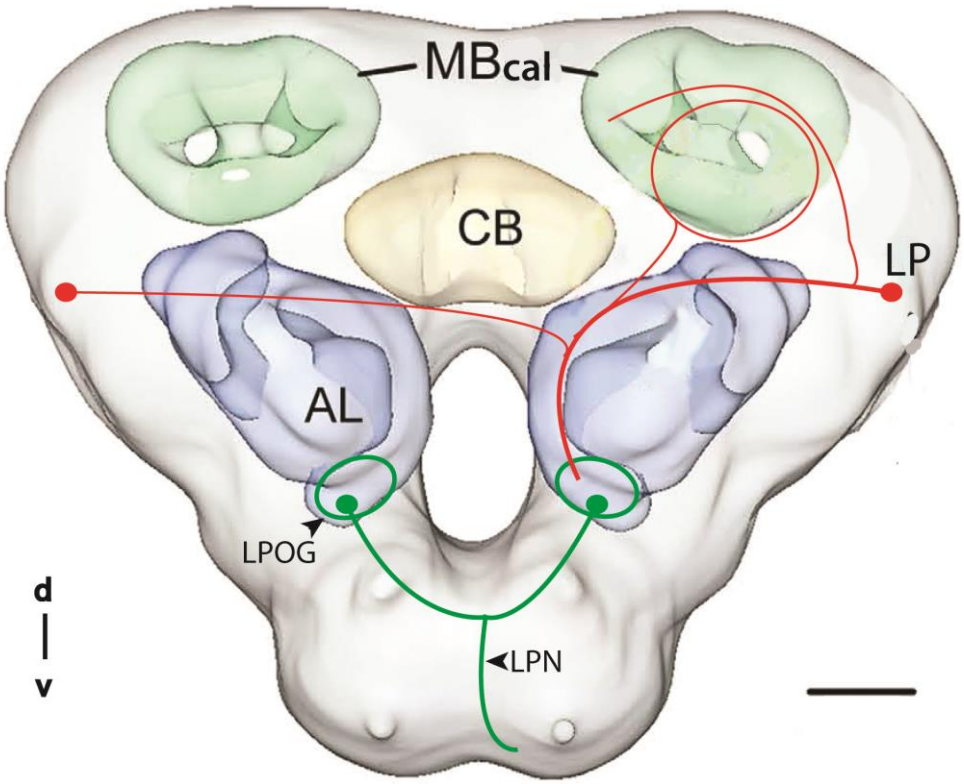

Figure 3.3: Schematic presentation of the primary and secondary projection pathways of the CO_2 system in the brain of the moth *H. virescens*. **Green:** The primary sensory pathway. The labial pit nerve (LPN) projects bilaterally to the labial pit organ glomerulus (LPOG) in the antennal lobe (AL) of both hemispheres. **Red:** Projection neurons following the medio-lateral antenno-protocerebral tract. The main termination area is in the ipsi-lateral protocerebrum. Three additional sub-bundles branch off from the common tract. One projects to the LP of the contralateral hemisphere. The two others terminate in slightly different areas of the superior protocerebrum, just anterior to the mushroom body calyces (MBcal). The central body (CB) is indicated for orientation.
Scale bar: 100 µm.

3.2.2 Location of somata

The somata of the stained PNs seem to be located in the lateral cell cluster. From the confocal images obtained in this study, it is difficult to determine the distinct connections between the individual fibers and their somata. However, stained somata located in both the LCI and the LCII area of the lateral cell cluster can be seen (figure 3.22). The confocal images show no stained somata in the medial or the anterior cell cluster. It is difficult to estimate the exact number of somata stained, because it differs between brain preparations and could in some cases be mistaken for autofluorescence. However, an assessed number of at least 3 stained axonal fibers in each tract indicate a minimum quantity of somata corresponding to the PN count.

3.3 Comparison between males and females

Comparisons of the brain preparations of males and females show no evidence for sexual dimorphism. The PNs originating from the LPOG follow similar protocerebral pathways and terminate in the corresponding target areas in the two sexes. Furthermore, there are no significant differences in the number of stained axonal fibers.

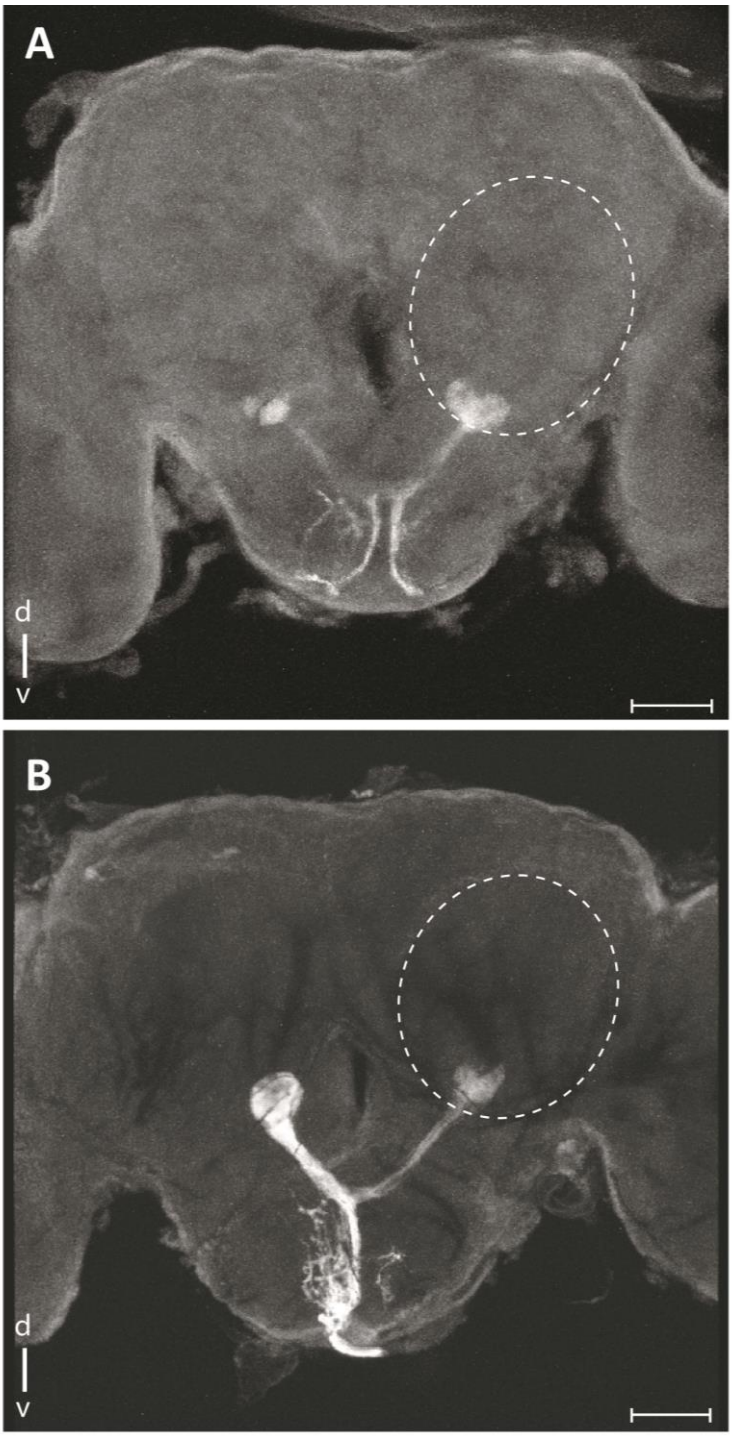

Figure 3.4: Confocal images (projection views) of two brain preparations showing staining of the labial-palp pit nerve (LPN) from the labial pit organ into the labial pit organ glomerulus (LPOG) in the antennal lobe (indicated by dashed circles). **A:** Brain preparation 101 (female) showing staining from both labial palps. **B:** Brain preparation 103 (female) showing staining from the right labial palp. The ipsi- and contralateral LPOG are stained in both preparations. **A-B** are frontally oriented and scanned with a 10x objective. Scale bars: 100 µm.

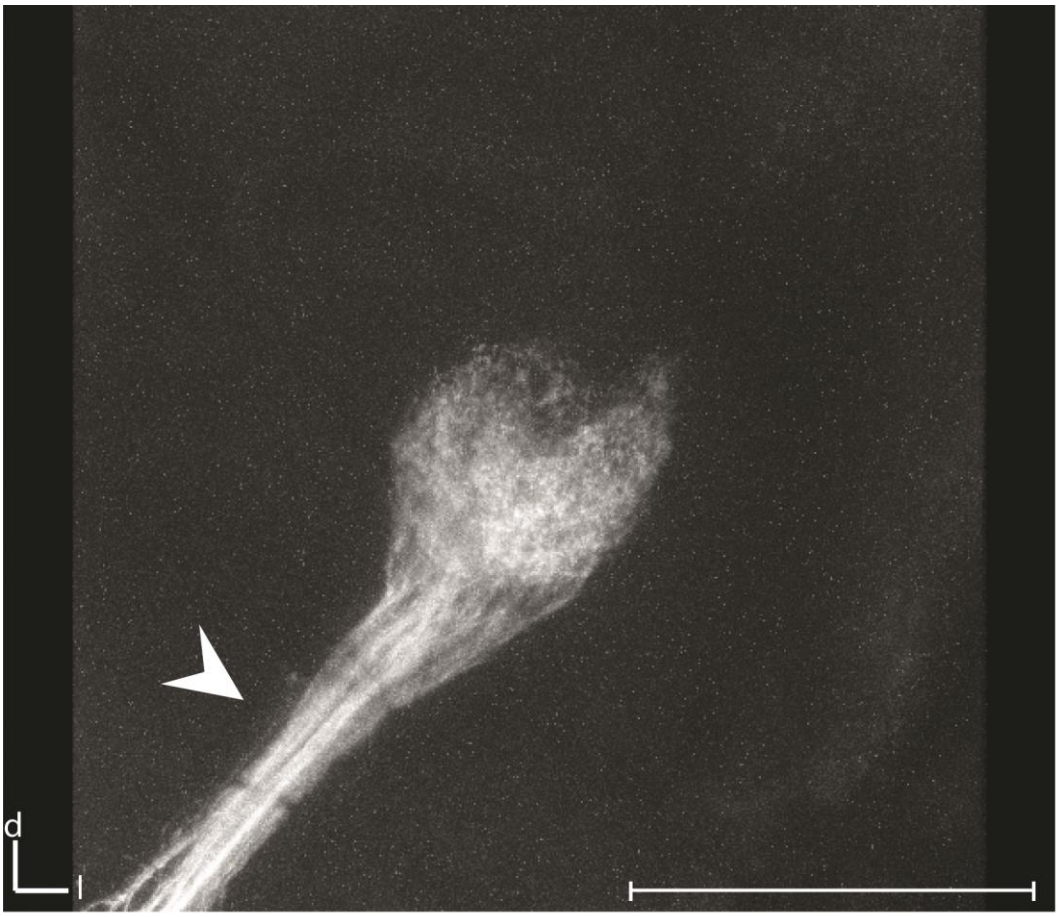

Figure 3.5: Confocal image (projection view) of the left labial pit organ glomerulus in brain preparation 103 (female) stained from the contralateral (right) labial palp. The arrowhead indicates the labial-palp pit nerve (LPN) with the axonal fibers of the sensory neurons originating from the labial pit organ. The LPN enters the ventral area of the glomerulus and the axon terminals innervate only the outer part, leaving the inner area empty. The preparation is frontally oriented and scanned with a 40x objective.
Scale bar: 100 µm.

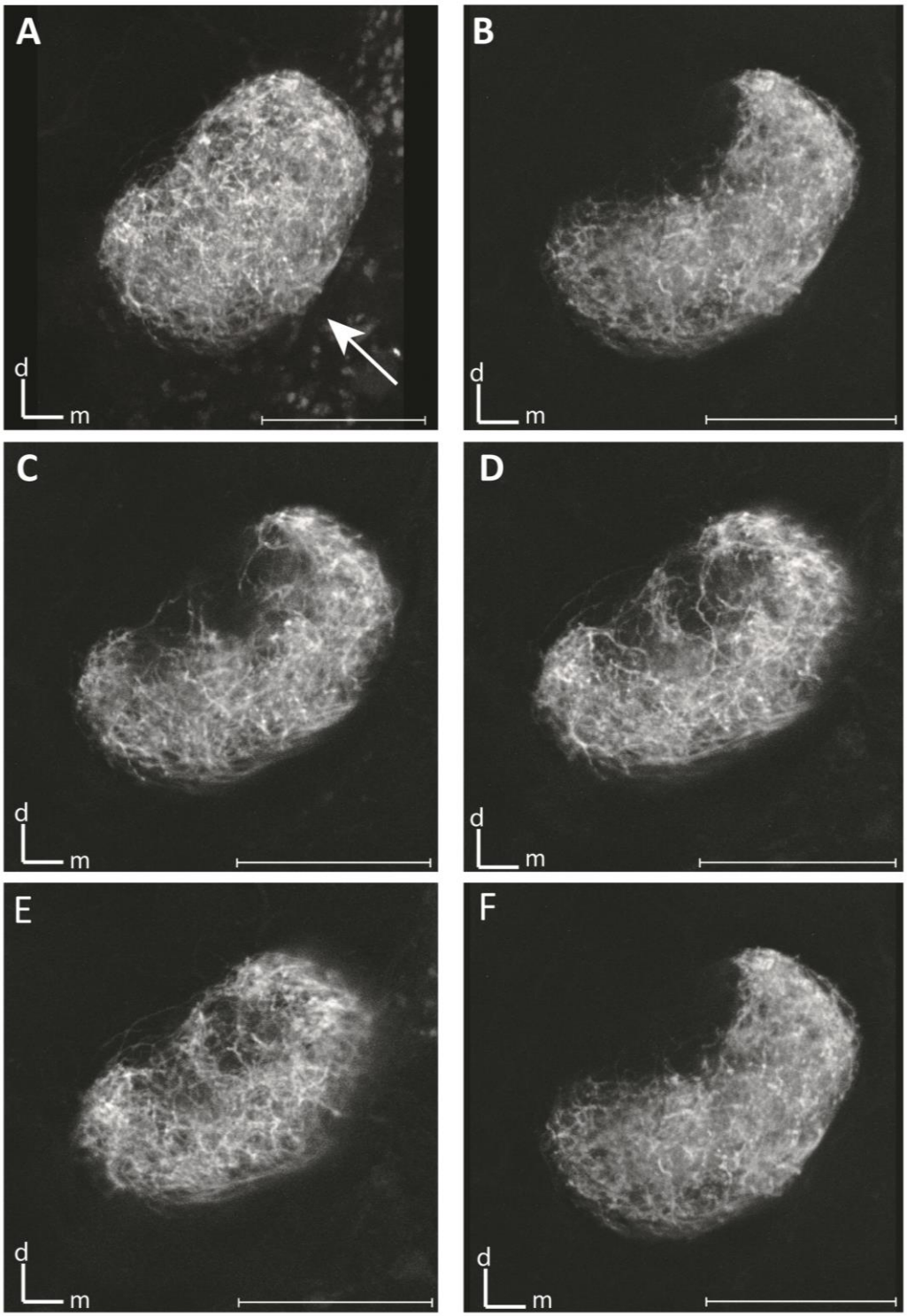

Figure 3.6: Confocal images of the right labial pit organ glomerulus (LPOG) in brain preparation 44 (female). All images are oriented in a frontal direction.
A: Projection view of the whole z-stack of confocal images. The white arrow indicates the direction in which the labial pit nerve enters the glomerulus.
B-F: Projection views of the different sections of the LPOG in a posterior-anterior direction. Scale bars: 100 µm.

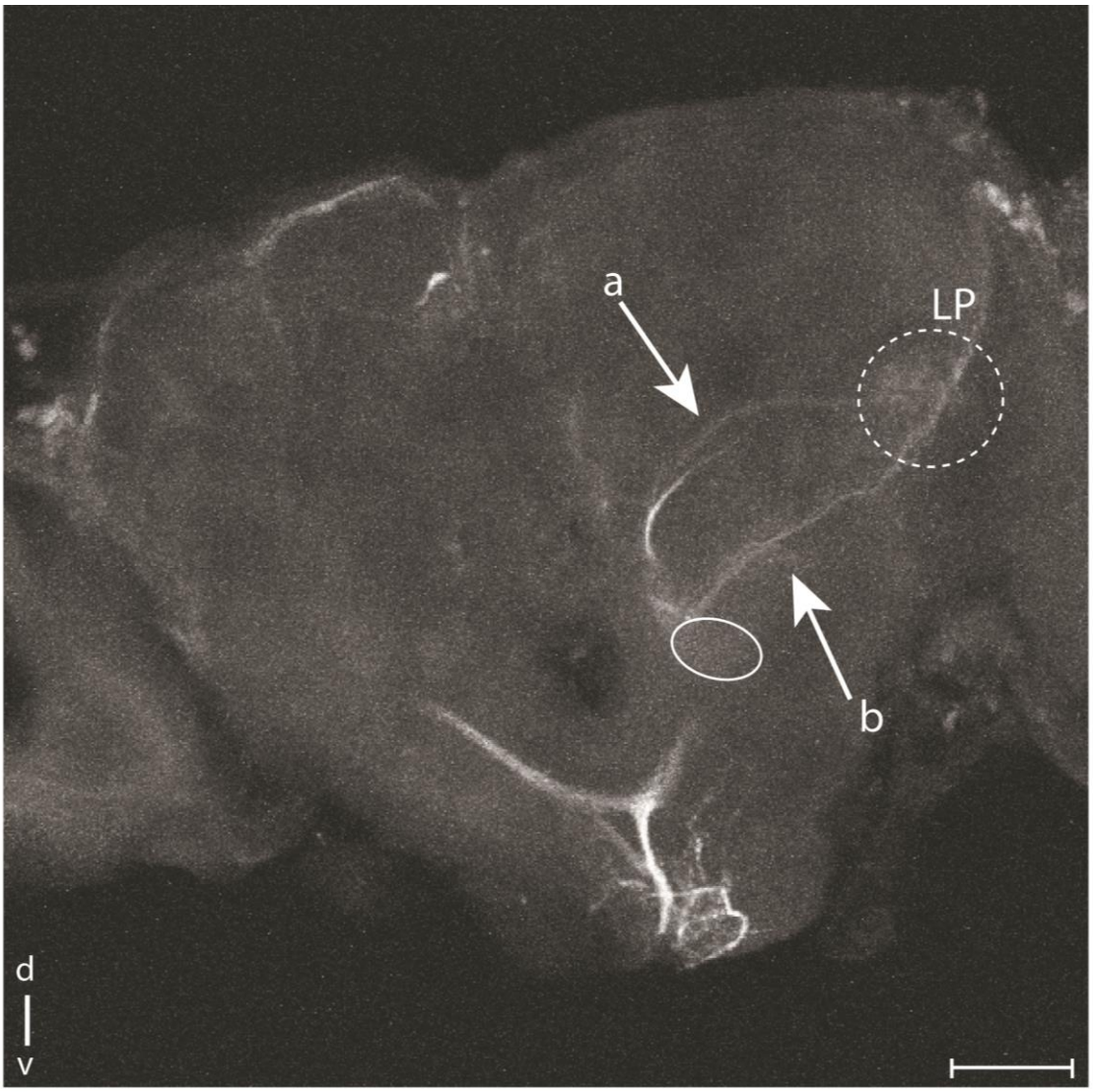

Figure 3.7: Confocal image (projection view) of brain preparation 52 (male) showing the main projection pathways (arrows) for projection neurons originating from the labial pit organ glomerulus (whole-lined circle) in the left hemisphere. **Arrow a:** Projection neurons following the medio-lateral antenno-protocerebral tract **Arrow b:** Projection neurons following the lateral antenno-protocerebral tract. The lateral protocerebrum (LP) is the main termination area for both tracts. The preparation is frontally oriented and scanned with a 10x objective. Scale bar: 100 µm.

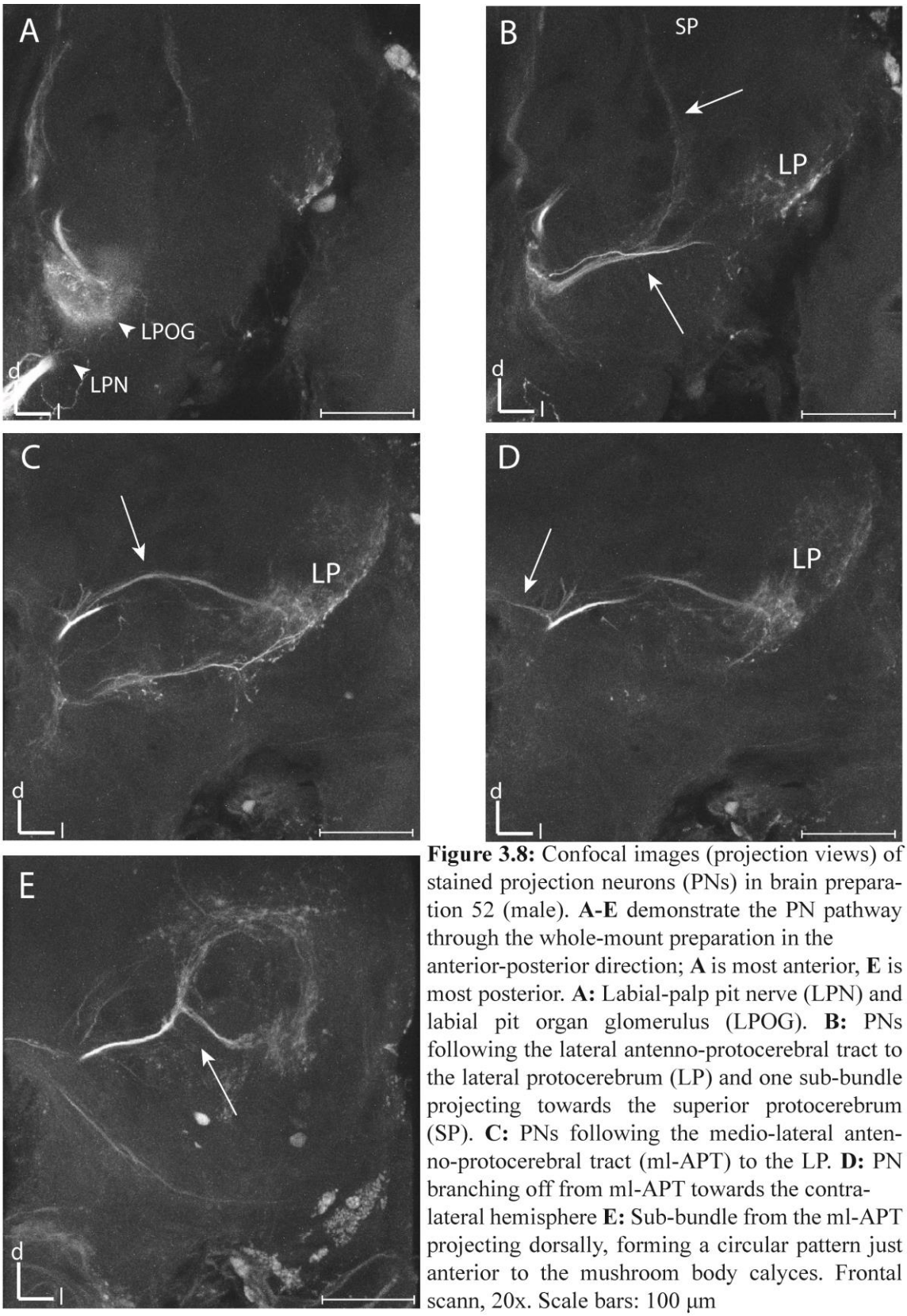

Figure 3.8: Confocal images (projection views) of stained projection neurons (PNs) in brain preparation 52 (male). **A-E** demonstrate the PN pathway through the whole-mount preparation in the anterior-posterior direction; **A** is most anterior, **E** is most posterior. **A:** Labial-palp pit nerve (LPN) and labial pit organ glomerulus (LPOG). **B:** PNs following the lateral antenno-protocerebral tract to the lateral protocerebrum (LP) and one sub-bundle projecting towards the superior protocerebrum (SP). **C:** PNs following the medio-lateral antenno-protocerebral tract (ml-APT) to the LP. **D:** PN branching off from ml-APT towards the contralateral hemisphere **E:** Sub-bundle from the ml-APT projecting dorsally, forming a circular pattern just anterior to the mushroom body calyces. Frontal scann, 20x. Scale bars: 100 µm

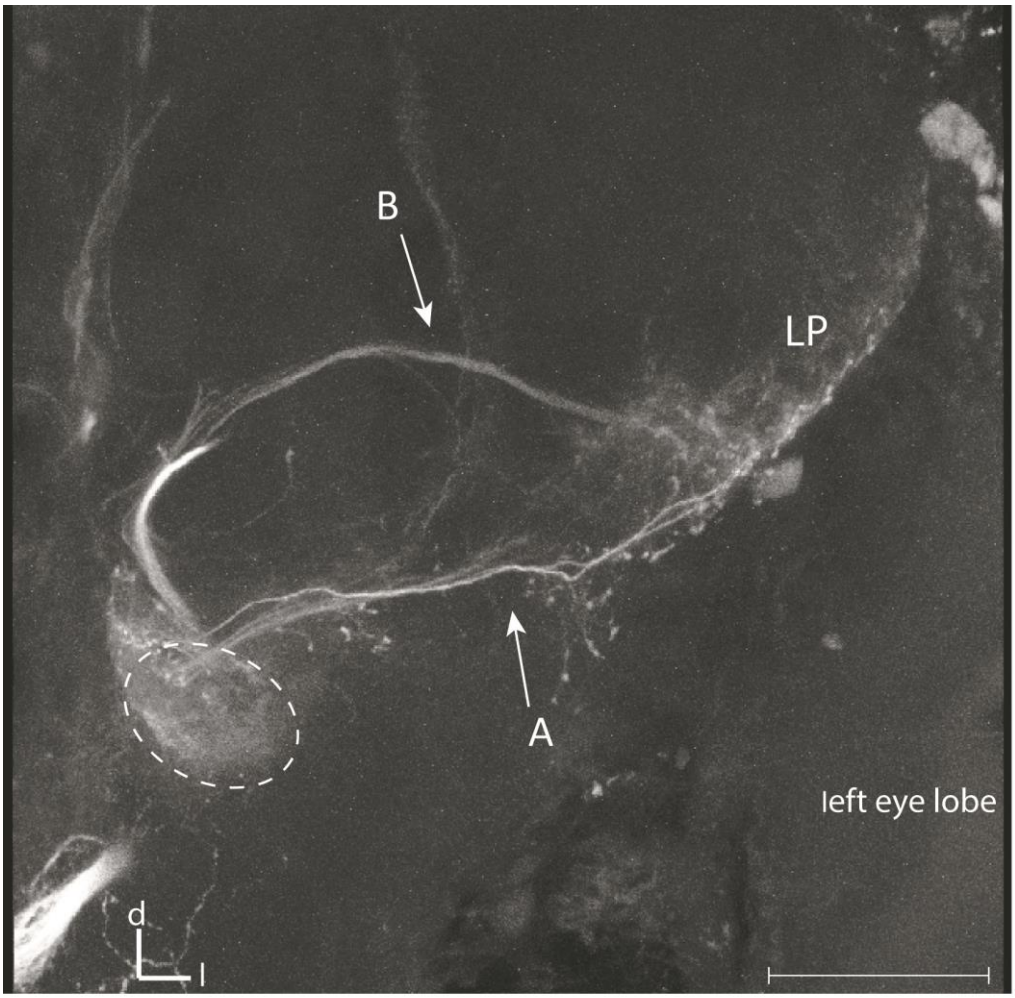

Figure 3.9: Confocal image (projection view) of brain preparation 52 (male), left hemisphere, demonstrating an overview of the main projection neuron (PN) pathways stained from the LPOG (dashed circle). **Arrow A** indicates PNs running in the lateral antenno-protocerebral tract. **Arrow B** indicates PNs running in the medio-lateral antenno-protocerebral tract. The lateral protocerebrum (LP) is the main terminal area for both tracts. The images are frontally oriented, and scanned with a 20x objective. Scale bar: 100 µm.

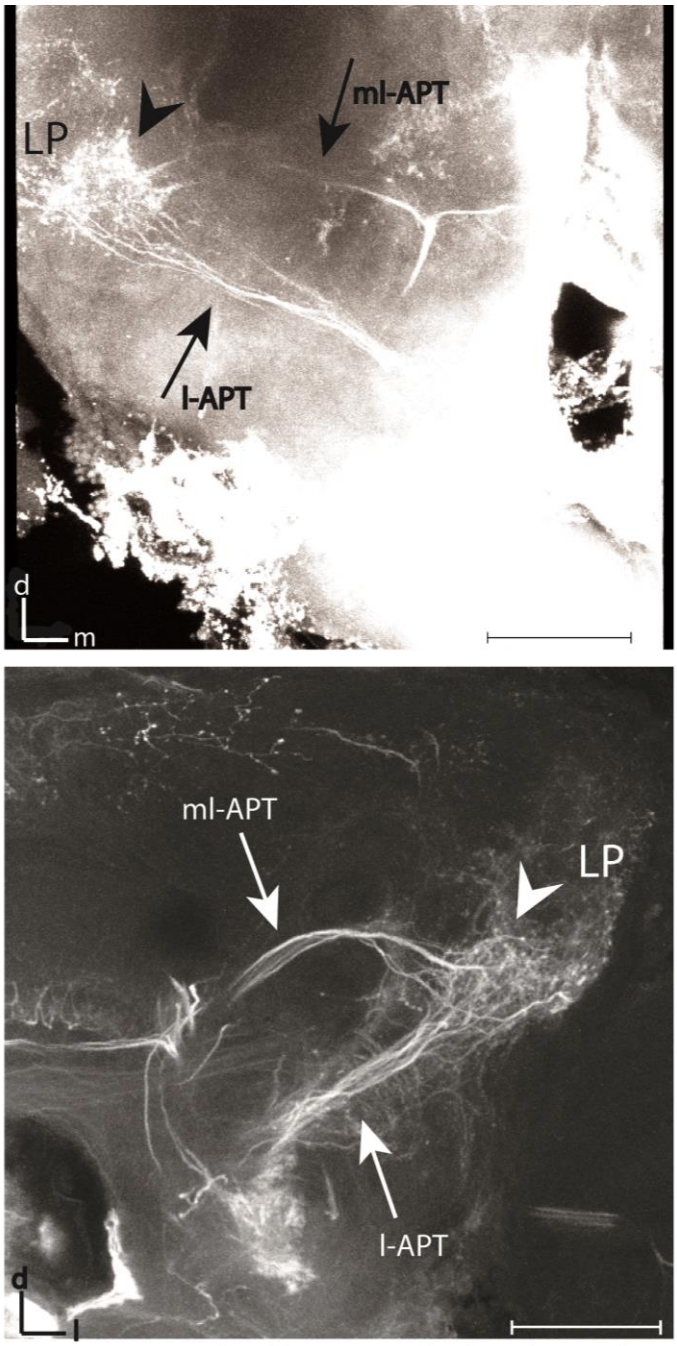

Figure 3.10: Confocal images (projection views) of two brain preparations showing projection neurons (PNs) originating from the labial pit organ glomerulus. The PNs follow the lateral antenno-protocerebral tract (l-APT) and the medio-lateral antenno-protocerebral tract (ml-APT) to their termination areas in the lateral protocerebrum (LP). **A:** Brain preparation 18 (female), right hemisphere, treated with an intensification protocol. **B:** Brain preparation 83 (female), left hemisphere. **A-B** are frontally oriented and scanned with a 20x objective. Scale bars: 100 µm.

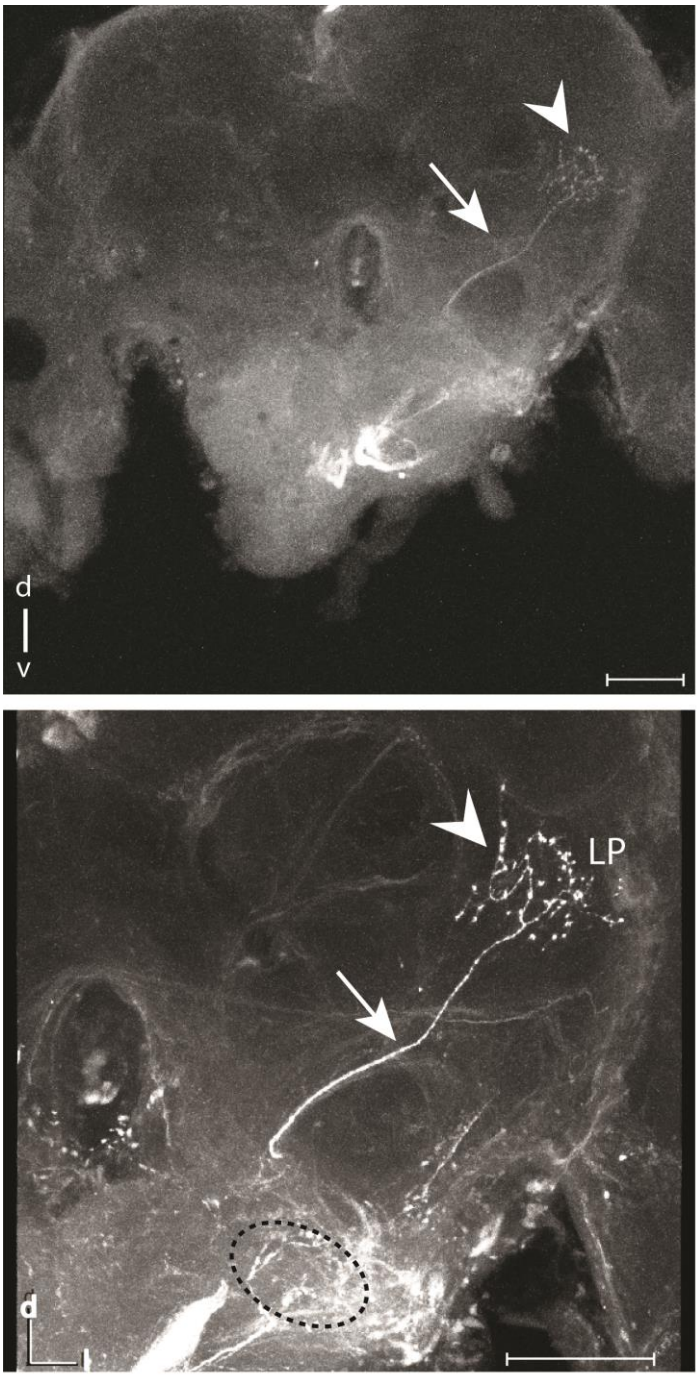

Figure 3.11: Confocal images (projection views) of brain preparation 55 (female) showing a projection neuron stained from the labial pit organ glomerulus (dashed circle) following the lateral antenno-protocerebral tract. Arrows indicate the axonal fiber and the arrow heads indicate the terminal area in the lateral protocerebrum (LP). **A:** Scanned with a 10x objective. **B:** Left hemisphere scanned with a 20x objective. **A-B** are frontally oriented. Scale bars: 100 µm.

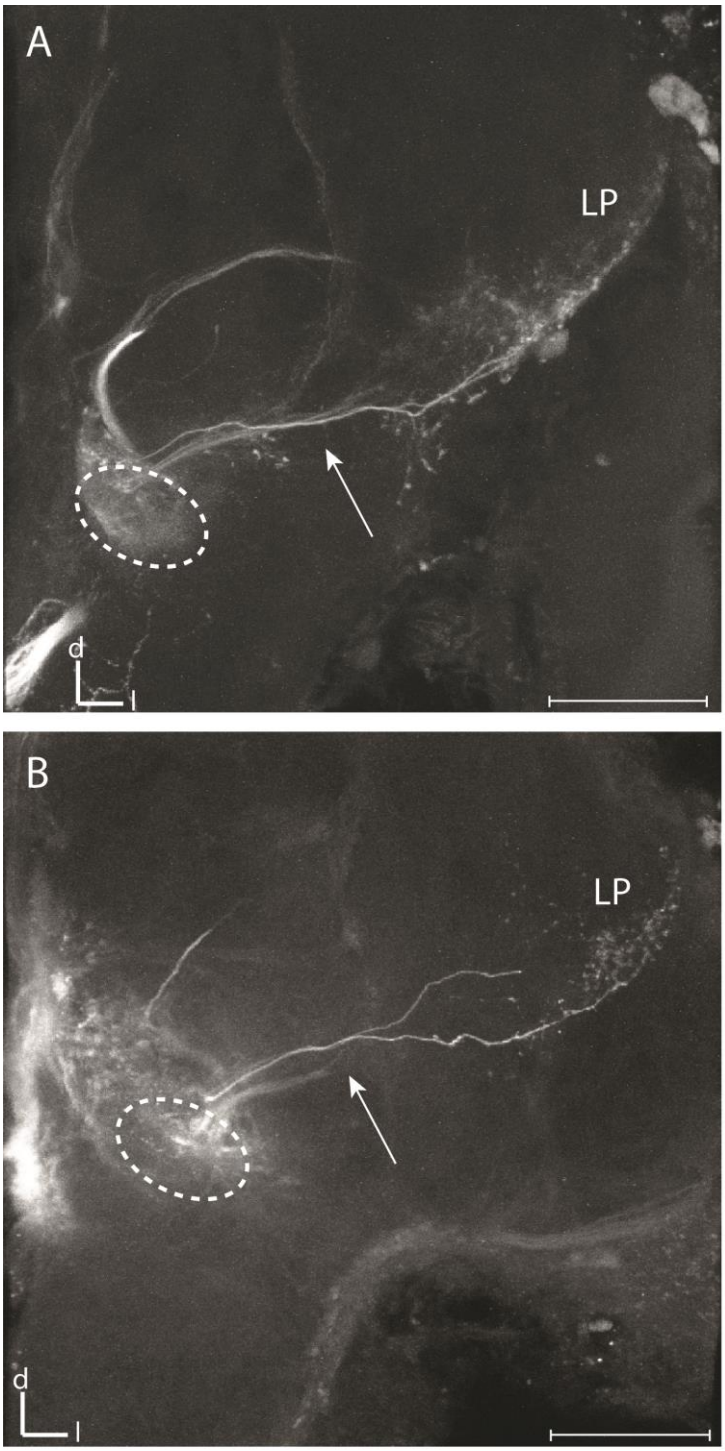

Figure 3.12: Confocal images (projection views) of two brain preparations, showing projection neurons stained from the labial pit organ glomerulus (LPOG) following the lateral antenno-protocerebral tract in two different brain preparations and their terminal areas in the lateral protocerebrum (LP). The dashed circles indicate the location of the LPOG. **A:** Brain preparation 52 (male) **B:** Brain preparation 69 (female). **A-B** are frontally oriented, and scanned with a 20x objective. Scale bars: 100 µm.

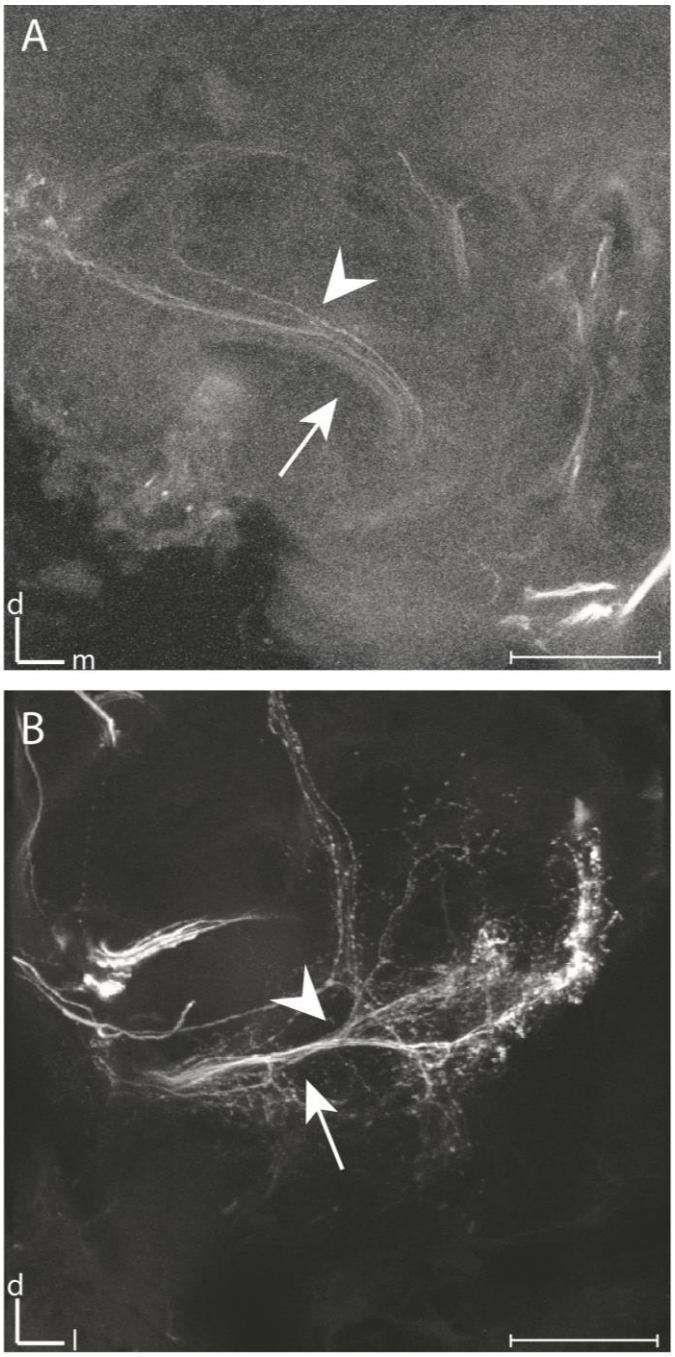

Figure 3.13: Confocal images (projection views) of projection neurons stained from the labial pit organ glomerulus in two brain preparations. Both images show axonal fibers following the lateral antenno-protocerebral tract (l-APT) (arrows). As demonstrated, the tract divides into two bundles (arrow heads), before terminating in the lateral protocerebrum. **A:** Brain preparation 73 (male), right hemisphere. **B**: Brain preparation 107 (male), left hemisphere. **A-B** are frontally oriented and scanned with a 20x objective. Scale bars: 100 µm.

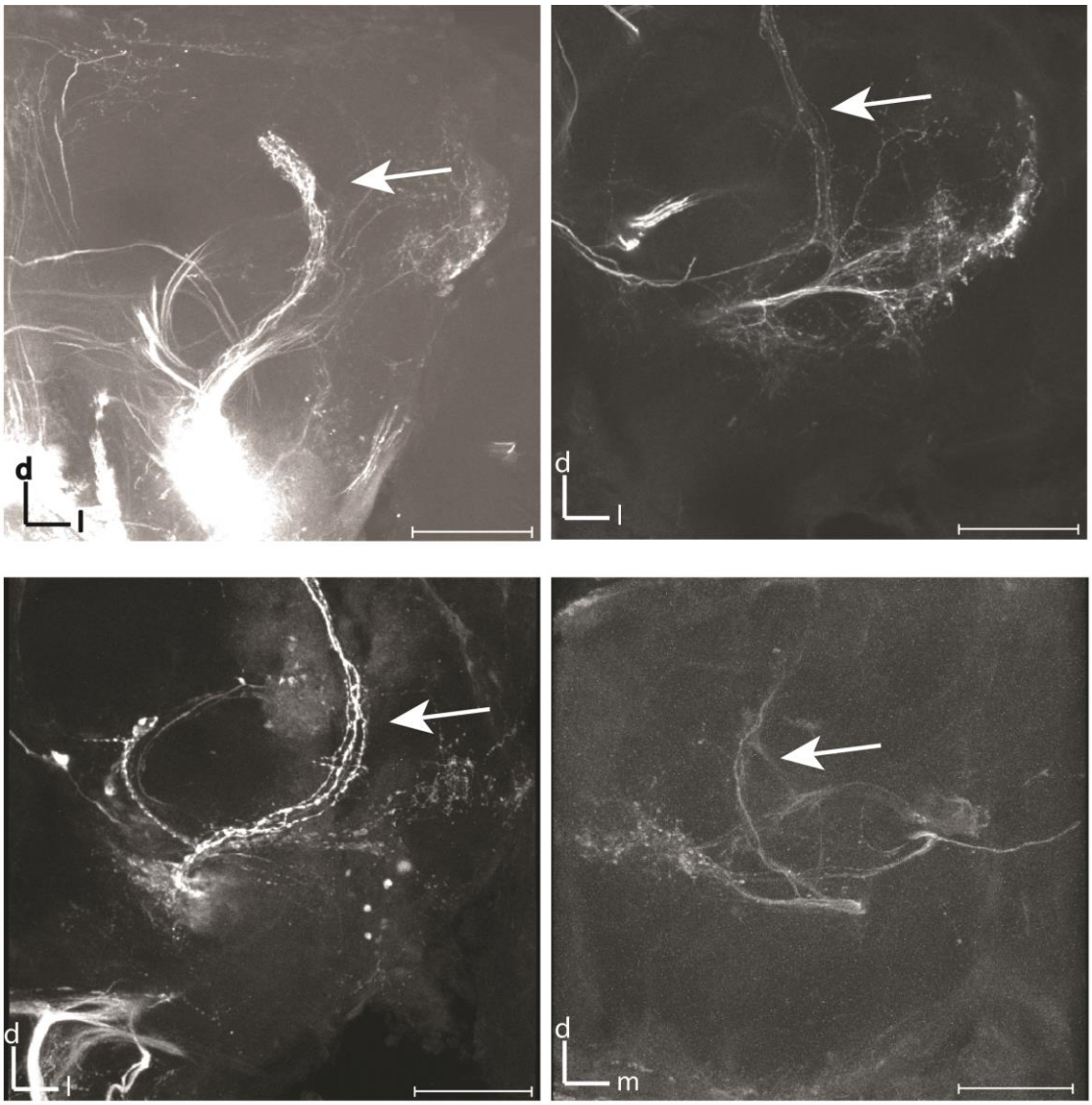

Figure 3.14: Confocal images (projection views) of four different brain preparations showing a distinct columnar bundle of projection neurons leaving the common lateral antenno-protocerebral tract, continuing dorsally towards the superior protocerebrum. **A:** Brain preparation 83 (female) **B:** Brain preparation 107 (male) **C:** Brain preparation 104 (female) **D:** Brain preparation 75 (female). **A-C** show projections in the left hemisphere, **D** show projections in the right hemisphere. **A-D** are frontally oriented and scanned with a 20x objective. Scale bars: 100 µm.

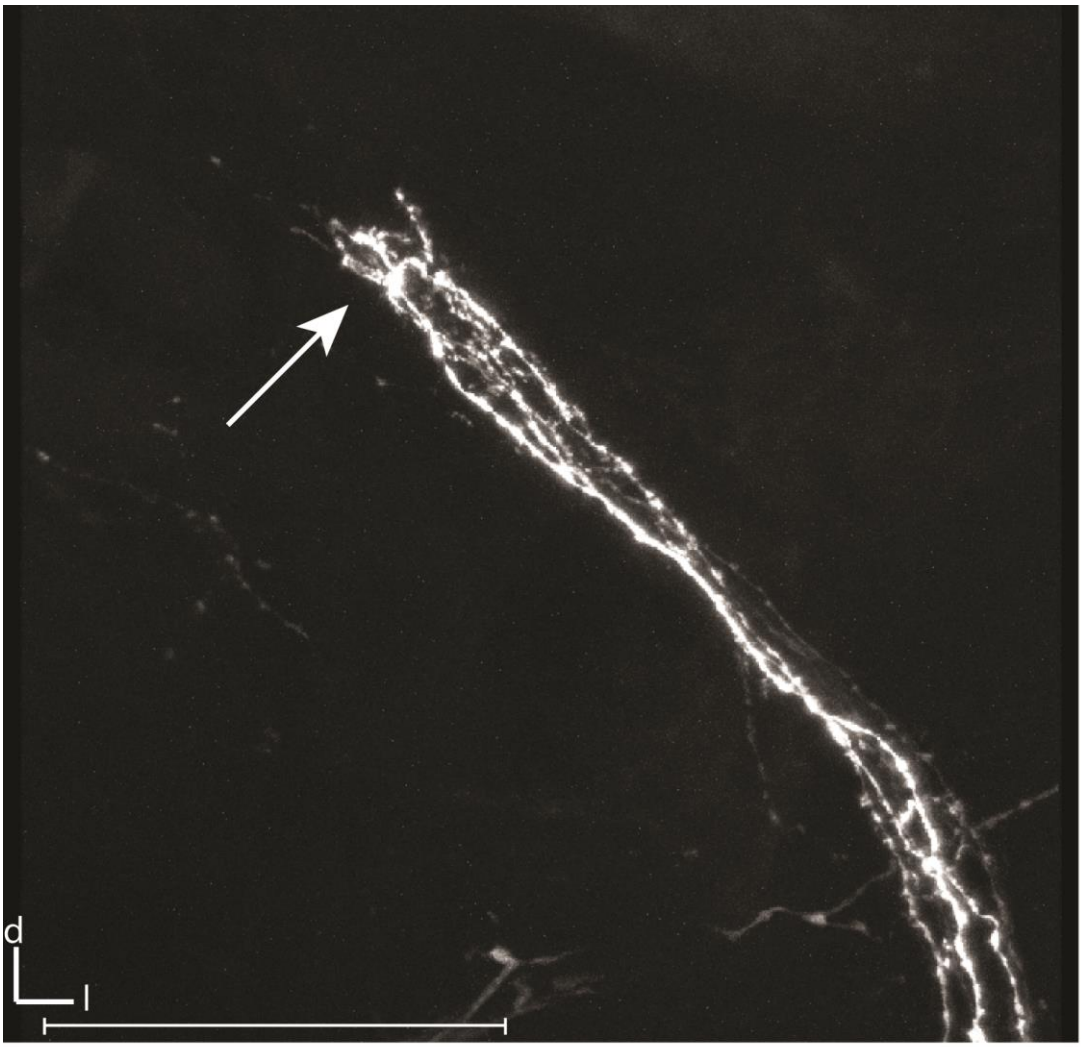

Figure 3.15: Confocal image (projection view) of brain preparation 104 (female), left hemisphere. It demonstrates a branch from the lateral antenno-protocerebral tract forming a columnar bundle of projection neurons (PNs) terminating in a narrow region of the superior protocerebrum. The arrow indicates its end point in the terminal area. The preparation is frontally oriented and scanned with a 40x objective.
Scale bar: 100 µm.

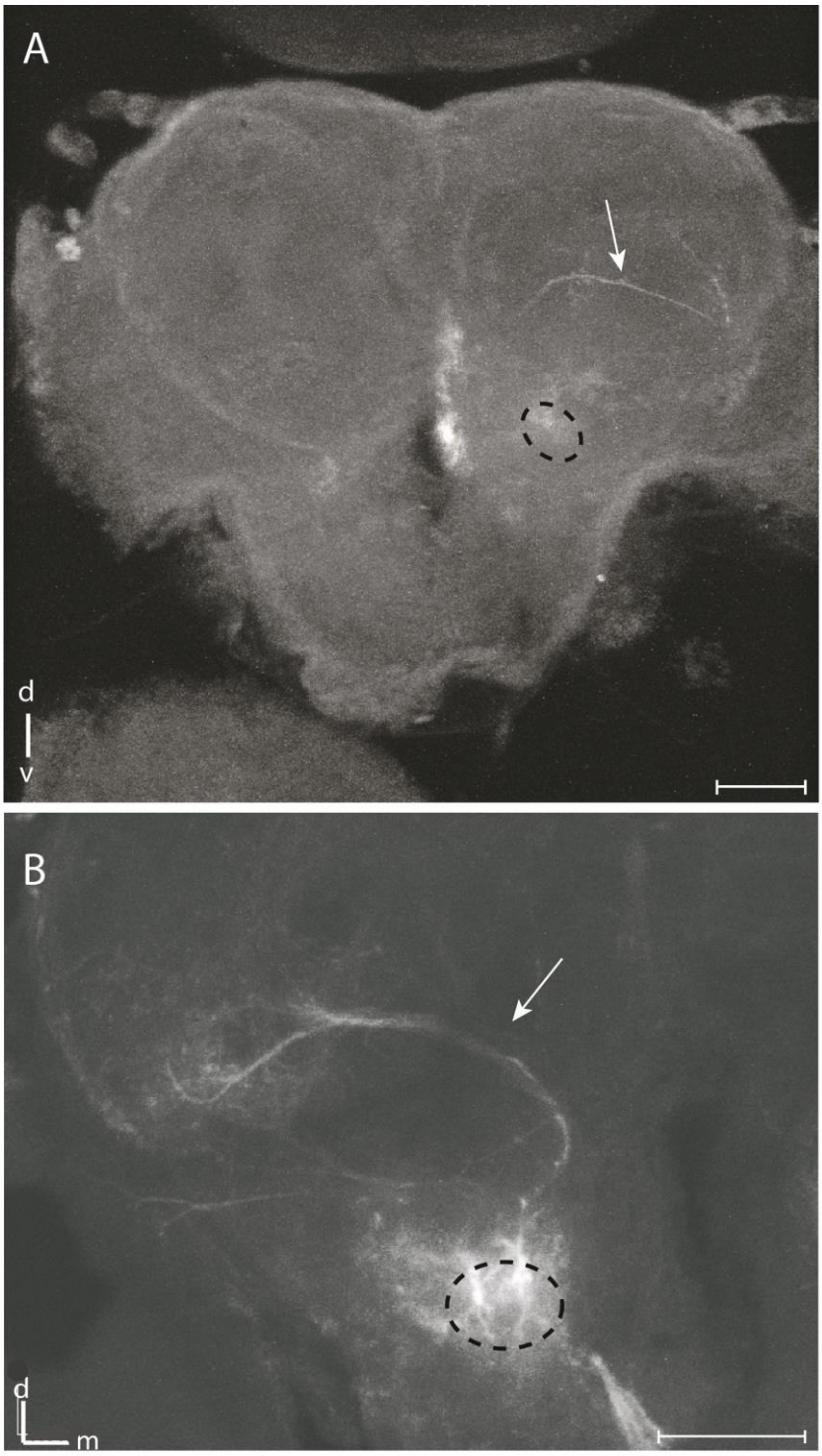

Figure 3.16: Confocal images (projection views) of projection neurons (PNs) originating from the labial pit organ glomerulus (LPOG) following the medio-lateral antenno-protocerebral tract. **A:** Brain preparation 69 (female) scanned with a 10x objective. **B**: Brain preparation 54 (male), right hemisphere, scanned with a 20x objective. Arrows indicate the PNs, and the dashed circles indicate the location of the LPOG. **A-B** are frontally oriented. Scale bars: 100 μm.

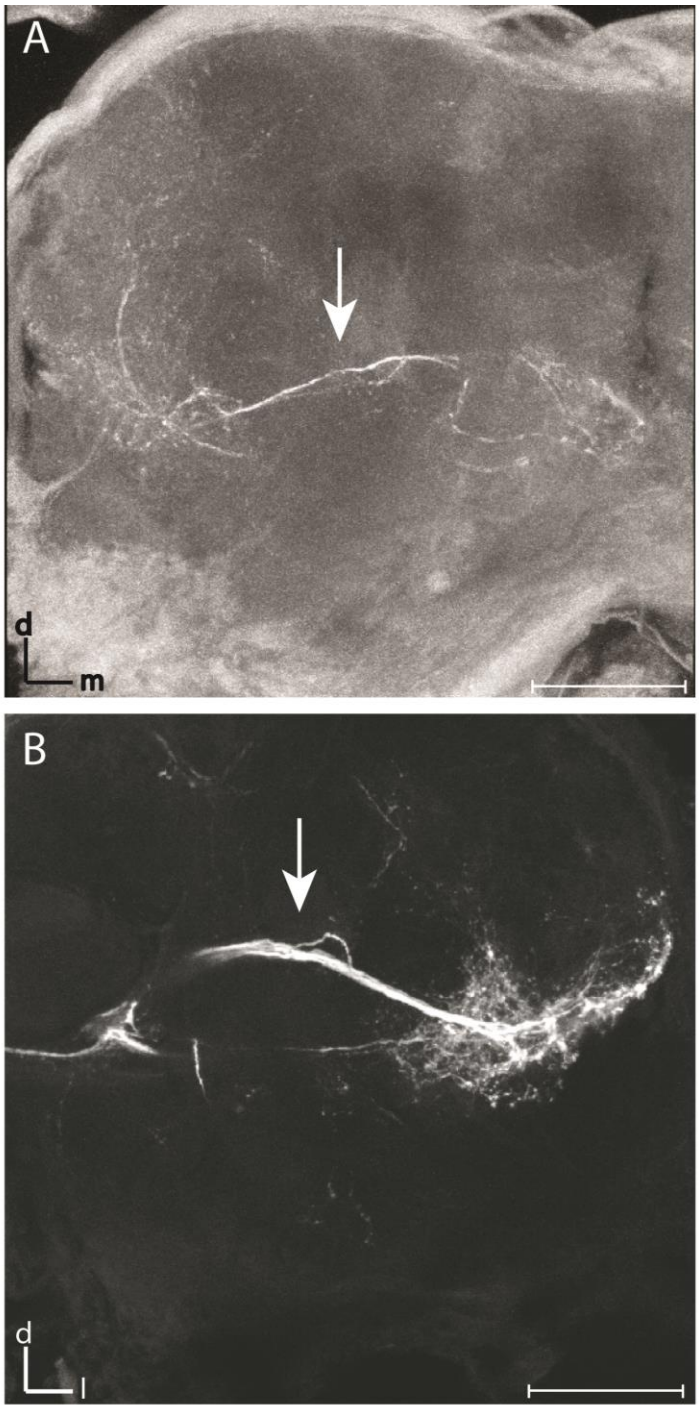

Figure 3.17: Confocal images (projection views) of projection neurons stained from the labial pit organ glomerulus following the medio-lateral antenno-protocerebral tract (indicated by arrows) and their main terminal area in the lateral protocerebrum (LP). **A:** Brain preparation 67 (male), right hemisphere. **B:** Brain preparation 107 (male), left hemisphere. In this preparation, the contralateral axonal fiber is indicated by an arrow head. **A-B** are frontally oriented and scanned with a 20x objective. Scale bars: 100 µm.

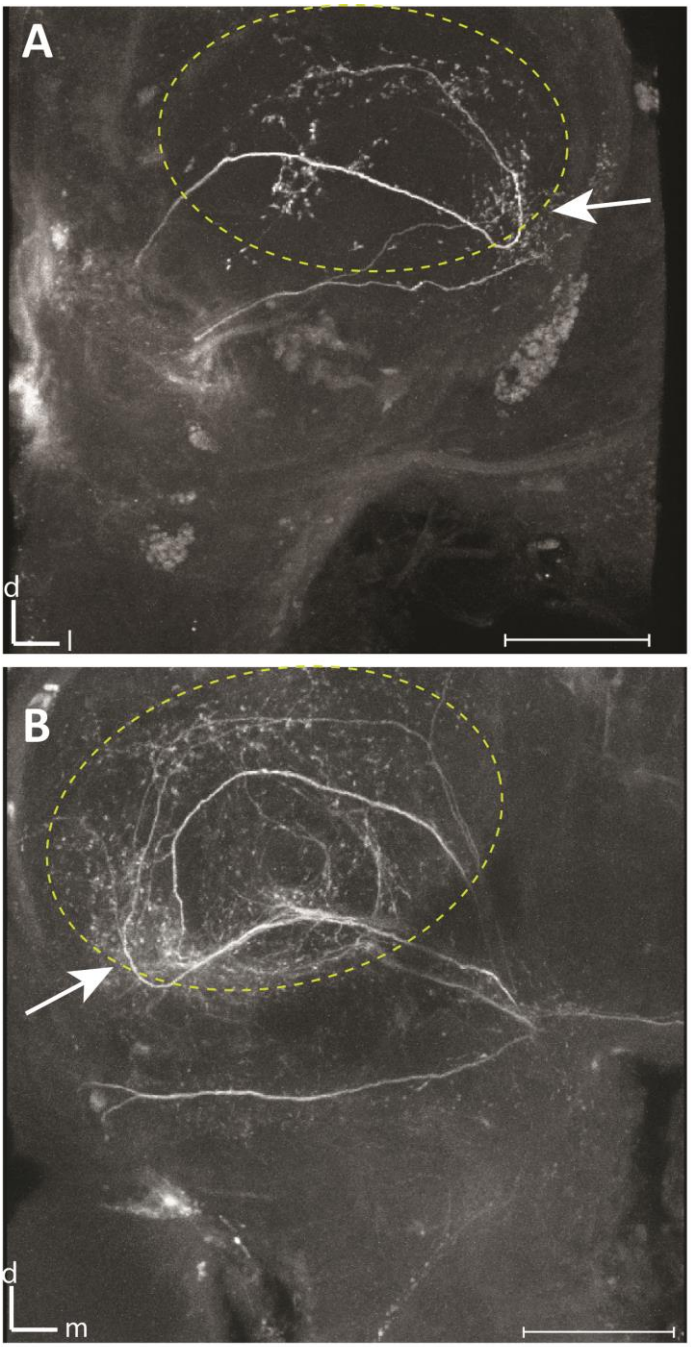

Figure 3.18: Confocal images (projection views) of two different brain preparations showing projection neurons (PNs) stained from the labial pit organ glomerulus. The PNs follow the medio-lateral antenno-protocerebral tract before they bend sharpely in a dorsal-medial direction and project to the superior protocerebrum, terminating just anteriorly to the mushroom body calyces (MBcal). The dashed circles indicate the position of the MBcal. **A:** Brain preparation 69 (female), left hemisphere **B:** Brain preparation 54 (male), right hemisphere. **A-B** are frontally oriented and scanned with a 20x objective. Scale bars: 100 µm.

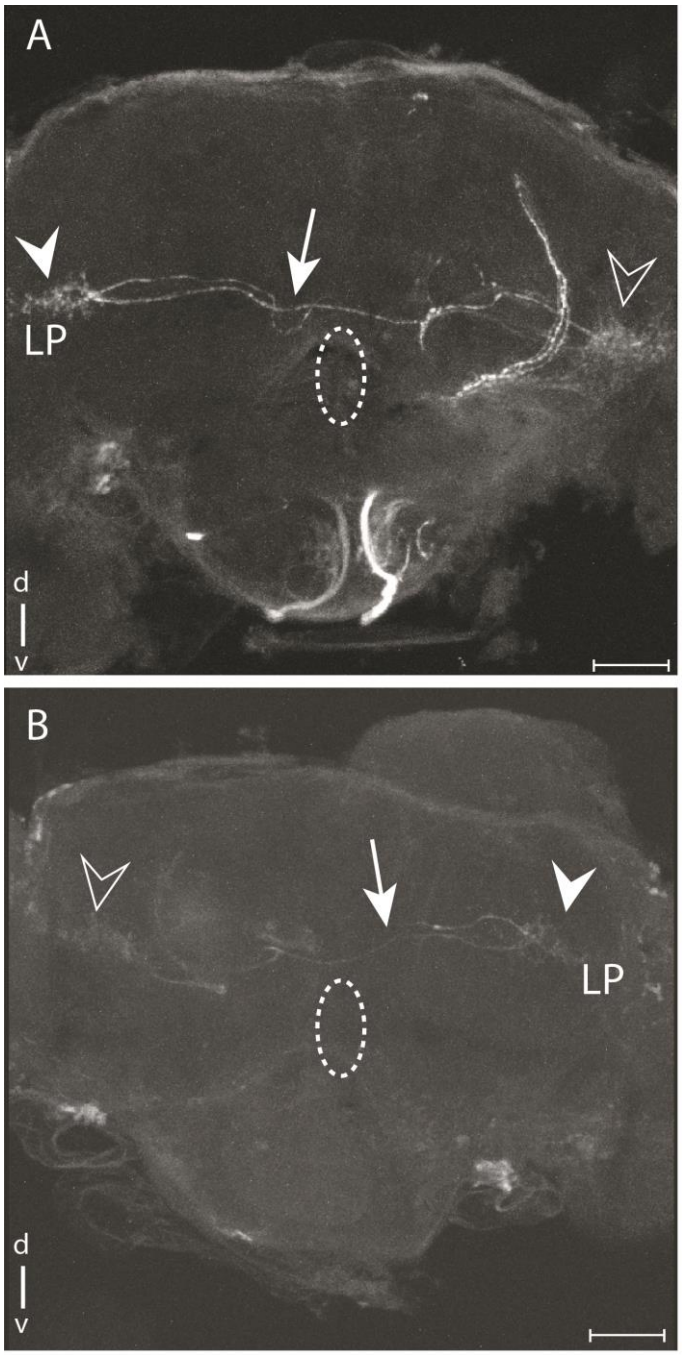

Figure 3.19: Confocal images (projection views) showing projection neurons (arrows) stained from the labial pit organ glomerulus constituting a contralateral branch from the medio-lateral antenno-protocerebral tract. The symmetrical ipsi- and contralateral terminal areas in the lateral protocerebrum are indicated by empty and filled arrow heads, respectively. Location of the oesophagus is indicated with a dashed circle for orientation purposes. **A:** Brain preparation 104 (female). **B:** Brain preparation 75 (female). **A-B** are frontally oriented and scanned with a 10x objective. Scale bars: 100 µm.

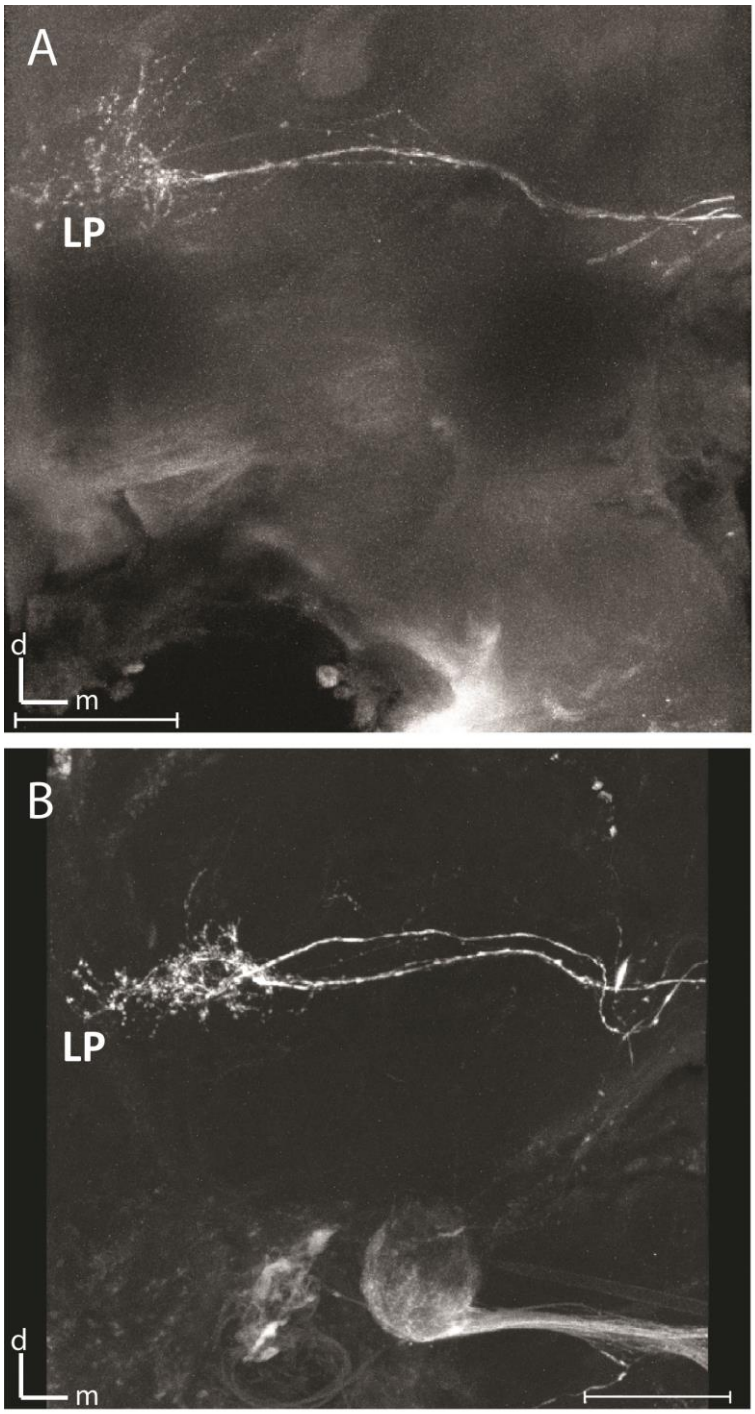

Figure 3.20: Confocal images (projection views) of two brain preparations showing the contralateral projection neuron branch stained from the left labial pit organ glomerulus, and its terminal area in the lateral protocerebrum (LP). **A:** Brain preparation 107 (male), right hemisphere.
B: Brain preparation 104 (female), right hemisphere.
A-B are frontally oriented and scanned with a 20x objective.
Scale bars: 100 µm.

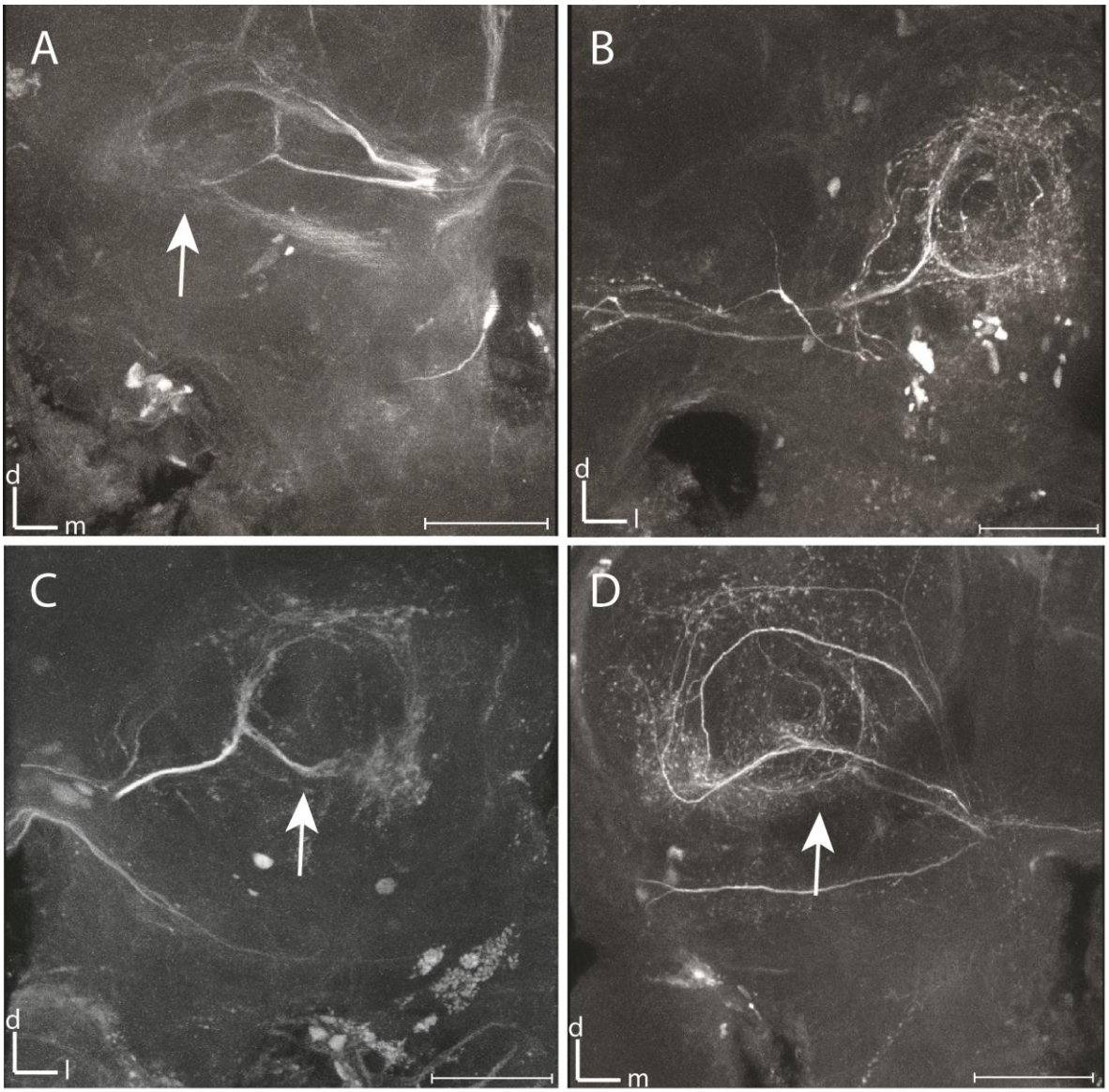

Figure 3.21: Confocal images (projection views) of four different brain preparations showing projection neurons stained from the labial pit organ glomerulus. The current projection neurons follow the medio-lateral antenno-protocerebral tract before they branch off from the common tract, and form a distinct circular pattern (white arrows) terminating in the superior proto-cerebrum, just anterior to the mushroom body calyces.
A: Brain preparation 38 (female), right hemisphere. **B:** Brain preparation 78 (female), left hemisphere. **C:** Brain preparation 52 (male), left hemisphere. **D:** Brain preparation 54 (male), right hemisphere. **A-D** are frontally oriented and scanned with a 20x objective.
Scale bars: 100 µm.

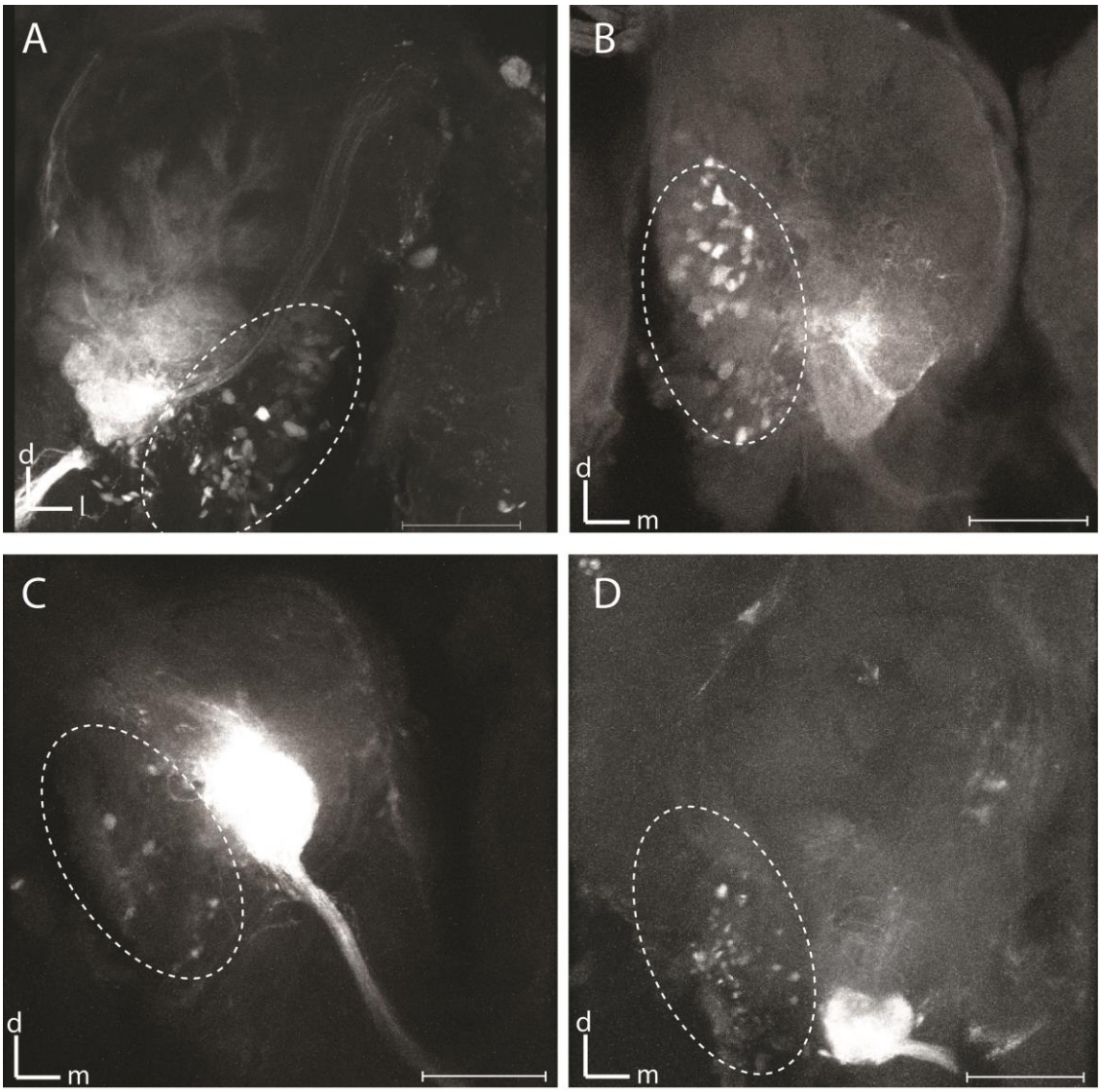

Figure 3.22: Confocal images (projection views) showing location of stained somata in the lateral cell cluster (LC), located in the outline of the antennal lobe. The LC is indicated by dashed circles. **A:** Brain preparation 52 (male), left hemisphere. **B:** Brain preparation 67 (male), right hemisphere. **C:** Brain preparation 75 (female), right hemisphere. **D:** Brain preparation 73 (male), right hemisphere. **A-D** are frontally oriented and scanned with a 20x objective. Scale bars: 100 µm.

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4 Discussion

The present Master's project is the first investigation intending to map the second order pathway of the CO_2 system of the moth *H. virescens*. Applying dye into the labial pit organ glomerulus of the antennal lobe revealed a distinct staining pattern of the CO_2 projection neurons and their target regions in the protocerebrum. Previous knowledge about the general olfactory pathway combined with the accessibility of dye application into one identifiable antennal-lobe glomerulus receiving input from the CO_2-receptive organ, made up a good basis for the design of this project. The results obtained from this study are considered to be of particular interest and importance since the current knowledge on this topic is limited.

4.1 Result summary

This morphological study elucidates that antennal-lobe PNs originating from the LPOG project via two main antenno-protocerebral tracts; the ml-APT and the l-APT. The fibers from the two tracts terminate in distinct brain regions, including the lateral protocerebrum (both in the ipsi- and contralateral hemisphere) and particular areas of the superior protocerebrum located anterior to the mushroom body calyces. The PN bundles following each of the tracts show extensive branching in the protocerebrum. There is no obvious arborization of PN dendrites in other AL glomeruli than the LPOG. A schematic summary of the CO_2 pathways is presented in figure 3.1. The projection pattern of the second order neurons originating from the LPOG seems to be similar in males and females.

4.2 Mapping of the LPOG in living individuals

Application of dye into the LPO resulted in staining of the LPOG in both antennal lobes (figure 3.4). The LPOG is previously found to be the biggest of the ordinary glomeruli both in males and females of heliothine moths, constituting a landmark in the AL with its position posterior and most-ventrally (Berg et al., 2002, Løfaldli et al., 2010, Skiri et al., 2005). The location and size of the glomerulus makes it a feasible target for mass staining of its neural innervations. Though, no study has previously attempted to specifically stain this glomerulus

with the intention to map its PNs. One of the main study aims of this Master's project was to establish a method for retrograde staining of the LPOG PNs by injection of dye into the relevant glomerulus in *H.virescens*. The method established for staining of the LPOGs in this study can be used in general, however, as the presence and location of the distinct glomerulus seems to be similar among different insect species. There are shown some variations in the number of glomeruli and their relative position to each other between lepidopterans, but the presence and location of the LPOG seems to be highly conserved. Thus, previous studies on several moth species, as *M.sexta*, *B.mori*, *Rhodogastria* moths, and *Antherae polyphemus*, have described the ventrally positioned LPOG by staining of the sensory neurons (Bogner et al., 1986, Kent et al., 1986, Kent et al., 1999). The findings in the current study, demonstrating bilateral projections from the sensory neurons to both antennal lobes (figure 3.4), are in compliance with the previous studies.

It is important to emphasize that the present study did not include electrophysiological recordings, thus the physiological properties of the PNs are not known, neither whether they respond to other volatiles besides CO_2. It is however suggested that all of the stained PNs indeed originate from the LPOG, and hence they are designated as CO_2 PNs in the consequent discussion.

4.3 Distribution and morphology of projection neurons originating from the labial pit organ glomerulus

In comparing the second order pathway of the CO_2 system, as described in the present study, with the corresponding part of the general olfactory system, it becomes evident that the two show both similarities and disparities. The knowledge about higher order processing of CO_2 information in lepidopterans is currently very limited. The most central contribution on this topic is a study on *M. sexta* by Guerenstein et al. (2004a). Together with two anatomical studies on the antenno-protocerebral pathways of the moth by Homberg et al. (1988) and Rø et al. (2007), the three mentioned studies constitute important and interesting information upon which the results of the present study is being compared and discussed.

Injection of micro-Ruby into the LPOG of *H. virescens* revealed that all stained PNs originating from this glomerulus follow two main antenno-protocerebral tracts, the l-APT or the ml-APT (figure 3.7., 3.8, 3.9, and 3.10). The total lack of labeled neurons in the m-APT,

as found in the current study, differs substantially from previous findings dealing with the general olfactory system reporting about numerous antennal-lobe projection neurons passing in this tract (Homberg et al., 1988, Rø et al., 2007). As mentioned previously, the m-APT, targeting the calyces before terminating in the lateral protocerebrum, is assumed to play a key role for learning and memory. The indication that information about CO_2 does not follow the experience-dependent pathway via the mushroom body calyces in *H. virescens,* is interesting. One might speculate that CO_2 is not suitable as a conditioned stimulus, as all plants release CO_2 in respiration. Hence, it might be difficult to use this molecule as a cue in search for adequate food sources. Most of the AL PNs that has been physiologically described in previous studies include medial tract PNs responding to specific odor substances (Berg et al., 1998, Christensen et al., 1995, Vickers et al., 1998). In these cases it seems more logical that the information is being processed by the mushroom body calyces, as the specific odor signals can be used to detect a favorable host plant or a potential mate.

The data presented in the current investigation is contradictory to the previous findings from *M.sexta*, however, as the second order neurons responding to CO_2 in this species are reported to project in the m-APT. This may indicate that the organization of the CO_2 system with regard to the projection pattern of the LPOG PNs is species-specific. Though, as the only knowledge on this subject is restricted to the above mentioned moth species, it is not possible to conclude whether this is true for all moth species, or whether groups of near-related species might show the same pattern of organization. Also, it is important to consider the scale of the studies in respect to the number of PNs stained. In both *M. sexta* and *H. virescens*, it might be other PN types originating from the LPOG than those so far described. The suggestion that the PNs innervating the LPOGs of the moth *H. virescens* do not in any extent follow the m-APT to the mushroom body calyces, is supported by current work in an ongoing Master's study describing the nervous branching patterns of the AL; thus anterograde staining of nerve fibers following the m-APT indicates that all ordinary glomeruli in the AL are stained, except for the LPOGs (Berg, A. 2013, personal communication, unpublished data).

Also, it should be mentioned that new data dealing with the second order pathway of the CO_2 system in *D .melanogaster* is in close correspondence with the results in the current study. Thus, a recent investigation in the fruit fly has demonstrated that no stained PNs of the CO_2 system were present in the m-APT. Similar to the present findings in *H.virescens,* the stained PNs projected in the l-APT and ml-APT. More precisely, 4 LPOG PNs in each hemisphere where identified in *Drosophila*; two in the ml-APT, bypassing the calyces to the lateral

protocerebrum, and two in the l-APT. The two latter are bilateral PNs with projections to both the lateral protocerebrum and the calyces, and with cell bodies lateral to the suboesophageal ganglion (Bräcker et al., 2013, personal communication with Ilona C. Grunwald Kadow). Hence, in comparing the findings in *D. melanogaster* and *H. virescens*, there are some differences in regards to the projection patterns of the individual neurons in the two tracts, but overall, the PNs follow the same antenno-protocerebral tracts and terminate in partly corresponding areas in the protocerebrum.

4.3.1 Projection neurons following the l-APT

Axonal fibers of at least 4 LPOG PNs were found to follow the l-APT in this study. Their target regions include both the lateral- and the superior protocerebrum of the ipsilateral hemisphere. The projection pathway consists of two distinct sub-bundles of PNs which separate shortly after leaving the LPOG. One branch targets a relatively large area of the lateral protocerebrum that is situated close to the border of the optic lobe (e.g. figure 3.12, 3.13). The projection pattern of this sub-bundle is comparable with that of a previously described PN type passing in the l-APT; thus both Homberg et al. (1988) and Rø et al. (2007) describe so-called POb neurons targeting the very lateral areas of the lateral protocerebrum via the l-APT. The other branch described in the present study, leaves the common l-APT by projecting dorsally and making up a distinct columnar structure from the more ventral to the superior protocerebrum (figure 3.14 and 3.15). The same characteristic structure linked to the l-APT is found in an ongoing Master's project performing mass staining of PNs in the various tracts of *H. virescens* (Lillevoll, S., 2013, unpublished data). Also, it should be mentioned that some of the POa fibers in the same species, described by Rø et al. (2007), showed similar branching patterns including a columnar shaped target region in the superior protocerebrum.

All stained CO_2 PNs running in the l-APT were uniglomerular. Previous studies have described both uni-and multiglomerular PNs in this tract. From the knowledge about the morphologically different PN subtypes in the l-APT, as described in *M. sexta* and *H. virescens,* it becomes apparent that the stained LPOG PNs following the l-APT in the present investigation shows some morphological correspondence with the formerly identified uniglomerular neurons. However, none of the stained l-APT PNs presented in the current study shows innervations in the mushroom body calyces.

The finding of different thicknesses of the stained PN fibers running in the l-APT versus the ml-APT corresponds to previous observations (Homberg et al., 1988, Rø et al., 2007). Besides, the observation that the thin axons in the l-APT form a relatively loosely connected bundle is in agreement with the report by Homberg et al. (1988).

4.3.2 Projection neurons following the ml-APT

The ml-APT was in addition to the l-APT the other tract found to contain stained PNs originating from the LPOG. At least 3 PN axons were stained in this tract, projecting to target areas in both the lateral- and superior protocerebrum after extensive branching in the medial protocerebrum (figure 3.16, 3.17, and 3.18). Previous studies have pointed out that the ml-APT differs from the m-APT in being more directly linked to the assumed premotoric areas in the brain, as the lateral protocerebrum, since this tract does not innervate the mushroom body calyces (Rø et al., 2007). The morphological properties of the ml-APT PNs described in the present study show clear similarities with former findings on the corresponding part of the general olfactory system. Thus, projection neurons passing directly to termination areas in the lateral protocerebrum via the ml-APT is in correspondence with previous findings in both *H. virescens* (Rø et al., 2007) and *M. sexta* (Homberg et al., 1988). Also, at least one axonal fiber running in the ml-APT in the current study projected further from the LP, targeting an area of the superior protocerebrum just anterior to the mushroom body calyces (figure 3.18). It is particularly interesting to notice that both Homberg et al. (1988) and Rø et al. (2007) describe one distinct PN type with the same morphological properties.

An obvious distinction from the previous findings is the presence of uniglomerular LPOG PNs in the ml-APT. Earlier studies on PNs present in this particular tract, both in *H.virescens* and other moth species, have found only multiglomerular PNs (Homberg et al., 1988, Rø et al., 2007). Hence, the present study might be the first to describe uniglomerular PNs in the ml-APT.

What might also be considered to be especially interesting in the current study is the staining of additional branches of PN fibers from the ml-APT that has, to my knowledge, not been described previously in any moth species. These processes include one branch sending one or two axonal fibers to the contralateral hemisphere (figure 3.19 and 3.20) and, also, one branch

bending off dorsally from the common ml-APT, terminating in a circular pattern in the superior protocerebrum, just anterior to the mushroom body calyces (figure 3.21). In a former study on structure and response patterns of central olfactory neurons in the honeybee, *Apis mellifera*, Abel et al. (2001) described a ring-formed neuropil surrounding the area of the α-lobe of the mushroom bodies, which was innervated by PN arborizations following the ml-APT. The PNs did not innervate the mushroom body calyces and it was suggested that the arborizations in the ring-formed neuropil might form connections with the extrinsic neurons of the mushroom body α-lobe (Abel et al., 2001). As for the contralateral branch described in the present study, which is not previously described, it terminated in a region of the lateral protocerebrum symmetrical to that innervated by ml-APT PNs in the ipsilateral hemisphere. One might ponder over the functional meaning of such an organization, and one possible purpose could be some kind of involvement in experience-independent orientation behavior. Since there is no comparative data available at the present time, however, this is highly speculative. What might be important to take into consideration, is that CO_2 information is processed bilaterally already in the pathway of the sensory neurons, i.e. from the LPO to the LPOGs (figure 3.4). This is different from the arrangement at the corresponding level of the olfactory pathway of most insect species, which projects only ipsilaterally (Anton and Homberg, 1999). Regardless of the function, it seems as if the bilateral representation of the CO_2 information is ensured also by particular projection neurons passing in the ml-APT.

A previous immunocytochemical study on the AL of *H. virescens* showed that numerous PNs following the ml-APT are in fact GABA immunoreactive, meaning that they provide inhibitory input from the AL (Berg et al., 2009). It would be of great interest to investigate whether the PNs from the LPOG belong to this category.

Having seen that information from the LPOG is conveyed to higher brain centers through two antenno-protocerebral tracts might support idea that the different tracts of *H. virescens* are not designated to pass on information about different odors, but rather to mediate information about different aspects of the same stimulus.

4.3.3 Location of stained somata

From figure 3.22 it is evident that several somata in the lateral cell cluster of the AL, were stained. There was no staining of somata in any of the other AL cell clusters, neither in areas of the protocerebrum or the suboesophageal ganglion. It is thereby likely that the somata belonging to LPOG PNs in *H.virescens* are present in the lateral cell cluster only. Considering the relevant two tracts in which the PNs follow (the ml-APT and l-APT), this observation is in accordance with suggestions in previous studies implying that the medial- and anterior cell cluster are innervated by PNs in the m-APT only (Berg, A., Master's study, unpublished data, Homberg et al., 1988). Both Homberg et al. (1988) and Rø et al. (2007) described somata in the lateral cell cluster for olfactory PNs following the ml- and the l-APT. It has also been proposed that the PN somata seem to belong in the cell cluster positioned closest to the glomerulus housing the arborizations, which is in correspondence with the present findings showing that the somata of LPOG neurons are located in the adjacent positioned cell cluster. Unfortunately, it is not possible to state which soma belongs to which axonal fiber as the neural connections are not perceptible in the confocal images. The number of both stained somata and PNs differs somewhat between the brain preparations, but this is likely due to differences in the number of axonal fibers that were penetrated and injected with dye during the experimental procedures.

4.4 Innervation of the LPOG projection neurons in higher brain centers in the protocerebrum

The majority of the LPOG PNs stained in this study show innervations in the lateral protocerebrum. As mentioned previously, this secondary brain center is considered to be one of the premotoric areas in the moth brain, receiving information via all antenno-protocerebral tracts from the AL, either directly through axonal fibers following the ml-APT and l-APT, or indirectly via the m-APT (Løfaldli et al., 2010, Menzel, 2001, Tanaka et al., 2004). In the current study the termination area in the lateral protocerebrum for each of the stained axon bundles passing in the two tracts do not seem to completely overlap; rather, there seems to be a distinct distribution of axonal terminals of the ml-APT that are being located more posteriorly and slightly more dorsally as compared to the target region of the l-APT. The differences in thickness of the axonal fibers in the two tracts indicate that neural signals

following the ml-APT, being carried by relatively thick axons, may reach the target area in the lateral protocerebrum prior to signals following the l-APT. When considering the possibility of the ml-APT neurons being inhibitory, this might - together with the signal speed - influence the information processing in the area covered by overlapping terminals. The current form of parallel processing could impact the behavioral responses to information about CO_2 elicited by the moth. The fact that a significant number of the LPOG PNs in *H.virescens* target the lateral protocerebrum without projecting to the calyces, leads to the suggestion that the information about CO_2 is processed by an experience-independent pathway.

Also the superior protocerebrum receives information from stained PNs following both the ml-APT and l-APT. However, their terminals are positioned in different regions. The termination areas for the ml-APT sub-bundles are located just anterior to the mushroom body calyces, with the ring-formed structure connected to the dorsally passing branch, positioned slightly more ventrally than the terminal processes of the axon arriving from the lateral protocerebrum. The columnar-shaped target region from the l-APT is positioned more anteriorly, medially, and dorsally than those mentioned above, linked to the ml-APT (l-APT: figure 3.14, 3.15; ml-APT: figure 3.18, 3.21). Little is known about the functional properties of the superior protocerebrum, but findings in both *H. virescens* and other species indicate that SP neurons receive olfactory input from both the mushroom body complex, the lateral protocerebrum, and the antennal lobe (Kirschner et al., 2006, Li and Strausfeld, 1997, Løfaldli, 2012, Rø et al., 2007, Tanaka et al., 2008). Based on studies performed on *H. virescens*, Løfaldli et al. (2012) suggests that neurons in this area may integrate information from all three mentioned regions via parallel-processing channels. It might therefore also be that CO_2 information mediated by the ml-APT and l-APT is integrated with other olfactory information in the superior protocerebrum, though this is only speculations.

4.5 No sexual dimorphism at the second order level of the CO_2 system

The results of the present study show no apparent sexual dimorphism at the level of output-neurons from the LPOG in the moth *H. virescens*. Both males and females show the same main PN branching patterns, and there are no obvious differences in the number of axonal fibers stained between the two sexes. As mentioned previously, studies on different insects have shown some inequalities in the CO_2 system between males and females. In the moth

C. cactorum, sexual differences in the development of the CO_2 sensory organ have been found, leading to the suggestion that it is involved in oviparous behavior (Stange et al., 1995, Stange, 1997). Use of CO_2 in oviposition has also been suggested for the hawkmoth *M. sexta* (Abrell et al., 2005), though no sexual dimorphism of the LPO of this species has been reported (Kent et al., 1986). A new study on *H.armigera* have shown that the labial pit of females is slightly deeper than that of males, but it is not known whether there is a difference in the number of sensilla between the two sexes. Also, it was observed in the same species that the terminal segment of the labial palps of many females was not covered with scales, which may indicate a role of the LPO in oviparous behavior (Zhao et al., 2013).

4.6 Methodological considerations of the study

The main aim of this thesis was to stain projection neurons originating from the LPOG in the AL. This means that the dye is to be placed in a very specific area, with the complications this might cause. All experimental methods used in this study were exposed to several test runs for ensuring optimization of the procedures and protocols. The methods used depend among others factors upon visual cues and motoric skills. As previously mentioned, there were no electrophysiological recordings confirming the correct site for the injected dye.

The LPOG is the biggest of the ordinary olfactory glomeruli in *H.virescens*, and its location is favorable for performing this kind of experiment. Its position in the ventral-most area of the AL makes it available for dye injection. But it is also located posteriorly in the AL, being partly hidden behind other glomeruli. Fluorescent dye applied to the sensory neurons in the labial palp enlightens the LPOG, but gives no indication about adjacently located glomeruli. Because of this insecurity, the injection site was chosen in regard to what was thought to be most suitable, taking the mentioned concerns into consideration. By studying earlier mapping of the AL glomeruli (Berg et al., 2002, Løfaldli et al., 2010), it was possible to determine which area of the particular glomerulus would be most suitable for dye injection, and the ventral area of the LPOG, close to the entrance of the LPN was chosen. Since only one distinct area of the LPOG was targeted for injection in most of the brain preparations, this might have limited the types of PNs stained.

Using both manual and iontophoretic staining made it possible to adjust towards the most optimal procedure. Both experimental procedures gave positive and similar results. The manual procedure gave more frequent success, while the iontophoretic stainings gave more

precise results. The time needed for preparation and staining is short when using the manual method, thereby exposing the moth to less stress and reducing the time the brain is not sufficiently moist. The use of dye crystals usually ensures stronger labeling than a solution of micro-Ruby and potassium acetate, but the crystals are also easier to spill and thereby increase the risk of staining adjacently located glomeruli, trachea, or other intracranial structures. The advantage of iontophoretic staining is that the needle can remain in the glomeruli for a longer time period, facilitating uptake. Also, it provides a more precise injection site, without the high risk of color spill. The experimental procedure evaluated to be the most favorable, was iontophoretic staining because it clearly visualized the PNs with minimal fluorescent disturbance in the confocal images. However, the injection of dye into the LPOG is not guaranteed to provide successful staining of all innervating PNs, so there might be e.g. multiglomerular LPOG PNs that have so far not been described.

Previous studies on olfactory PNs in *H. virescens* have stained PNs having arborizations in glomeruli positioned close to the LPOG. None of these stainings have shown projection patterns similar to those described in the present investigation (Rø et al., 2007; Løfaldli et al., 2010). This supports the assumption that the PNs described in this study originates from the LPOG and not any of the neighboring glomeruli in the AL.

Micro-Ruby was chosen as dye both for staining the sensory fibers of the LPN and the PNs arborizing in the LPOG. This might have caused complications in studying the arborizations in the AL as it makes it difficult to separate the two staining steps. The reason why this was chosen in spite of the unfavorable outcome was that none of the other dyes tested gave successful staining in either of the steps. The different procedures tested are listed in Appendix II.

Nevertheless, the established method for pre-staining of the LPOG in a living preparation, followed by retrograde staining of the LPOG, using a stereo microscope equipped with fluorescence filter, have proven to work very satisfactory.

5 Conclusion

To understand how sensory information is coded in the nervous system of the moth, it is inevitable to gain knowledge about the morphology of central cell types and how they are organized relatively to each other. This encompasses the projection pathways of PNs from the AL to higher brain centers. The present Master's project has provided new knowledge about sensory encoding mechanisms by mapping the central pathways for CO_2 information in the brain of the moth *H. virescens.* By injection of dye into the LPOG, stained PNs were seen to follow two of the main antenno-protocerebral tracts; the l- and the ml-APT. All stained PNs were uniglomerular. Extensive bifurcations were present from the PN bundles of both tracts. No PNs were stained in the m-APT. Two main termination areas were shown to receive information from the LPOG; the lateral and the superior protocerebrum. The particular projection pattern, lacking axons in the prominent m-APT, indicates that information from the LPOG to higher olfactory brain centers follow an experience-independent processing pathway. None of the brain preparations showed obvious differences between males and females, thus these results suggest that there is no sexual dimorphism at the level of PNs originating from the LPOG. All successfully stained brain preparations show the same branching patterns in the protocerebrum, which substantiate the proposed morphological properties of the antenno-protocerebral pathways linked to the CO_2 system in *H. virescens.*

The results of this thesis indicate that there are significant differences between the second order levels of the general olfactory system and the CO_2 system in *H. virescens.* It would therefore be of great interest to further expand our knowledge about both sensory systems. I suggest that the stained neurons presented in this thesis should be reconstructed and integrated in the standard brain atlas of *H. virescens* in order to study their possible connections with olfactory neurons described in previous studies, and for comparing the localization of specific target regions, as for instance the superior protocerebrum, with well-known neuropil structures in the brain. This might provide a better understanding on how CO_2 information is integrated in higher brain regions. Although the current study has gained new knowledge about the central pathway mediating CO_2 information, much work is still needed. In order to fully understand the physiological properties of this system, electrophysiological studies exploring the response characteristics of the projection neurons linked to the LPOG is required.

6 Abbreviations

AL - Antennal lobe

AN – Antennal nerve

CB - Central body

GABA - Gamma amino butyric acid

KC - Kenyon Cell

LN - Local interneuron

LP - Lateral protocerebrum

LPN – Labial-palp pit nerve

LPO – Labial pit organ

LPOG – Labial pit organ glomerulus

l-APT - lateral antenno protocerebral tract

MBcal - Mushroom Body Calyces

MGC - Macroglomerular Complex

ml-APT - medio-lateral antenno protocerebral tract

m-APT - medial antenno protocerebral tract

OR - Olfactory receptor

ORNs - Olfactory receptor neurons

PN - Projection neuron

SOG - Suboesophageal ganglion

SBA - Standard Brain Atlas

7 References

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Appendix I

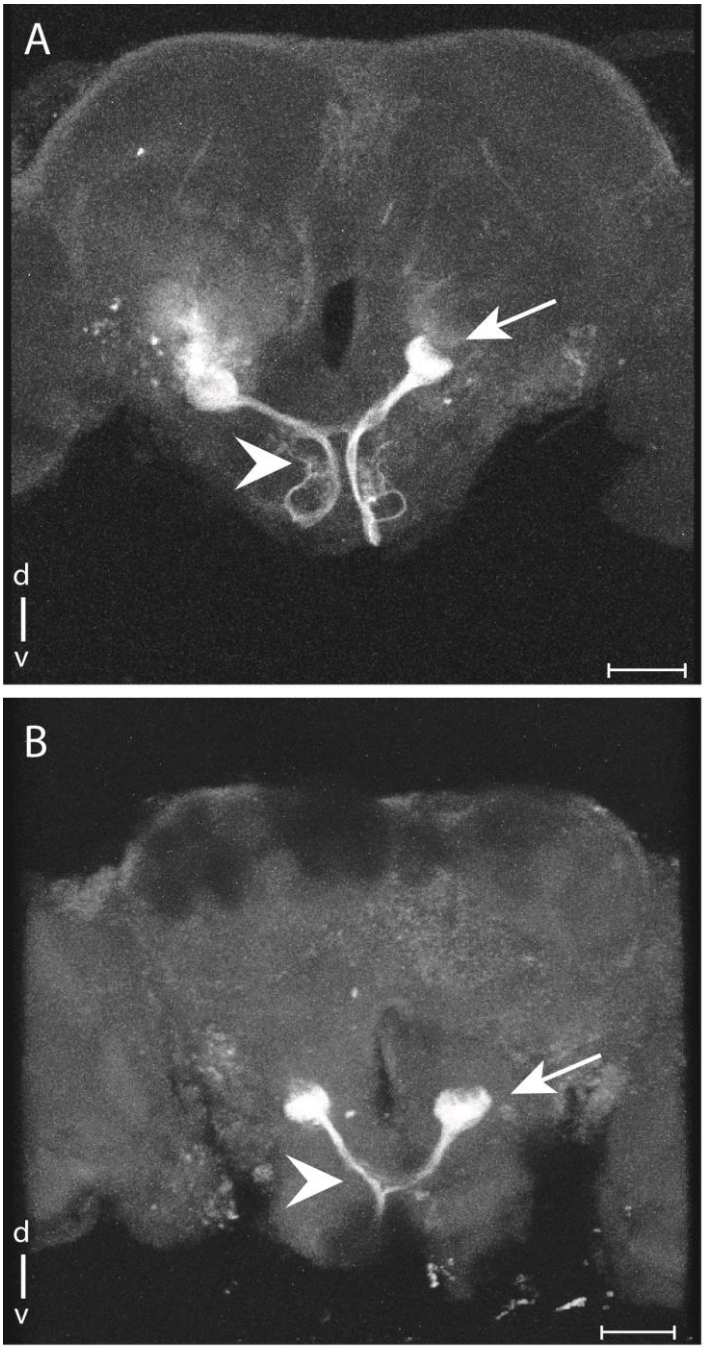

Figure A1: Confocal images (projection views) of two brain preparations showing the primary sensory pathway for CO_2 information; the labial-palp pit nerve (arrow head) and labial pit organ glomerulus (arrow).
A: Brain preparation 13 (female), stained from both labial palps. **B:** Brain preparation 19 (female), stained from the right labial palp. **A-B** are frontally oriented and scanned with a 10x objective. Scale bars: 100 µm.

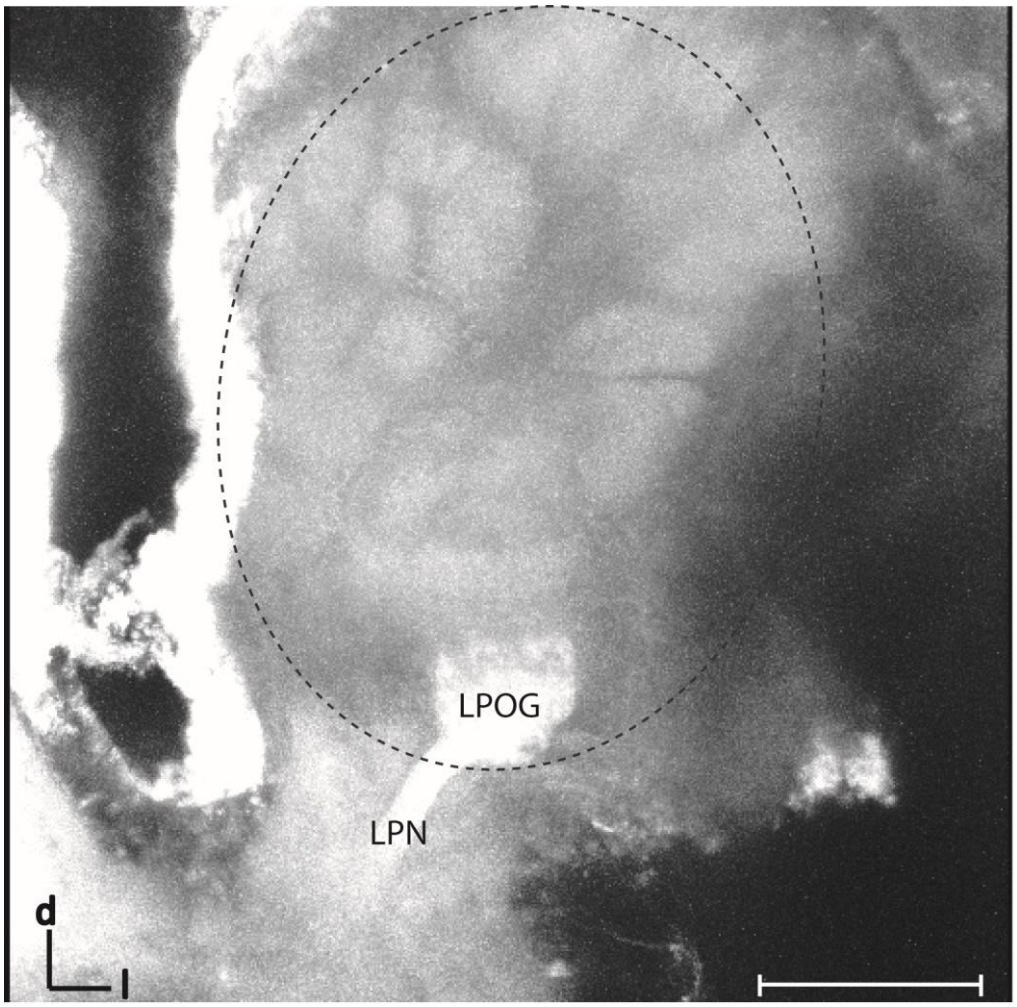

Figure A2: Confocal image (projection view) of the labial-palp pit nerve (LPN), antennal lobe (indicated by dashed circle), and the labial pit organ glomerulus (LPOG) in brain preparation 18 (female). It shows the position of the LPOG in the ventral-most and posterior area of the antennal lobe. The preparation is frontally oriented and scanned with a 20x objective. Scale bar: 100 µm.

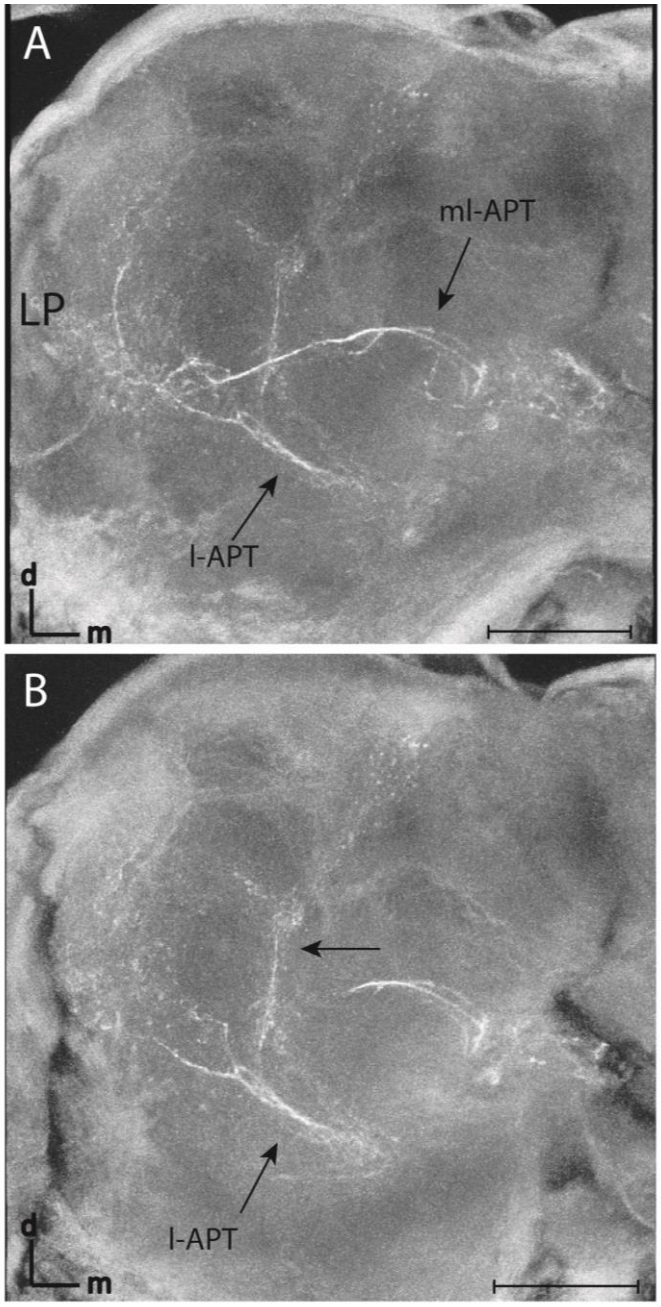

Figure A3: Confocal images (projection views) of brain preparation 67 (male) showing projection neurons (PNs) following the medio-lateral antenno-protocerebral tract (ml-APT) and the lateral antenno-protocerebral tract (l-APT). **A:** Shows both the ml-APT and l-APT and the termination areas in the lateral protocerebrum (LP). **B:** Shows the l-APT and a sub-bundle branching off from the common tract, turning dorsally, and projecting towards the superior protocerebrum. The preparation is frontally oriented and scanned with a 20x objective.Scale bars: 100 µm.

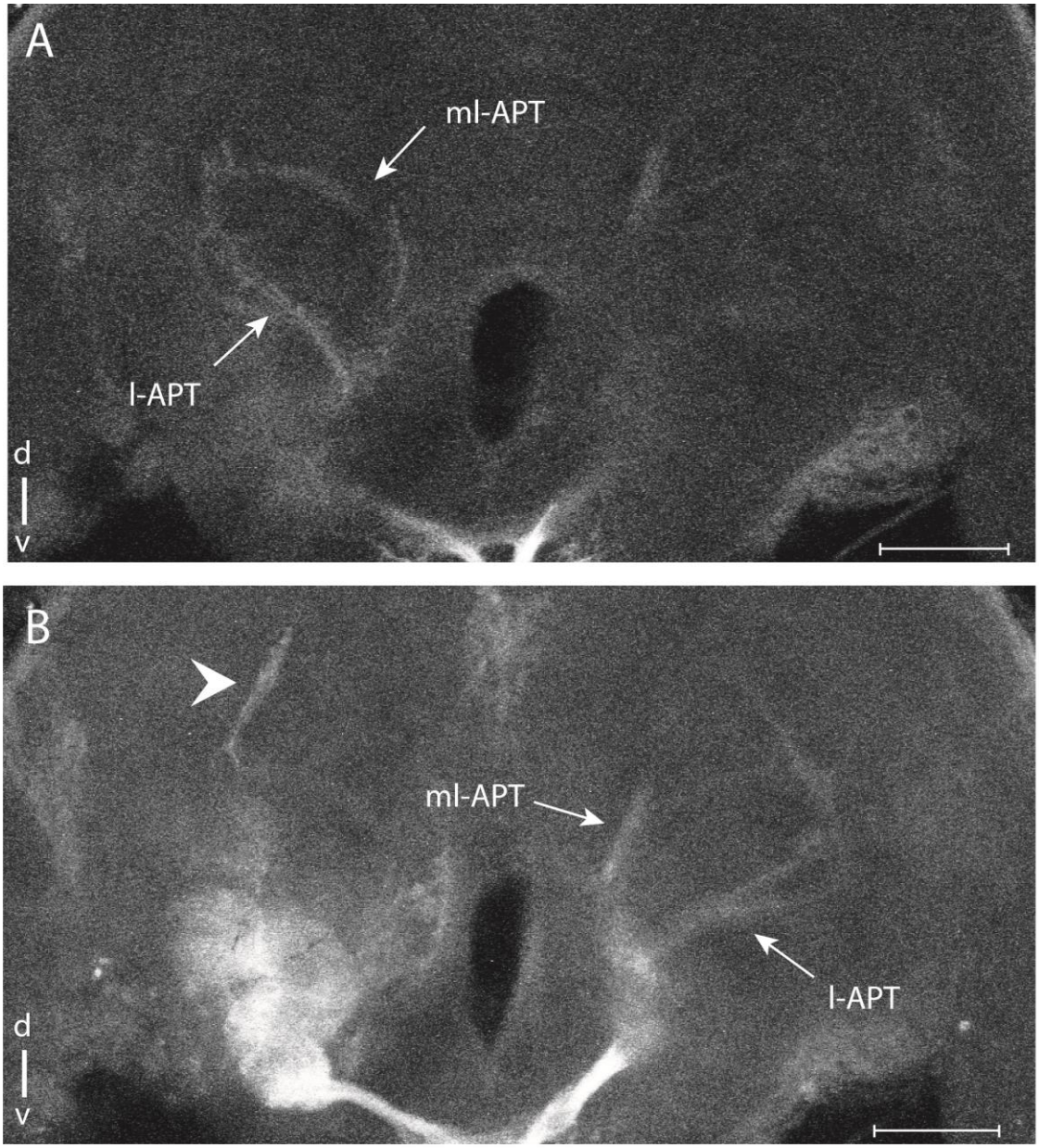

Figure A4: Confocal images (projection views) of brain preparation 13 (female), showing projection neurons (PNs) following the medio-lateral antenno-protocerebral tract (m-APT) and lateral antenno-protocerebral tract (l-APT) in both hemispheres. **A:** PNs following the ml-APT and l-APT in the right hemisphere. **B:** PNs following the ml-APT and l-APT in the left hemisphere. A branch of the l-APT projects dorsally and terminate in the superior protocerebrum (arrow head). The preparation is frontally oriented and scanned with a 10x objective. Scale bars: 100 µm.

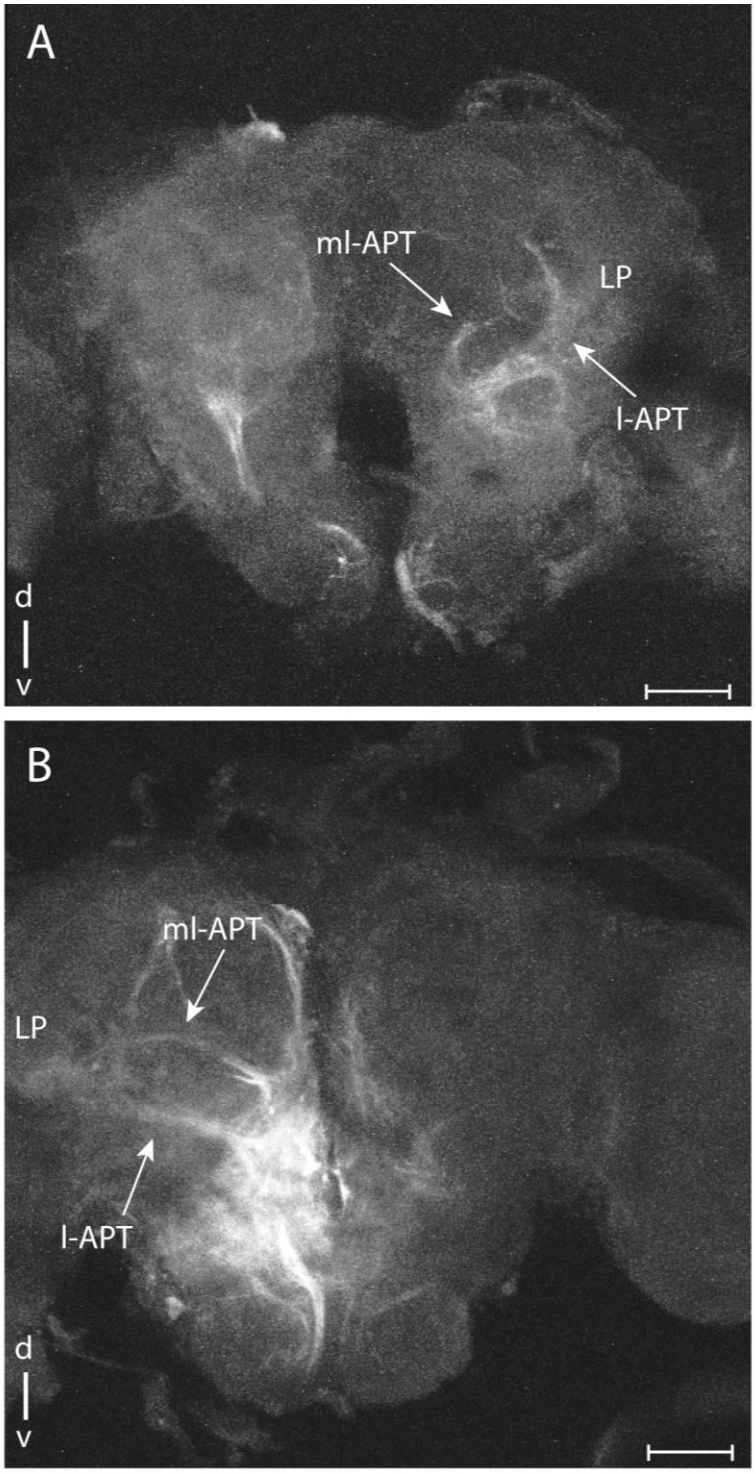

Figure A5: Confocal images (projection views) of two brain preparations showing projection neurons following the medio-lateral antenno-protocerebral tract (ml-APT) and the lateral antenno-protocerebral tract (l-APT). The lateral protocerebrum (LP) is the main terminal area for both tracts. **A:** Brain preparation 78 (female). **B:** Brain preparation 38 (female). **A-B** are frontally oriented and scanned with a 10x objective. Scale bars: 100 µm.

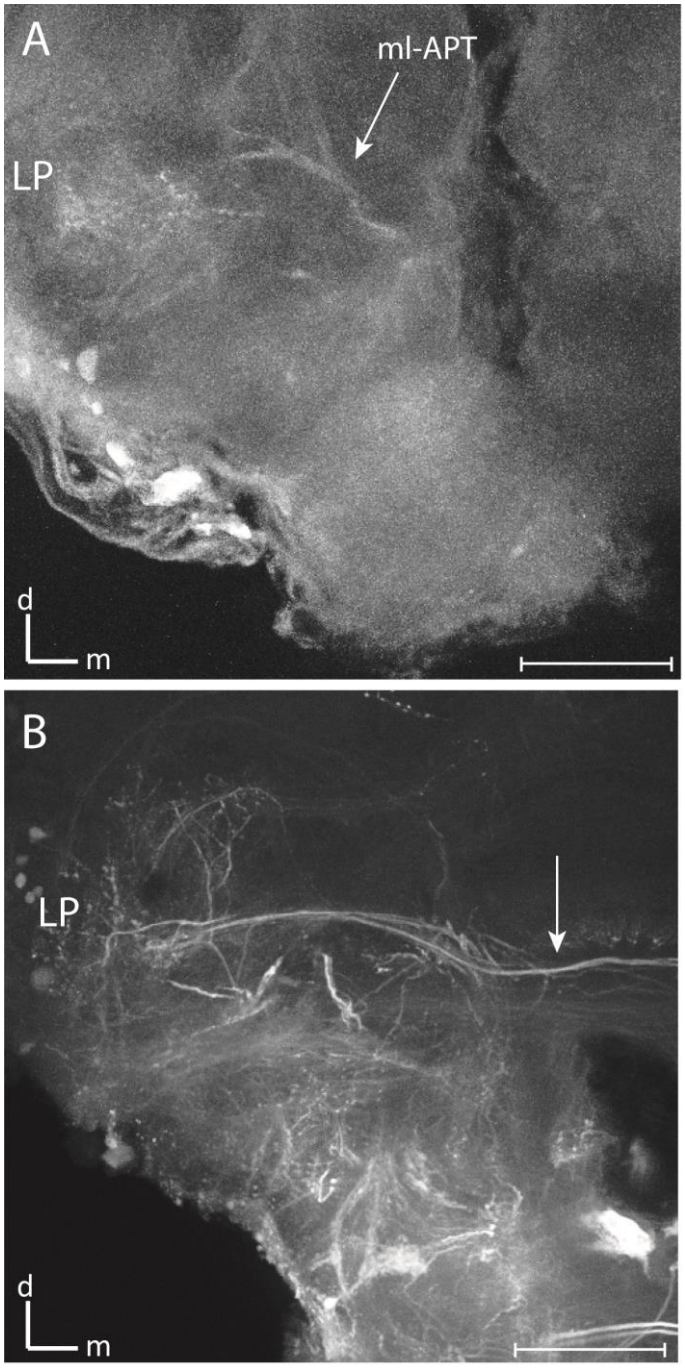

Figure A6: Confocal images (projection views) of two brain preparations showing projection neurons (PNs) stained from the labial pit organ glomerulus (LPOG). **A:** Brain preparation 63 (male) show PNs following the medio-lateral antenno-protocerebral tract (ml-APT), to the termination area in the lateral protocerebrum (LP). **B:** Brain preparation 83 (female) show a contralateral PN stained from the left LPOG terminating in the LP of the right hemisphere. **A-B** are frontally oriented and scanned with a 20x objective. Scale bars: 100 μm.

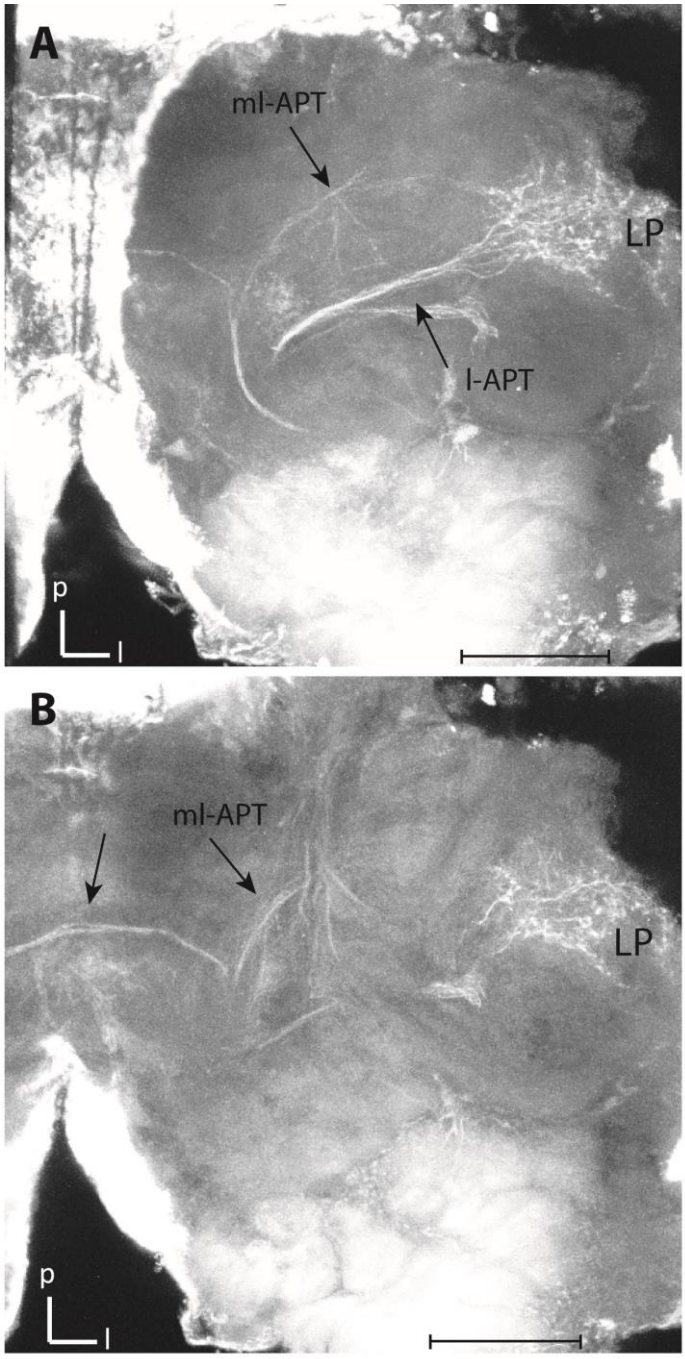

Figure A7: Confocal images (projection views) of brain preparation 18 (female) showing projection neurons following the medio-lateral antenno-protocerebral tract (ml-APT) and the lateral antenno-protocerebral tract (l-APT). **A:** The most prominent tract in this image is the l-APT, and its termination area in the lateral protocerebrum (LP) is indicated. **B:** This image clearly show the contralateral branch originating from the ml-APT crossing the midline of the brain. **A-B** are dorsally oriented and scanned with a 20x objective. Scale bars: 100 µm.

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Appendix II

The materials and methods presented in the main section were results from several test runs which aimed to optimize the protocols in establishing a method for retrograde staining of the LPOG. Several trials excluded both dyes and injection procedures and these are presented in the following section.

Dissection:

Several dissection procedures were performed in order to find the most suitable method:

- Opening a cuticula window between the antennae and down to the base of the proboscis. This procedure did not provide a sufficient access to the LPOG.
- Opening a window from one eye, keeping most of the cuticula, and all mouth parts intact. This provided access to the LPOG, but it was difficult to maneuver the electrode in a favorable direction and the procedure gave limited results.
- Opening of a big cuticula window, between the antennae and alongside the inside of the compound eyes, removing also the mouthparts and proboscis, as described in the main method section. This procedure provided successful results and was considered to be the most optimized dissection protocol.

Injection:

Before using glass electrodes, special made insect needles were used to apply dye into the LPOG. They were found to be too big for this purpose, and gave limited and imprecise results.

Dyes tested:

- Alexa-Fluor 488: Dextran, Alexa-Fluor (Invitrogen, Molecular Probes, Eugene, Oregon, USA). Ex/em: 495/519
- Micro-Ruby: Dextran, tetramethylrhodamine and biotin (Invitrogen, Molecular Probes, Eugene, Oregon, USA). Ex/em: 555/580
- Dextran, Fluorescein, 3000 MW, anionic, Nonfixable, D3305 (Invitrogen, Molecular Probes, Eugene, Oregon, USA). Ex/em: 494/521

Different dye combinations in the two staining steps:

a) micro-Ruby fixable in LPO + micro-Ruby fixable in LPOG
b) micro-Ruby nonfixable in LPO + micro-Ruby fixable in LPOG
c) micro-Ruby fixable in LPO + Alexa-fluor 488 in LPOG
d) Alexa-fluor 488 in LPO + micro-Ruby in LPOG

Only combination a) gave successful results with sufficient staining of both the primary sensory pathway (LPO and LPOG), and higher order pathways (projection neurons originating from the LPOG). It was desirable to stain with two different dyes, in order to be able to better separate the two staining steps in the visualization of the antennal lobe. It is unknown why these dye combination did not succeed, and the reason is only up for speculation.