Preface and acknowledgements

The present work was carried out at the chemosensory laboratory at the Department of Psychology, Norwegian University of Science and Technology (NTNU). The author of the current thesis has been responsible for collecting and analyzing the data. The supervisor developed the research question, and the thesis is a part of a larger project in the lab.

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Abstract

The ability of animals to detect volatile molecules in their environment is universal, and in an evolutionary perceptive, ancient. In many species a contact with the chemical world through the sense of smell is crucial for survival. Typical for the olfactory sensory system is the presence of sub-systems that process distinct odor stimuli. In the present study, the anatomy of the carbon dioxide sensory system in the moth *Helicoverpa armigera* was investigated. By applying fluorescent dye to the labial pit organ the total axonal assembly of first order neurons was mapped. Further, synapsin immunostaining allowed for detection of central neuropil areas. In accordance with previous reports, this double staining confirmed that the carbon dioxide sensitive neurons project to three main areas of the moth central nervous system: 1) one large glomerulus in each antennal lobe, 2) the subesophageal zone, and 3) the ventral nerve cord. In addition to mapping the target region of labial-palp axons passing in the ventral nerve cord −, which is documented for the first time −, the high resolution confocal images obtained here revealed several novel findings. These include an arrangement of several sub-tracts targeting the labial pit organ glomerulus and an extensive pattern of axon terminals in the subesophageal zone including projections in the antennal mechanosensory and motor center. Based on the anatomical organization of these pathways, as well as unique qualities typifying carbon dioxide as an olfactory cue, putative functions of this system is discussed.

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Introduction

The external world is a tremendous chaos of possible stimuli. Our senses have evolved to extract relevant information from the environment that surrounds us. Specifically, properties of the physical world, light waves for instance, are detected by specialized sensory organs with cells that have the ability to translate stimuli into the language of the brain; electrical signals. Internal neuronal networks in the brain of an organism enable representation of external stimuli, making it possible to respond in an adaptive manner to the requirements of the environment. Tailored solutions for sensing the world have evolved to ensure the survival and reproduction of different species (Axel, 2005).

In the present study, a sensory system for detection of carbon dioxide (CO_2) molecules in air is investigated. Various insects have evolved CO_2 sensing receptors and organs, functioning in parallel with their main sensory system for smell (Galizia & Rössler, 2010). These insects rely on CO_2 as an olfactory signal to navigate their environment (Guerenstein & Hildebrand, 2008). For instance, several moth species use information about fluctuations in local CO_2 levels to find nutritious food sources and suitable locations for laying eggs (Stange, 1997; Thom, Guerenstein, Mechaber, & Hildebrand, 2004). As several moth species are destructive agricultural pests, knowledge about their olfactory system and behavior may facilitate development of non-toxic and food safe pesticides.

Furthermore, by studying the anatomy of such functional neuronal networks we can get an understanding of how sensory information generating diverse behaviors is sent via specific brain circuits. In this context, insect olfaction is ideal to study because of the existence of sub-systems generating diverse behaviors that are critical for survival. Interestingly, the majority of animals probe the world mainly by using their chemical senses (Ache & Young, 2005; Axel, 2005).

The chemical senses

The chemical senses, taste and smell, are the oldest and most conserved sensory systems. Virtually all organisms, even bacteria have the ability to detect and react to chemical stimuli in the external environment. Many animals, both terrestrial and aquatic, rely on olfactory cues in order to identify and locate desirable items like food, as well as dangers to avoid. Olfaction creates possibilities for communication via chemical signals, so called pheromones, which can convey individual identity, social status, and group membership. Consequently, odorants have the potential to trigger aggression as well as mating behaviors (Ache & Young, 2005).

In humans, the sense of smell is closely related to memory, emotions, and learning. However, olfaction is rarely appreciated for the significant role it plays in human life. Retronasal smell (molecules reaching the nasal cavity through the mouth) contributes substantially to the perception of taste, actually more than the taste buds on the tongue. A loss of smell will therefore result in significant decrease in the perception of taste, and thus a decrease in quality of life (reviewed by Shepherd, 2006).

The discovery of the gene superfamily coding for olfactory receptors in mammals led to a revitalization of research on olfaction (Buck & Axel, 1991). The relatively large groups of olfactory genes, not only in mammals but also in other organisms, emphasize the importance of smell in the animal kingdom. In addition, the presence of numerous odor receptor types indicates how it is possible to recognize such a broad range of structurally diverse odor molecules (Axel, 2005). Nevertheless, the olfactory system is still the least understood among the senses (Galizia & Rössler, 2010).

A model system to investigate olfaction

The insect nervous system has been popular to use as a model for investigating the encoding principles of olfaction. Insects are often described as the most successful class of animals in the history of life and evolution. They vastly outnumber other groups of animals, both with respect to the number of species and the number of living organisms. As the product of 400 million years of evolution, insects are diverse, well adapted, and found to inhabit almost all environments on earth (Grimaldi & Engel, 2005). Accordingly, they possess a great repertoire of complex behaviors, many of which are evoked by sensitivity to chemical signals (Martin et al., 2011).

Compared to vertebrates the nervous system of insects is of modest complexity, with relatively few and large neurons. As their nervous system is easily accessible for experimental research, the use of insects as model organisms makes it possible to study, in a brain highly devoted to olfaction, distinct neuronal networks that have the potential to initiate immediate behavioral responses (Haupt, Sakurai, Namiki, Kazawa, & Kanzaki, 2010).

Similarities in olfactory signaling across phyla

Interestingly, striking similarities in the organization of olfactory pathways across a broad array of phyla are observable (Ache & Young 2005; Hildebrand & Shepherd, 1997; Kaupp, 2010). The most noteworthy commonality is the organization of the primary olfactory center of the brain in round, synapse-dense structures termed glomeruli, and complex

interconnections between neurons in these functional units. This kind of arrangement is evident both in the insect antennal lobe (AL) and the human olfactory bulb (Kaupp, 2010). In addition, the principle of combinatorial coding, meaning that every olfactory stimulus leads to a specific pattern of activated glomeruli, has been conserved in the primary olfactory center. This indicates that the primary olfactory center plays a critical role in processing olfactory information (Ache & Young, 2005).

These commonalities cannot be the result of a common ancestor, as the olfactory receptors of insects and mammals are structurally different and genetically unrelated (Kaupp, 2010). Indeed, the organization of the olfactory pathways is an instance of convergent evolution, where the same strategies for processing olfactory input developed independently of each other. Similarities in the olfactory pathways imply that there to some degree exists an optimal way to detect and process information from chemical stimuli (Ache & Young, 2005). Another remarkable similarity is the consistent presence of parallel olfactory subsystems, observed across insect and vertebrate species (Galizia & Rössler, 2010).

Parallel olfactory systems

Parallel processing of sensory information is a common principle in biological systems across different species. In humans for example, visual information is processed in parallel. Information about different visual features, like color and movement, for example, are conveyed along distinct pathways from retina via thalamus to the visual cortex, and are finally carried in two separated streams to higher brain areas. This is an example of so-called dual parallel processing, where different properties of the same type of stimuli are processed in parallel pathways. (Galizia & Rössler, 2010).

A second category of parallel systems is segregated parallel systems, where distinct stimuli are processed either by a separate organ or pathway. This is most common in the olfactory system (Galizia & Rössler, 2010). Many mammals possess an accessory olfactory organ, called the vomeronasal organ, with specific pathways dedicated to detection and processing of pheromone information (Brennan & Zufall, 2006; Haplern, 1987; Liberles, 2014). Also in the male moth two anatomically segregated pathways exists in the olfactory system, one for plant odors and one for pheromones (Berg, Zhao, & Wang, 2014). These types of olfactory subsystems processing input from specific chemical stimuli are best understood in moths and flies. In addition to the pheromone system, several insects have a specialized sensory system for detection of CO_2 (Galizia & Rössler, 2010).

CO_2 as an olfactory cue

CO_2 is an ever-present, unspecific gas that is a significant element of the atmospheric air. While plants take up CO_2 for photosynthesis, animals release it into the environment during cellular respiration. In fact, nearly all organisms create a local concentration gradient of CO_2 because of their metabolic activity. In the immense disarray of CO_2 sources and sinks, rapidly changing temporal and spatial gradients of CO_2 can convey important information about the proximate environment (Jones, 2013). The ambiguous nature and high background concentration of CO_2 make it a curious olfactory signal (Stange & Stowe, 1999). While humans are unable to smell this gas, various insect species have evolved CO_2 sensing organs and rely on CO_2 as a chemical signal to navigate their ecological niche (reviewed by Guerenstein & Hildebrand, 2008).

Social insects, like ants and bees, use CO_2 cues in sophisticated ways to evaluate and regulate the climate in their densely populated nests. Fanning their wings to push out air if CO_2 levels in the hive are too high, the working bees are facilitating labor extensive air conditioning (Jones, 2013; Seeley, 1974). Hematophagous (blood-sucking) insects like mosquitos rely on CO_2 cues both from a long range, to locate hosts for feeding, and short range, for penetrating skin (Gilles, 1980; Guerenstein & Hildebrand, 2008). Knowledge of the mosquito CO_2 detection system is of medical relevance, as many serious diseases are transferred to humans by mosquito bites (Jones, 2013).

Several moth species are remarkably sensitive to fluctuations in ambient CO_2 levels. In the first study demonstrating that CO_2 influences the behavior of moths, Rasch and Rembold (1994) showed that the *Helicoverpa armigera* larva is attracted to CO_2. The behavioral significance of CO_2 in adult moths has been demonstrated as well. For instance, forging behavior linked to CO_2 was found in *Manduca sexta.* Possibly CO_2 can reveal the profitability of plants to moths (Thom et al., 2004). In addition, CO_2 dependent oviposition behavior has been reported in *Cactoblastis cactorum,* emphasized by sexual dismorphism of the CO_2 sensing organ in this species (Stange, 1997). However CO_2 levels do not seem the affect oviposition behavior in *M. sexta* (Abrell et al., 2005).

As a chemical signal CO_2 is both species-specific and context-specific. The biological meaning of CO_2 is dependent on the ecological niche of the animal. For instance, different CO_2 gradients have different values for moth species whose hostplants are CO_2 sinks, and species that use hostplants that are CO_2 sources (Guerenstein & Hildebrand, 2008). Furthermore, the fruit fly, *Drosophila melanogaster*, will avoid CO_2 when conspecific individuals that are stressed release it, but are attracted when CO_2 is emitted from rotten fruit.

This indicates that interactions with other odorants are critical for determining the behavioral relevance of CO_2 (Turner & Ray, 2009).

Predicted effects of rising CO_2

Increased level of CO_2 in the atmosphere, caused by human activities, is contributing to climate change. While the atmospheric CO_2 levels are estimated to have been on an average of 280 ppm (parts per million) before the industrial revolution, the present levels are measured at about 400 ppm (IPCC, 2007). Several observations have documented both direct and indirect influence of elevated CO_2 levels on moths. For instance, the CO_2 sensory cells of *H. armigera* are reported to respond to temperature as well as CO_2 under conditions of elevated CO_2 levels (Stange & Wong, 1993). This probably makes the CO_2 system less precise (Stange & Wong, 1993). Another example is from *C. cactorum,* where sensory neurons adapted and stopped responding to higher levels of CO_2 over time (Stange, Monro, Stowe, & Osmond, 1995). These reports may imply that the CO_2 sensitive organs developed and are adjusted to environments with pre-industrial levels of CO_2 (Guerenstein & Hildebrand, 2008).

In several studies on indirect effects of raised CO_2 levels, interactions between insects and plants are addressed (Guerenstein & Hildebrand, 2008). Indeed, the chemistry of plants changes, as they are grown in elevated CO_2 levels (Cotrufo, Ineson, & Scott, 1998). For example, decrease of the nutritional value of plants under such conditions is well documented (Stiling & Cornelissen, 2007). Among reports, are changes in the moths' preferred food sources, and increased consumption as a compensation for loss of nutrition (Yin, Sun, Wu & Ge, 2010). One study on *C. cactorum* showed that the female laid fewer eggs on a plant grown under conditions including elevated CO_2 concentration, suggesting that atmospheric CO_2 may influence oviposition in Lepidopterans (Stange, 1997). In the following section an overview of the moth CO_2 olfactory sub-system and its close integration with the main olfactory system is presented.

A distinct CO_2 pathway closely connected with the main olfactory system

The CO_2 sensitive neurons of moths are located on a distinct sensory organ, separate from the main olfactory organ, the antennae. Still, a significant number of the CO_2 sensory neurons project directly to the primary *olfactory* center in the insect brain, the AL. For this reason, the CO_2 system is considered to be a part of the olfactory system, and is presented accordingly here.

The peripheral part of the CO_2 pathway

While the central olfactory pathways are well described, much is unknown about the CO_2 sub-system. In insects, CO_2 sensitive cells are always located exclusively on either the mouthparts or the antennae, never on both in a single species (reviewed by Stange & Stowe, 1999). Several species of moths and butterflies possesses an organ on their mouthpart called the labial pit organ (LPO; Kent, Harrow, Quartararo, & Hildebrand, 1986; Lee, Selzer, & Altner, 1985), which is dedicated to detection of CO_2 (Bogner, 1990; Guerenstein, Christensen, & Hildebrand, 2004). The LPO is to be found on the third and most distal segment of each labial palp, forming a pair of upward-oriented appendages on each side of the proboscis (Figure 1). Scales densely cover the third segment, and at the tip is a narrow opening into a depression, groove or pit (Figure 2). Sensilla, hairike cuticular structures within this cavity constitute the LPO (Zhao et al., 2013a).

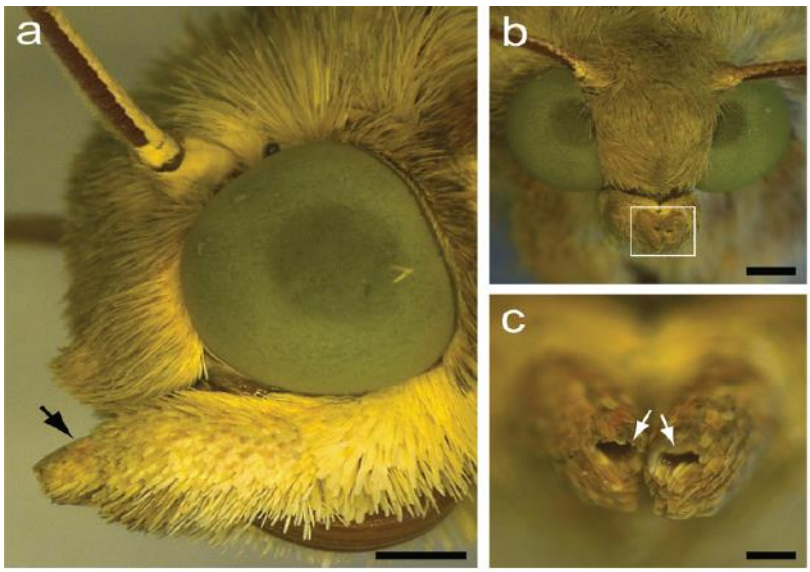

Figure 1. Pictures showing the location of the labial palps and the opening of the labial pit organ in the moth *H. armigera*. (Figure copied from Zhao et al. 2013).

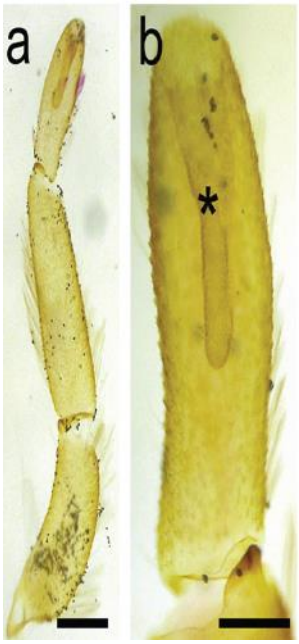

Figure 2. The CO2-sensitive labial pit organ (indicated by star) is located in the third and outermost segment of the labial palp. (Adopted from Zhao et al. 2013).

Zhao et al., (2013a) identified two morphologically different LPO sensillum types in *H. armigera*, trichoid (hair-like) and basiconic (peg-shaped) sensilla. The number of sensilla in the LPO varies greatly between different Lepidopteran species. It is reasonable to believe that this diversity is correlated with the considerable differences in feeding habits observed across moth species (Guerenstein & Hildebrand, 2008; Kent et al., 1986). The LPO of *M. sexta* is reported to contain approximately 1750 sensilla, while species of *Antheraea* possess only ca. 40 (Kent et al., 1986). In *H. armigera*, on the other hand, the LPO houses approximately 1200 sensilla (Zhao et al., 2013a).

Sensilla within the LPO contain specialized sensory neurons that selectively respond to CO_2 (Bogner, 1990; Guerenstein et al., 2004). Stange (1992) reported that the LPO of *H. armigera* is capable of detecting fluctuations in ambient CO_2 levels of 0.5 ppm, making it one of the most responsive CO_2 sensory systems known (Jones, 2013). Generally, the sensilla contain a single sensory neuron, but the existence of two neurons in one sensillum has also been reported (Bogner, Boppré, Ernst, & Boeckh, 1986; Kent et al., 1986; Lee et al., 1985). The CO_2 sensory neurons have unique physiological characteristics that distinguish them from the olfactory sensory neurons on the antennae. As they do not adapt to prolonged or repeated stimulus, they are assumed to monitor background levels of CO_2 continuously (Bogner et al., 1986; Stange & Wong, 1993. Simultaneously, the responses of sensory neurons to CO_2 are reported to correspond to changes in CO_2 gradients. That is, when the local concentration increases, the spiking of the neurons increase at the same rate of change, and vice versa when the concentration decrease (Guerenstein et al., 2004).

These physiological properties are similar to qualities of neurons that respond to temperature and humidity, and it has therefore been suggested that CO_2 neurons should be included in the group of neurons that monitor environmental variables such as temperature and humidity (Guerenstein & Hildebrand, 2008). Interestingly the stimuli quality is also comparable, as temperature, humidity and CO_2 all are ubiquitous stimuli (Bogner et al., 1986).

Receptor proteins on the sensory neurons enable transduction of chemical stimuli to neural signals. The first insect CO_2 receptors identified were those in the model organism *D. melanogaster*. Surprisingly, these proteins constitute gustatory receptors (GRs), not olfactory receptors (Jones, Cayirlioglu, Kadow, & Vosshall, 2007; Kwon, Dahanukar, Weiss, & Carlson, 2007). Therefore, CO_2 receptors have been described as "gustatory receptors expressed in olfactory organs" (Jones, 2013). Similarly to the olfactory receptors, the CO_2 GRs are G-protein coupled proteins. In *D. melanogaster*, two GR genes, Gr21a and Gr63a, are co-expressed in CO_2 sensitive cells on the antennae. Together they mediate CO_2 responses (Jones et al., 2007). Flies with genetically silenced GR21a fail to detect CO_2 and show no innate CO_2 responses, demonstrating that activation of the neurons expressing this receptor is necessary for proper CO_2 processing (Suh et al., 2004). In moths (including *H. armigera*), beetles, and mosquito three ortholog CO_2 GR genes (genes that share a common ancestry) have been identified (Robertson & Kent, 2009). In *H. armigera*, these genes are specifically expressed in the labial palps (Xu & Anderson, 2015). In sum, there is a subfamily of GRs that are sensitive to CO_2, and they are highly conserved between various insect species although the number of receptors differs. Interestingly, several insects, including bees, might employ other CO_2 detection mechanisms, as the CO_2 sensitive GR genes mentioned above are not present in their genome (Robertson & Kent, 2009).

Like in other insects, the moth antennae hold the primary sensory organ for olfaction. The flagellum, comprising the third segment of the antennae, is densely covered with sensilla (Kaupp, 2010). Based on morphology, several types of antennal sensilla have been identified. For instance, the long, male-specific *sensilla trichodea*, are tuned specifically to female-produced sex pheromones (Berg et al., 2014). Simultaneously, other types of sensilla house neurons detecting plant odors. Each olfactory sensillum houses the dendritic branches of several olfactory sensory neurons (Haupt et al., 2010). Similarly to the mammalian olfactory sensory neurons, these are bipolar neurons, directly connecting the chemical environment and the brain. Both insect and mammalian olfactory receptors olfactory receptors are 7-transmembrane G-protein coupled receptors (Kaupp, 2010). However, the insect olfactory receptors include an odorant binding unit and a co-receptor unit, thus making up a

heterodimeric complex (Sato et al., 2008). Each olfactory sensory neuron expresses only one receptor type, a principle which has been named the “one neuron, one receptor” principle of olfaction (Kaupp, 2010).

Olfactory information from the antennae is mediated via the antennal nerve to second-order neurons in the ipsilateral AL (Galizia and Rössler, 2010). However, the antenna is not only dedicated to processing of olfactory stimulus. In addition to the olfactory sensilla, there are both taste and mechanosensory sensilla on this sensory organ (Homberg, Christensen, & Hildebrand, 1989; Jørgensen, Kvello, Almaas, & Mustaparta, 2006). Mechano sensitive neurons on the moth antenna are reported to be crucial in maintaining stability during flight (Sane, Dieudonné, Willis, & Daniel, 2007). The axons of mechanosensory cells on the antennae run in the antennal nerve, together with the olfactory neurons that target the AL. These, however, project to an area ventral and posterior to the AL called the antennal mechanosensory and motor center (AMMC; Figure 3). In addition, the AMMC is innervated by the dendrites of motor neurons that control the muscles in the antennae (Homberg et al., 1989; Zhao et al., 2016). The gustatory sensilla on the antennae allow for simultaneous perception of volatile odorants molecules and nonvolatile tastants. The projections of gustatory neurons also bypass the AL, and target the dorsal part of the gnathal ganglion (GNG; Jørgensen et al., 2006).

Axons of sensory cells in the LPO also project to the GNG, that is, close to the taste area. However the majority of the CO_2 neurons project to the AL, the first relay station for processing of olfactory information in the insect brain (reviewed by Mustaparta, 2002).

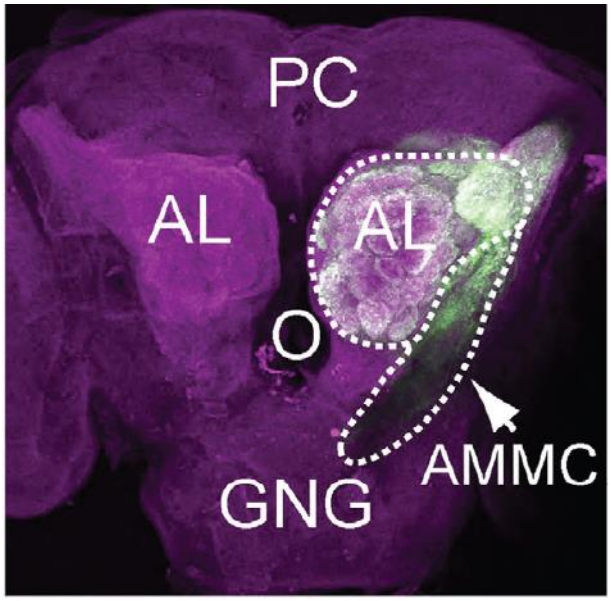

Figure 3. Confocal projection image showing the projection pattern of antennal sensory neurons in the antennal lobe (AL) and antennal mechanosensory and motor center (AMMC). Gnathal ganglion GNG, protocerebrum PC, oesophagus O. (Adopted from Zhao et al. 2016).

A substantial portion of the CO_2 sensitive neurons target the LPOG in the AL

Kent et al. provided, in 1986, the first evidence that the AL actually is the primary processing center for CO_2 information in the moth brain. Later, Guerenstein et al. confirmed this with electrophysiological studies from AL projection neurons (2004). Thus, the sensory cells of the CO_2 detecting LPO send their axons via the labial palp nerve to a large glomerulus in each AL named the labial pit organ glomerulus (LPOG; Kent et al., 1986). That is, the CO_2 responding neurons project bilaterally, unlike the olfactory sensory neurons on the antennae. The LPOG receives no input from the antennae, and therefore seems to be specialized for processing information about CO_2 (Bogner et al., 1986; Lee & Altner 1986).

Like the mammalian olfactory bulb, the AL is characterized by glomerular neuropil organization. The axon terminals of the numerous sensory neurons from the antennae and the labial palps converge and make synaptic contact with projection neurons in the glomeruli (Hildebrand & Shepherd, 1997). The number of glomeruli varies between different insect species and sex, but not between individuals. Work on males of the species *H. armigerea* has revealed that this species holds an AL consisting of ca. 78-80 glomeruli. This number is probably similar in other moth species (Zhao et al., 2016).

The AL of male moths consists of two parts, a collection of ordinary glomeruli and a few enlarged glomeruli called the macroglumerular complex (Homberg et al., 1989). The numerous ordinary glomeruli, which are analogous to those of the female moth, receive input from sensory neurons that respond to general odors, like plant odors (Mustaparta, 2002). The macroglomerular complex, on the other hand, is specific for males and receive information from pheromone sensitive olfactory sensory neurons. The male-specific glomeruli, often comprising 3-4 units, are located dorsally in the AL, by the entrance of the antennal nerve (Berg et al., 2014). These compartments are reported to process information from different pheromone components that elicit distinct behaviors (Berg, Almaas, Bjaalie, & Mustaparta, 1998). In addition to receiving input about pheromone substances released by the conspecific female, which induces attraction, distinct parts of the macroglomerular complex receive information about substances released by heterospecific females, inducing rejection (Berg et al., 2014; Zhao et al., 2014).

The AL projection neurons are responsible for carrying the olfactory information, including CO_2 information, to higher brain centers in the protocerebrum (Galizia and Rössler 2010; Homberg, Montague, & Hildebrand, 1988). A large number of olfactory sensory neurons synapse on a significantly smaller number of projection neurons, indicating a pronounced convergence, similarly to that found in the primary olfactory center of mammals (Hildebrand & Shepherd, 1997). The projection neurons can be described as either uniglomerular or multiglomerular, meaning that their dendrites either innervate only one or several glomeruli. The projection neurons carry olfactory information, including CO_2, via three main AL tracts to the lateral horn and the mushroom bodies (Homberg et al., 1988; Rø, Müller, & Mustaparta, 2007). The lateral horn is an area associated with innate behaviors (Gupta & Stopfer; Zhao et al., 2014; Tanaka, Awasaki, Shimada & Ito, 2004), while the mushroom bodies are considered to be the site for associative learning in the insect brain (Belle & Heisenberg, 1994; Fahrbach, 2006; Menzel & Muller, 1996). The CO_2 projection neurons in moths seem to include mainly uniglomerular neurons. The CO_2 pathway displays a more bilateral projection pattern than the olfactory, also at the second level of processing (Guerenstein et al., 2004; Moe Dahl, 2013). In the heliothine moth it is found that the CO_2 projection neurons project in two of the AL tracts, the lateral and medio-lateral tract, and target the lateral horn (Moe Dahl, 2013). In an older study on *M. sexta* they were reported to project in the medial tract (Guerenstein et al., 2004).

In addition to the sensory neurons and the projection neurons, the glomeruli in the AL are innervated by local interneurons and centrifugal neurons. The local interneurons are restricted

to the AL, where they provide connections between individual glomeruli (Christensen, Waldrop, Harrow & Hildebrand, 1993; Haupt et al., 2010; Martin et al., 2011; Rø et al., 2007). Multiglomerular local interneurons in the AL innervate the LPOG proving that some integration of antennal and palp information does occur (Galizia & Rössler, 2010). This is also underscored by the previously mentioned context dependent nature of CO_2 evoked behaviors, indicating that the CO_2 system can be modulated by neurons encoding other odors.

The centrifugal neurons, the third category of second order neuron, are relatively few in numbers. Their dendrites and somata usually reside in the multisensory protocerebrum (Homberg et al., 1989; Rø et al., 2007), which implies that they provide modulatory feedback to the AL. It is assumed that the centrifugal neurons affect the afferent signals in the AL, based on the putatively multimodal input being achieved in the protocerebrum (Galizia & Rössler; Zhao, Pfuhl, Surlykke & Berg, 2013b).

CO_2 sensitive neurons project to the GNG and the ventral nerve cord as well

The axons of CO_2 sensory neurons enter the brain at the level of the GNG. While the majority of the axons target the LPOG, some divide and send off processes to the GNG and ventral nerve cord (Zhao et al., 2013a). The GNG is made up of three pair of fused ganglia (labial, maxillar, mandibular) that control the mouthparts (Ito et al., 2014). Actually, the GNG is the most anterior ganglion of the ventral cord, and connects the ventral cord to the tritocerebrum of the brain (Yack & Homberg, 2003).

CO_2 sensory neurons are reported to leave the brain through the cervical connective and project directly to the ventral nerve cord. The ventral nerve cord is the insect equivalent of the human spinal cord, and it is made up of cervical connectives and ganglia (Figure 4). Axons of descending neurons and ascending neurons run in the cervical connectives. The ganglia contain cell bodies of both motoric neurons and ascending neurons. Nerve pairs arise from these ganglia and control muscles and sensory organs. The thorax contains the prothoracic and pterothoracic ganglion, also called the first and second thoracix ganglia (T1 & T2). The pterothoracic is composed of the fused meso- and metathoracic ganglion. There are one pair of legs on the prothorax, and two on the ptherothorax. In addition, there are several abdominal ganglia (Yack and Homberg, 2003).

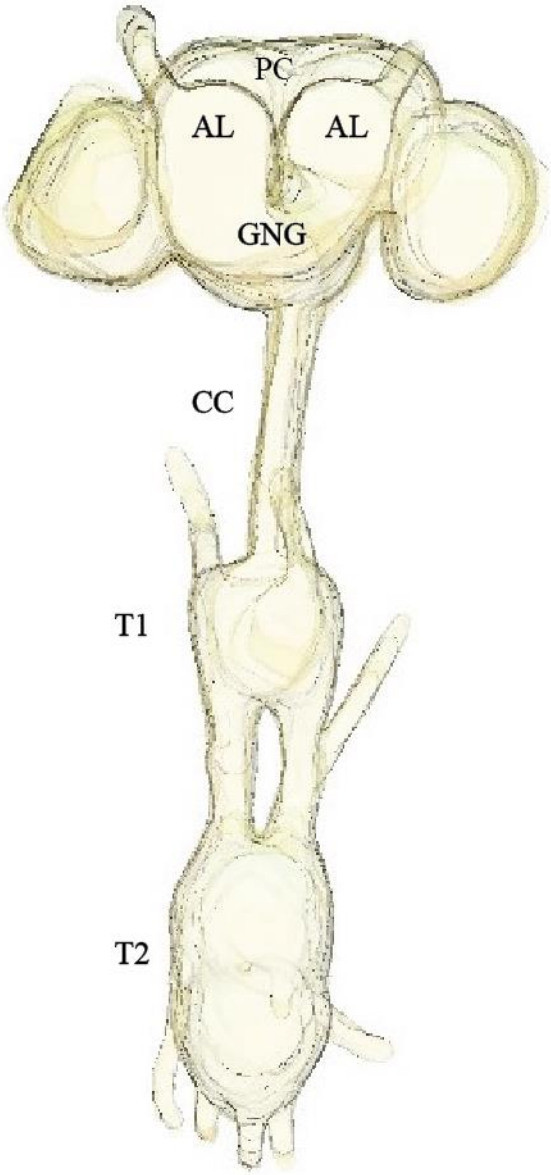

Figure 4. Reconstruction of the brain and thoracix ganglia of the ventral nerve cord in the moth *H. virescens*. Protocerebrum PC, antennal lobe AL, gnathal ganglion GNG, cervical connective (CC), first thoracic ganglion (T1), second thoracic ganglion (T2). (Adopted from Zhemchuzhnikov et al. 2014).

The current study

Little attention has been given to the CO_2 sensitive projections in the GNG and ventral cord. Generally, it is well-known that the sensory cells of the CO_2 detecting LPO send their axons to three main regions in the central nervous system of the moth: 1) a large glomerulus in both ALs named the LPOG, 2) the GNG, 3) and the ventral cord. The projection to the GNG has to some extent been visualized in previous articles (Kent et al., 1986; Lee and Altner, 1986). However, it is not known whether these terminal endings overlap with projections from taste and/or mechanosensory neurons from the antenna. Concerning the CO_2-projections descending to the ventral cord, there has been no knowledge about their distinct target region or innervation pattern.

Principal goal:

- To map the central projections of sensory neurons tuned to CO_2 in a functional neural system.

Specific goal:

- To perform anterograde staining of CO_2 sensitive neurons originating from the LPO combined with synapsin immunostaining in order to reveal new details about the total assembly of axonal projections in the central nervous system of the noctuid moth *H. armigera*.

- To specifically map the sensory axons' projection pattern in the subesophageal zone (SEZ) and the ventral nerve cord.

Materials and Methods

Insects

Males of the moth species *H. armigera* (Lepidoptera; Noctuidae, Heliothinae) were used in the study. The insects were bred in China, and arrived our lab as pupae. The pupae were kept in Plexiglas cages (18 x 12 x 17 cm). Upon hatching. the moths were transferred to Plexiglas cylinders installed with paper sheets to climb on, and a plastic cup with sucrose solution (10%) for feeding. The insects, in the cages and cylinders, were kept in climate chambers (Refitherem 6E and 200, Struers-Kebolab, Albertsund, Denmark) with a reversed light-dark cycle of 14 hours light and 10 hours dark. The chambers had a temperature of 23°C and 70% air humidity. To avoid space-related stress, a maximum of eight moths were kept in the biggest cylinders (12.5 x 19.5 cm) and a maximum of five in the smaller ones (9.2 x 19.5 cm). Once a week, the containers were cleaned, and paper and sucrose was renewed. Dead insects were removed continuously.

Anterograde tracing of the sensory neurons in the LPO

In order to map the central projections of the CO_2 sensory neurons, crystals of the fluorescent dye, micro-Ruby (tetramethylrhodamine dextran with biotin; Invitrogen, Eugene, OR, USA), was applied to the LPO. The dye was then carried, by means of axonal transport, to the terminal endings of the first order CO_2 sensitive neurons. The dye, which was stored in a freezer at -20°C, was kept in room temperature for a short while before the bottle was opened, ensuring that the crystals did not condensate. In addition, the bottle was kept under a black lid, to prevent deration of the fluorescent components.

Before the experiments, the moths were kept in the refrigerator for 5-15 minutes, to ensure that they were calm and sedated in time for the experimental procedure. Living moths were immobilized using wax (Kerr Corporation Romulus, MI, USA). With their ventral side facing up, the insect's wings were spread out and restrained by wax. In addition, the abdomen and the head were held in place by two strings of wax (Figure 5A). To provide access to the LPO, the tip of the third and outermost segment of one of the palps was cut off with micro-scissors (under a stereomicroscope, Leica M60). Micro-Ruby crystals were applied to the lesion by use of forceps. Before and in between several applications of dye, tap water was used to ensure uptake and dissolving of the crystals. This was repeated until the cut and the tissue around were colored pink (Figure 5B). After the experiments, the moths were kept in the fridge at 4°C for two nights, in a light impervious container to avoid bleaching. The

container was filled with wet paper to ensure humidity. This procedure allowed sufficient time for the dye to be transported in the nervous system of the insect, i.e. 40-56 hours.

Dissection, fixation and dehydration

The brain and ventral nerve cord were dissected as one unit under a stereomicroscope. The first part of the dissection was carried out while the moth was positioned the same way as during the staining procedure. Forceps and micro-scissors were used for dissection. First, the antennas, palps, and the proboscis were cut off. Then the thorax was opened and the second thoracic ganglion exposed. From here, the ventral cord was exposed all the way up to the GNG (Figure 5C). The pigmented layer of the compound eyes was removed. Then the cephalon (brain) and ventral cord were separated from the rest of the tissue and transferred to a dissection bowl, where cuticles, muscles, and trachea attached to the brain were removed. Ringer's solution (in mM: 150 NaCl, 2 CaCl2, 3 KCl, 10 TES buffer, 25 sucrose, pH 6.9) was applied to the neural tissue throughout the procedure to avoid dehydration.

After dissection the preparations were fixed in 4% paraformaldehyde (Roti, Histofix pH 7) either for 1 hour in room temperature or in the refrigerator overnight, i.e. 16-24 hours. This was done to stiffen and preserve the tissue. Then followed a dehydration of the preparations, by ascending ethanol series 50%, 70%, 90%, 96%, 100%, and 100%, 10 minutes each. After dehydrations, the brain/ventral cord preparation was embedded in methyl salicylate, ensuring a transparent preparation suitable for microscopy. Each preparation was embedded in methylsalicylate, on a punctured metal plate, in between two glass coverslips. A total of 46 moths were stained and dissected.

The preparations were checked for successful staining by means of a fluorescent light microscope (Leica DMC 4500), to decide which to scan in the confocal microscope. Those containing staining in relevant regions, i.e. at least one of the ALs, the GNG, and the ventral cord, were analyzed further in the confocal microscope.

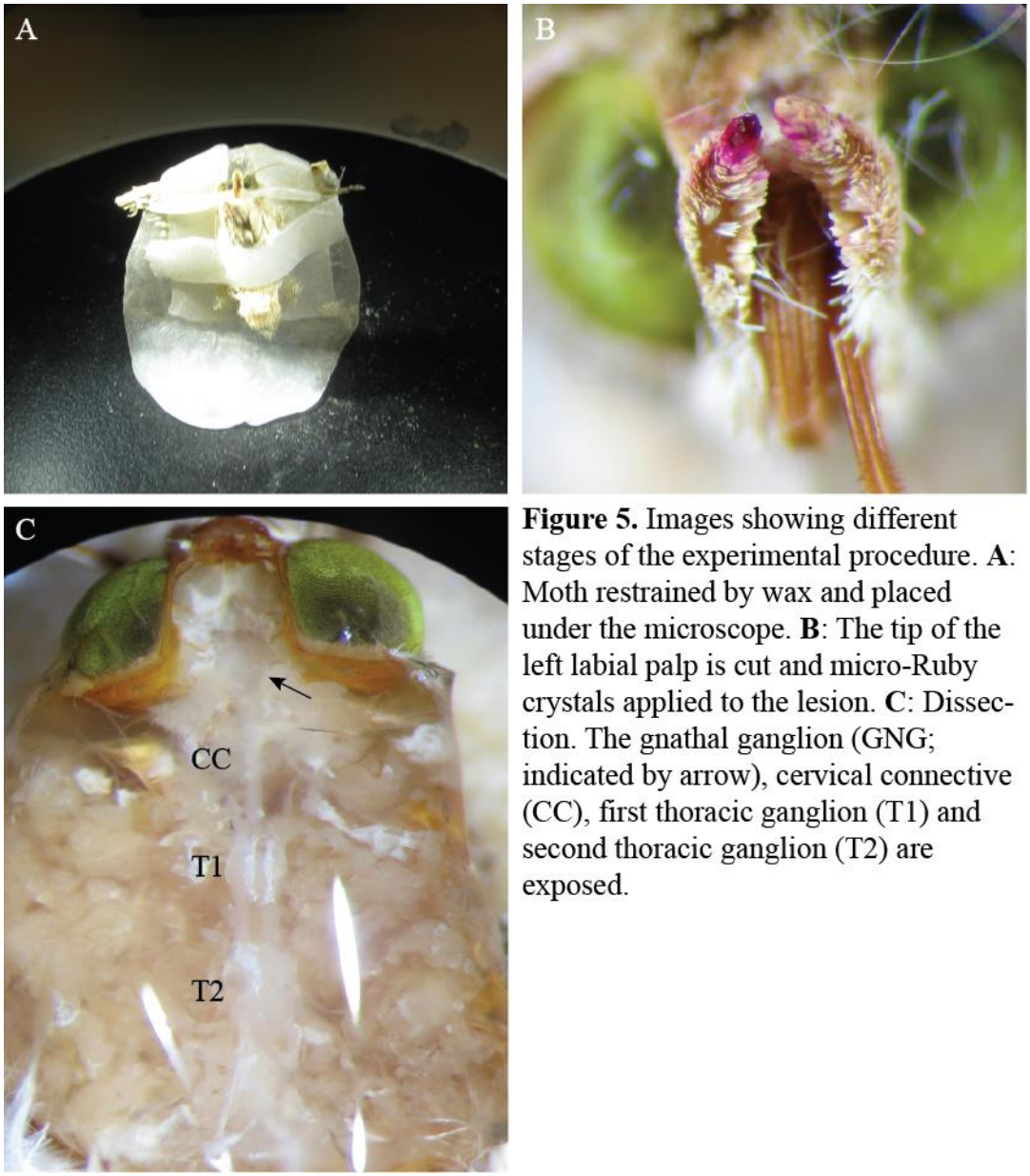

Figure 5. Images showing different stages of the experimental procedure. **A**: Moth restrained by wax and placed under the microscope. **B**: The tip of the left labial palp is cut and micro-Ruby crystals applied to the lesion. **C**: Dissection. The gnathal ganglion (GNG; indicated by arrow), cervical connective (CC), first thoracic ganglion (T1) and second thoracic ganglion (T2) are exposed.

Immunostaining

Immunostaining experiments were conducted on 18 of the 46 brain preparations labeled with micro-Ruby. This kind of double labeling allows visualization not only of anterogradely stained axons from the LPO, but of synaptic neuropil brain regions as well. It thus visualizes different sub-structures in the central nervous system being innervated by terminals of CO_2 sensitive axons. The immunostaining technique is based on an antibody named SYNORF1, binding specifically to a protein located in all presynaptic regions, i.e. synapsin (Klagges et a., 1996). Five of the 18 preparations that were used had already been dehydrated and scanned in the confocal prior to the immunostaining, and thus had to be rehydrated before the procedure could begin. This was done in a descending ethanol series

100%, 100%, 96%, 90%, 70%, and 50%, 10 minutes each. These preparations were selected because they were particularly well pre-stained.

After rehydration, the brains were rinsed in PBS (phosphate-buffered saline) for at least ten minutes. This was done to wash away the methyl salicylate. To prevent nonspecific binding of the antibody, the preparations were embedded in a blocking solution of 5% normal goat serum (NGS) in PBSX (PBS containing 0.5% Triton-X 100, pH 7.4), for 3 hours in room temperature. Following blocking, the preps were embedded in the primary antibody, anti-SYNORF1 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA), diluted (1:10) in PBSX containing 5% NGS for 5 days at 4°C. The preparations were then rinsed in PBS, 6 x 20 min at room temperature, before being embedded in the secondary antibody, Cy2-conjugated anti-mouse (Invitrogen, Eugene, OR, USA) (diluted 1:500 in PBSX), for 2-3 days at 4°C (fridge). This antibody binds to SYNORF1 and gives off fluorescent light when exited with a wavelength of 488 nm. Finally, the brains were dehydrated in an acending ethanol series, and embedded in methyl salicylate.

The rest of the preparations were immunostained immediately after fixation, following the same protocol as described above. Although this made it impossible to check for successful labeling before conducting the immunostaining, the risk of damage and deterioration on the tissue was minimized.

Confocal microscopy

The stained neurons in the brain and ventral cord were scanned via confocal microscopy (Zeiss LSM 800, Carl Zeiss Microscopy, GmbH, Jena, Germany), creating stacks of optical images. Different objectives were used depending on the area of interest and the quality of the labeling. A 10 x air (10x/0.30 Plan-Neofluar) and 10 x water (10x/0.45 W C-Apochromat) objective was used for obtaining an overview of the preparations. A 20 x air (Plan-Apochromat 20x/0.8) objective was used to reveal fine details of particularly interesting areas. Two different lasers were utilized to visualize the staining pattern in the double-labeled preparations. A 561 nm line of a helium neon laser was used to excite the micro-Ruby staining whereas the Cy2 immunostaining was excited by the 488-nm line of an argon laser. The image resolution was set to 1024 x 1024 pixels, with a pinhole size of 1 airy unit. The distance between each section was adjusted so it usually gave an overlap of 30-50%. The confocal laser-scanning microscopy was performed at the Department of Physics, NTNU. After microscopy, the samples were transferred to small plastic capsules filled with methyl salicylate to prevent them from drying out.

Data analysis and image processing

The serial optical images were analyzed by means of visual inspection in the ZEN software, which is developed specifically to process microscope images from the Zeiss confocal scanning microscope. Single optical sections and projection views were stored as JPG files, before the final figures were composed in Adobe Illustrator CS6 (Adobe Systems, San Jose, CA).

Ethical considerations

The use of Lepidopterans in research is not regulated under Norwegian law (Forskrift om bruk av dyr i forsøk, 2015). However, the utilization of animals for research purposes has been and continues to be a controversial subject. All life have an inherent value of their own, and humans are morally obliged to treat animals with respect. While the use of animals as model objects in research projects always will pose ethical concerns, there is no doubt that such an approach has been of fundamental importance for scientific progress, and thus human prosperity. The insects used in the current study were held in living conditions designed to ensure their wellbeing. Efforts were made to avoid inflicting unnecessary harm or stress upon the insects.

Nomenclature

The nomenclature suggested by Ito et al. (2014) is adopted. Here, the brain of the fruit fly, *D. melanogaster*, was utilized as a reference framework. Important to note in this thesis is the use of the terms “subesophageal zone” and “gnathal ganglion”, replacing the previously used subesophageal ganglion. The insect brain is made up of the protocerebrum, deutocerebrum, tritocerebrum and GNG (Ito et al., 2014), which are fused in moths (Haupt et al., 2010). Traditionally the three first have been referred to as the supraesophageal ganglion and the latter the subesophageal ganglion. Confusingly, subesophageal ganglion is used in the literature referring both to a specific neuromere, the GNG, and brain tissue below the esophagus in general. This is contradictory, as the location of the esophagus relative to the different neuromeres varies between insect species. In several species, including moths, the tritocerebrum and deutocerebrum also lie around and below the level of the esophagus. Therefore, it is more precise to use the term GNG than SOG, referring to a particular neuromere. Further, SEZ can be used to denote brain tissue below the level of the esophagus in general, based on an arbitrary boundary. In moths this term covers the GNG, tritocerebrum and parts of the deutocerebrum (Ito et al., 2014).

Results

In total, 46 insects were prepared. Of these, 21 were successfully stained and investigated in the confocal microscope. Most of the stained preparations showed labeling in three main regions of the central nervous system: 1) The LPOG in both ALs, 2) the SEZ, and 3) the ventral cord. The labeled axons of the CO_2 sensitive neurons entered the brain in the ventrolateral part of the GNG, via the labial palp nerve. Here, the axon bundle divided into numerous branches that projected to one of the three regions (Figure 6). The preparations being subject for additional synapsin immunostaining resulted in visualization of neuropil areas, making it possible to identify the target regions of the sensory afferents originating from the LPO.

Bilateral projections to the LPOG in each AL

The main target area of the CO_2 sensitive neurons was one large glomerulus in each AL, positioned most ventrally, medially to the lateral cell cluster, i.e. the LPOG. Two thick fiber bundles extending dorsally from the labial palp nerve innervated the ventrally located glomerulus in both ALs (Figure 7). The staining in the ipsilateral LPOG was consistently more prominent than in the contralateral one. In each AL, the entire glomerulus was densely innervated by the sensory axons (Figure 8).

Soon after entering the ventral part of the GNG, the labial nerve split up into numerous sub-branches (Figure 13). Two prominent sub-branches, forming a V-shaped structure in this region, targeted each of the two LPOGs. The ipsilateral branch was more prominent than the contralateral one. Both branches projected dorsally along the midline of the GNG, before turning slightly laterally when entering the prow (PRW; Figure 6). Each of these two sub-branches consisted of two fiber bundles, one thicker than the other. Ventral to the esophagus a small assembly of fibers created a transverse connection between these two sub-tracts, forming a U-shape (Figure 9). In addition, at least three other fiber bundles originating from the labial nerve were found to target the LPOGs (Figure 10). Two of these relatively thin bundles targeted the ipsilateral LPOG selectively, whereas one targeted the contralateral.

Innervation pattern in the SEZ

The ipsilateral GNG was the main target area of the sensory neurons in the SEZ. A dense network of terminal branches was identified along the midline of the GNG, close to the main ramification point of the labial-nerve axons. The main portion of stained processes terminating in the GNG targeted a region located relatively anteriorly. These terminals were

positioned alongside the ipsilateral tract targeting the LPOG, although more posteriorly and laterally (Figure 11). A few processes crossed the midline and terminated in the ventral part of the contralateral GNG. In addition to the innervations in the GNG, a few axon terminals targeted targeted a relatively wide region located posteriorly and ventrally to the ipsilateral AL, including the AMMC (Figure 12).

Generally, the complex projection pattern of these terminal branches made it difficult to identify their origin (Figure 13). However, most of them seemed to arise from the main branching point of the labial nerve ventrally in the GNG. A few splitting off from the tracts targeting the ALs could be seen as well.

Projection pattern in the ventral cord

An assembly of axons splitting off from the main branching point of the labial nerve left the brain through the ipsilateral cervical connective and terminated in the thoracic ganglia. Most of these axons targeted the prethoracic ganglion, named T1. Here, the terminals innervated a relatively large area located relatively ventrally, in the anterior and lateral region of the ipsilateral ganglion, nearby the nerve roots, IN1 and IN2 (Figure 15). A thin fiber was observed to cross the midline of the GNG before descending towards the ventral cord (Figure 16). Additionally, a separate projection was observed in the cervical connective, however, targeting the same area of T1 as the main descending branch (Figure 15). In two preparations, a couple of stained fibers projecting all the way down to the metathoracic ganglion, named T2, could be observed - still forming an ipsilateral labeling pattern (Figure 14). No axons were found to project towards the abdominal ganglion.

Figures

In the following section confocal images from six preparations are presented.

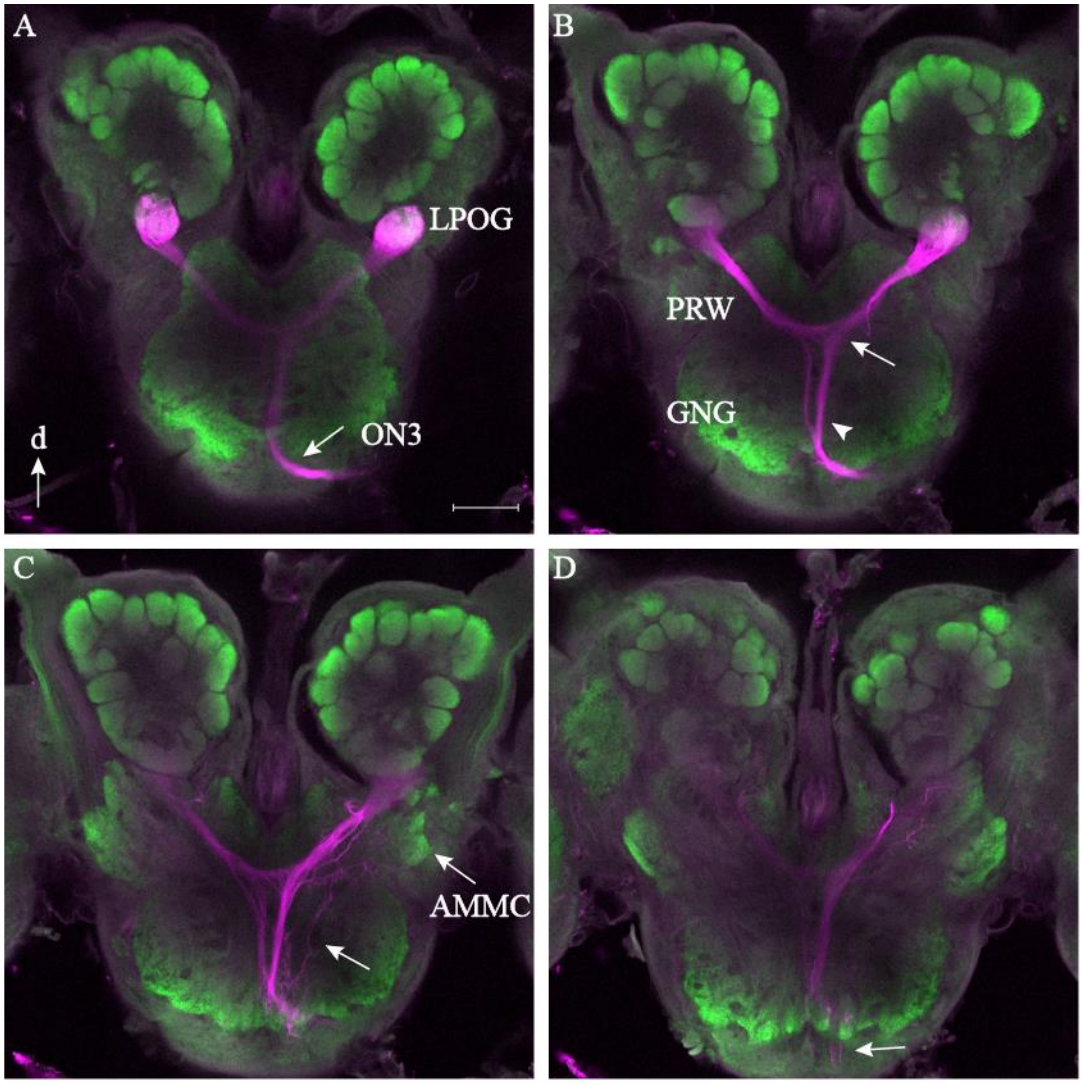

Figure 6. Schematic overview of the labeled axons of CO2-sensitive neurons originating from the labial pit organ (LPO). Single-slice confocal images of preparation no. 34 (from anterior to posterior). **A:** The labial palp nerve (ON3) enters the gnathal ganglion (GNG). **B:** The axons split up and form a V-shape in the ventral part of the GNG (arrowhead), before they pass through the prow (PRW) forming a U-shape in this region (arrow). **C:** Innervations in the GNG and antennal mechanosensory and motor center (AMMC) are indicated by arrows. **D:** Axons leave the brain through the cervical connective, indicated by arrow. 10x objective. Labial pit organ glomerulus (LPOG), Dorsal d. Scale bar = 100 µm

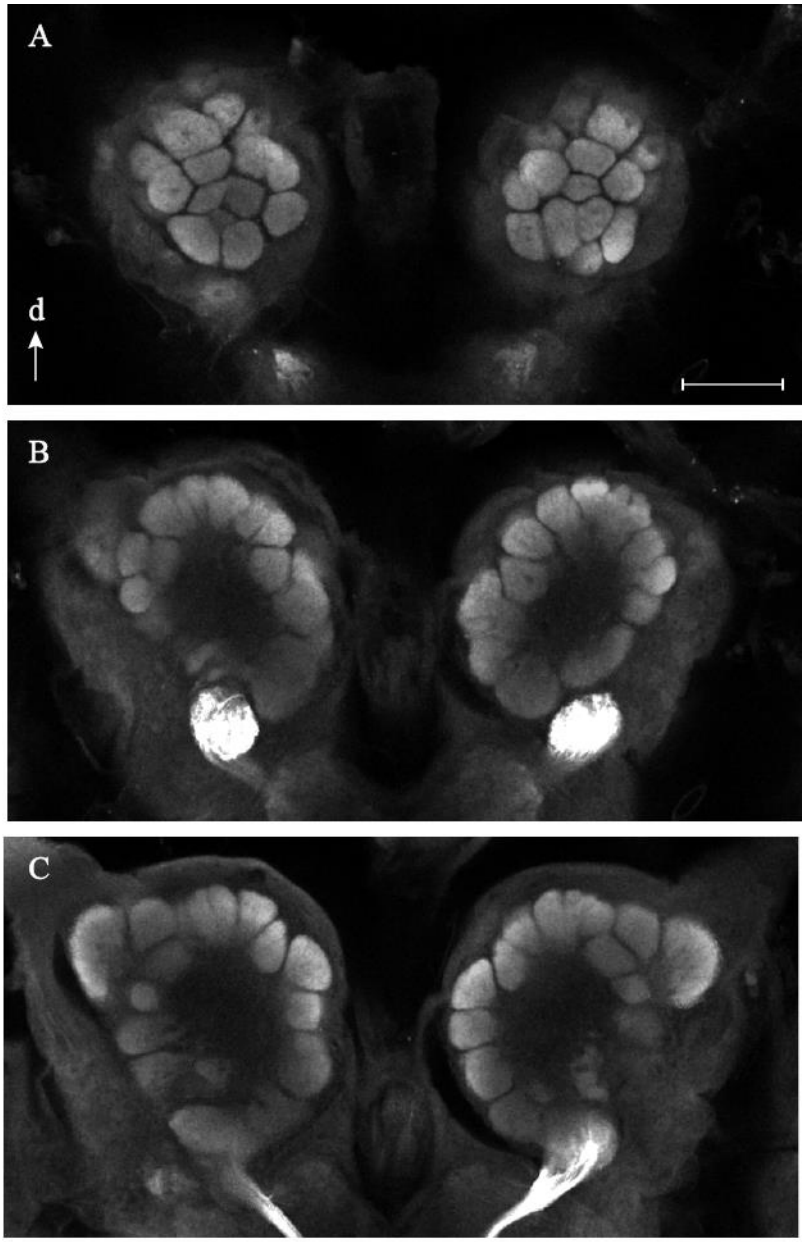

Figure 7. Axons from the labial pit organ forming bilateral projections to the labial pit organ glomerulus (LPOG) in both antennal lobes (ALs). Single-slice confocal images of brain preparation no. 34 (from anterior to posterior). **A-C:** Sections demonstrating the location of the LPOG most ventrally in the AL. Dorsal d. Scale bar = 100 µm

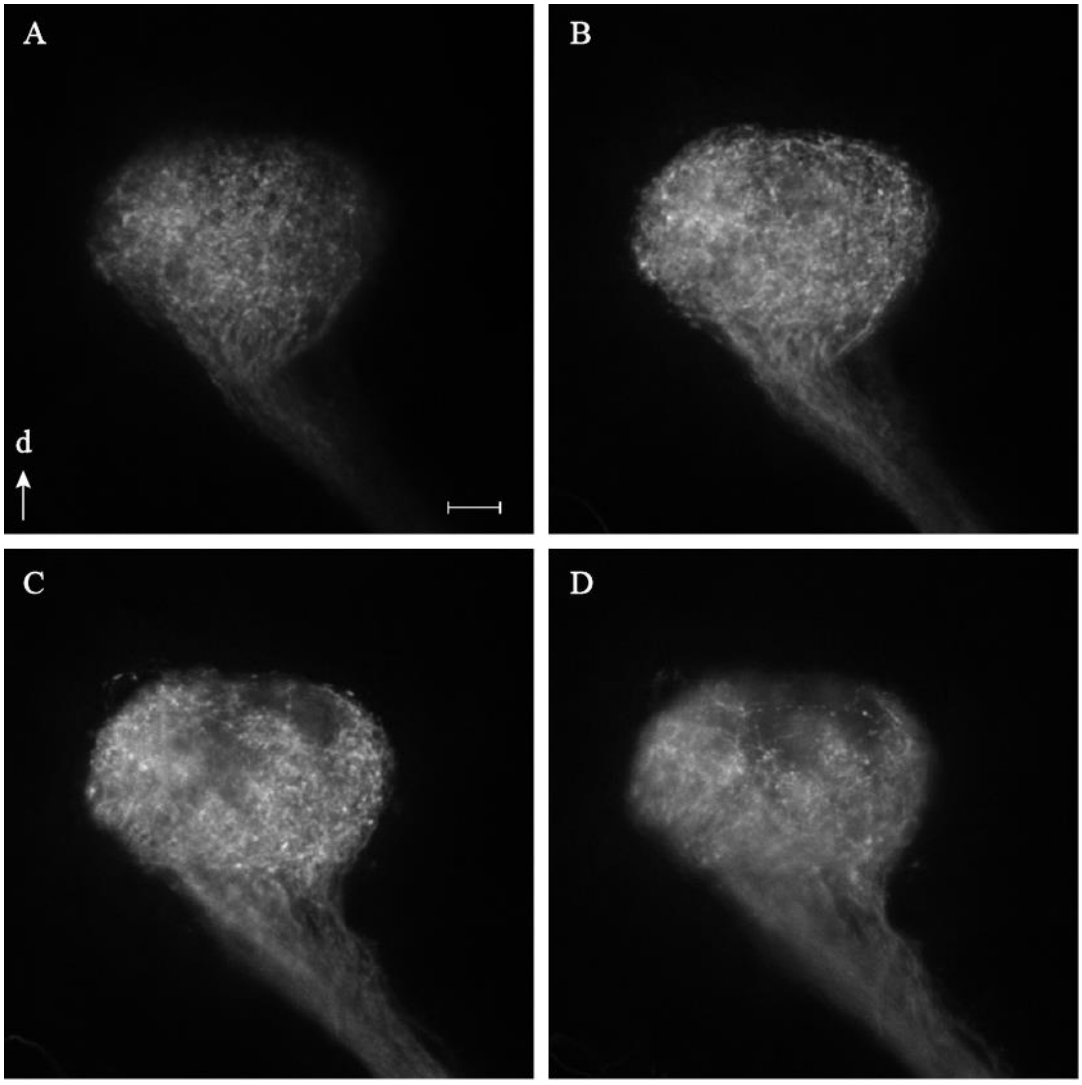

Figure 8. Innervation pattern of the labial pit organ glomerulus (LPOG). Single-slice confocal images of brain preparation no. 14 (from anterior to posterior). **A-D:** Sections demonstrating that the whole glomerulus is innervated by the axons of CO2-sensitive neurons. Dorsal d. Scale bar = 20 µm

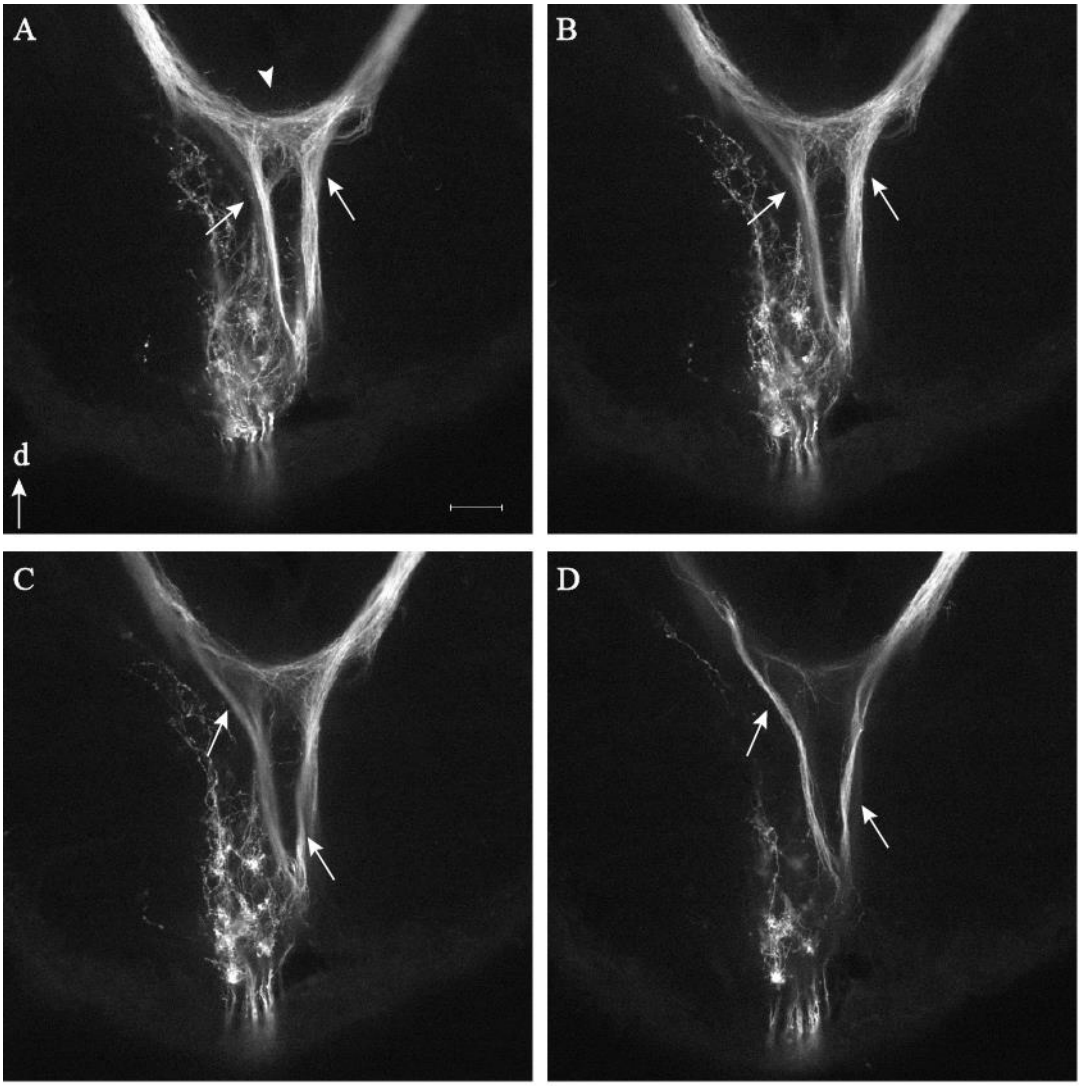

Figure 9. Close-up images from the subesophageal zone showing details of the two main branches that target the labial pit organ glomerulus (LPOG) in each antennal lobe (AL). As indicated by the arrows, each branch consists of two sub-bundles. Three additional fiber bundles are shown in figure 10. Single-slice confocal images of brain preparation no. 6 (from anterior to posterior). **A-B:** In each branch, the thickest and most anterior fiber bundles are shown. Fibers connecting these bundles form a U-shape (arrowhead). **C:** Two thinner and more posterior bundles emerge. **D:** The two thinner bundles are completely visible. 20x objective. Dorsal d. Scale bar = 50 µm

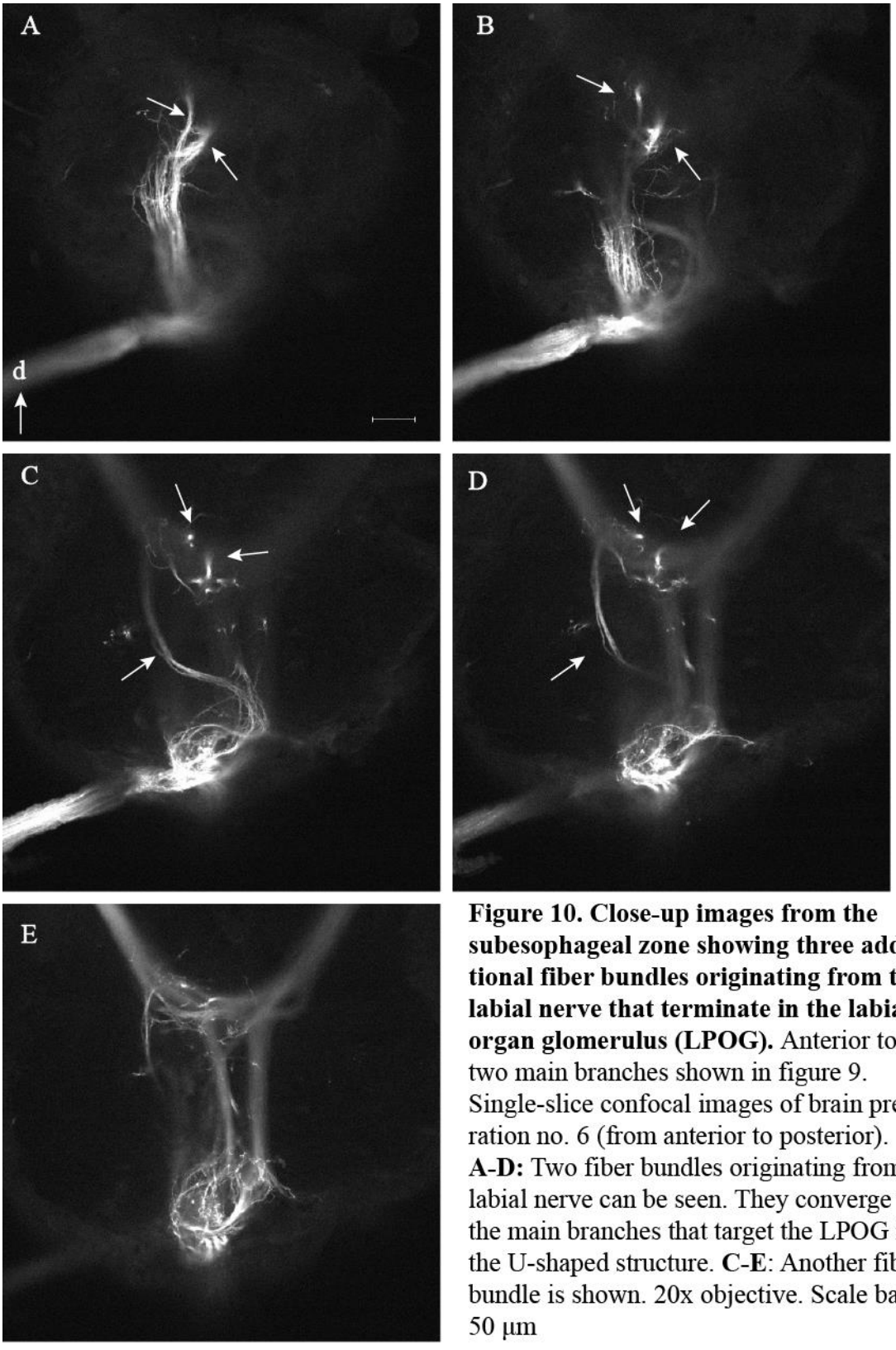

Figure 10. Close-up images from the subesophageal zone showing three additional fiber bundles originating from the labial nerve that terminate in the labial pit organ glomerulus (LPOG). Anterior to the two main branches shown in figure 9. Single-slice confocal images of brain preparation no. 6 (from anterior to posterior). **A-D:** Two fiber bundles originating from the labial nerve can be seen. They converge with the main branches that target the LPOG in the U-shaped structure. **C-E**: Another fiber bundle is shown. 20x objective. Scale bar = 50 µm

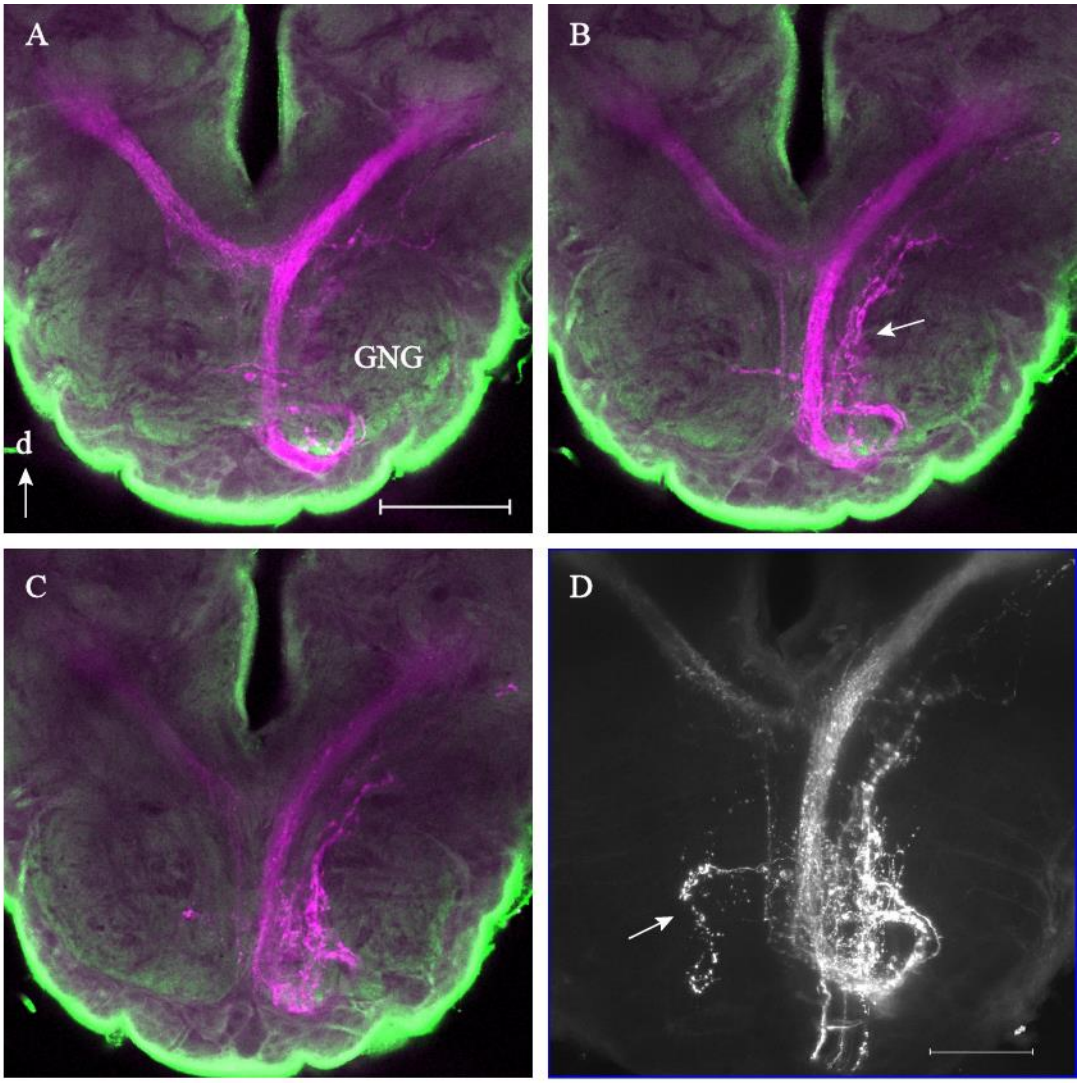

Figure 11. Overview of axon terminals in the gnathal ganglion (GNG). A-C: The main portion of processes are positioned posteriorly and laterally to the tract that target the ipsilateral labial pit organ glomerulus (LPOG). Single-slice confocal images of preparation no. 19 (from anterior to posterior). 10x objective. **D:** A few fibers cross the midline and terminate in the ventral part of the contralateral GN). Projection image of preparation no. 19. 20x objective. Dorsal d. Scale bar = 100 µm

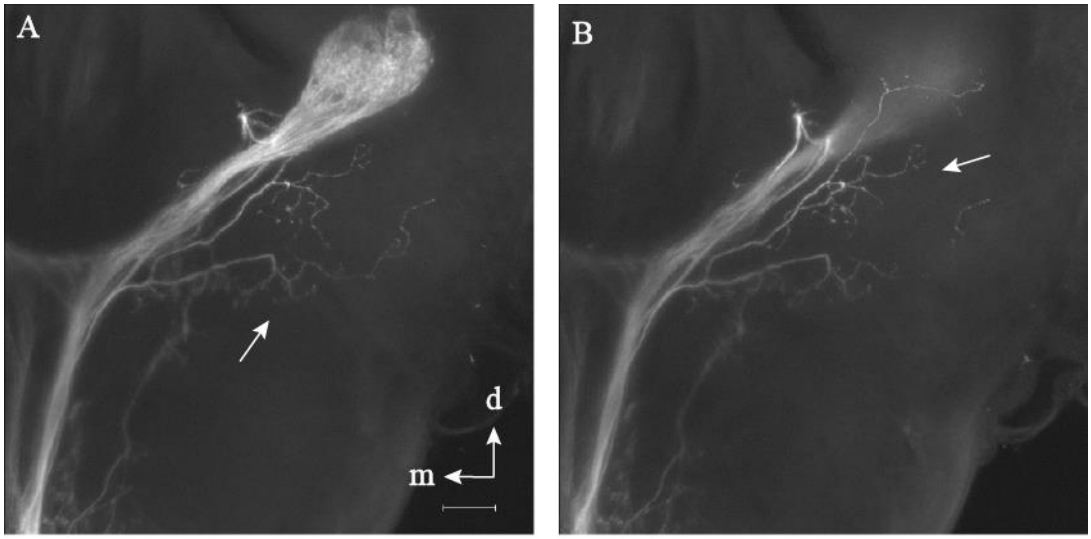

Figure 12. Innervation pattern of CO2 sensory neurons in the antennal mechanosensory and motor center (AMMC; arrows). A-B: Single-slice confocal images of brain preparation no. 34 (from anterior to posterior). 10x objective. Dorsal d, Medial m. Scale bar = 50 µm

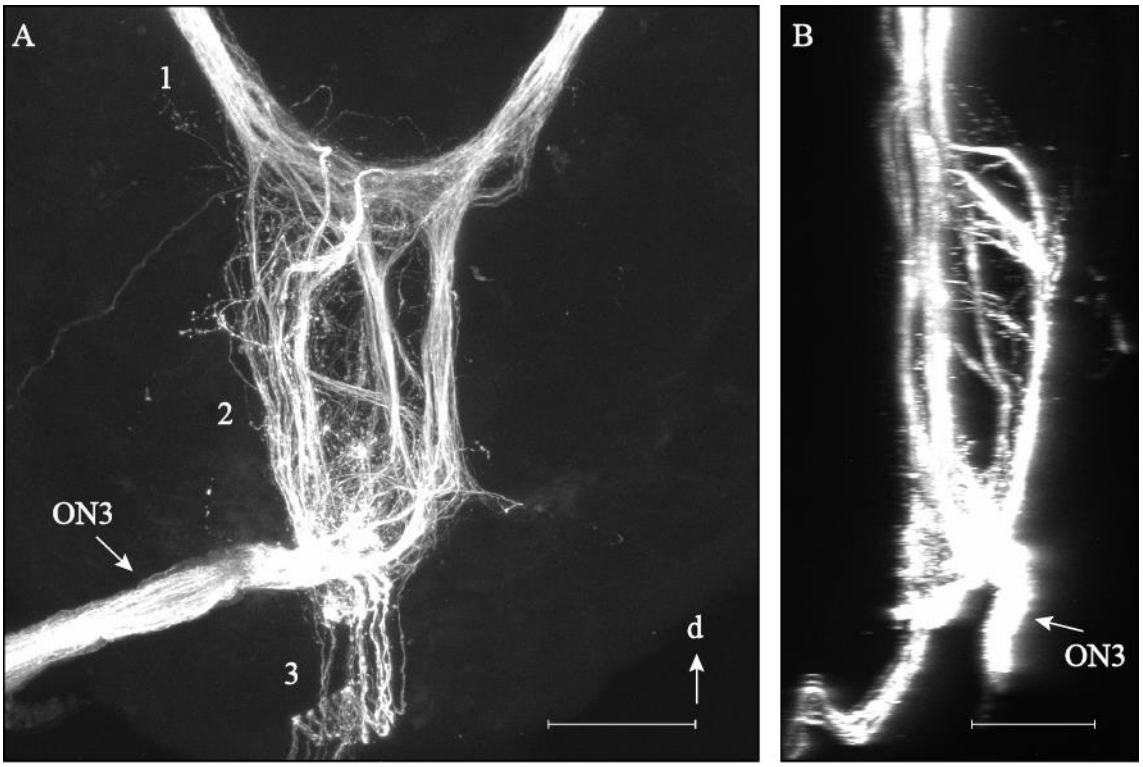

Figure 13. Overview of the complex network in the subesophageal zone (SEZ). The labial nerve (ON3) split up and project to three main areas: 1) the antennal lobes, 2) the SEZ, and 3) the ventral nerve cord. Confocal projection images of preparation no. 6. **A:** Frontal view. **B:** Sagittal view. Dorsal d. Scale bars = 100 µm

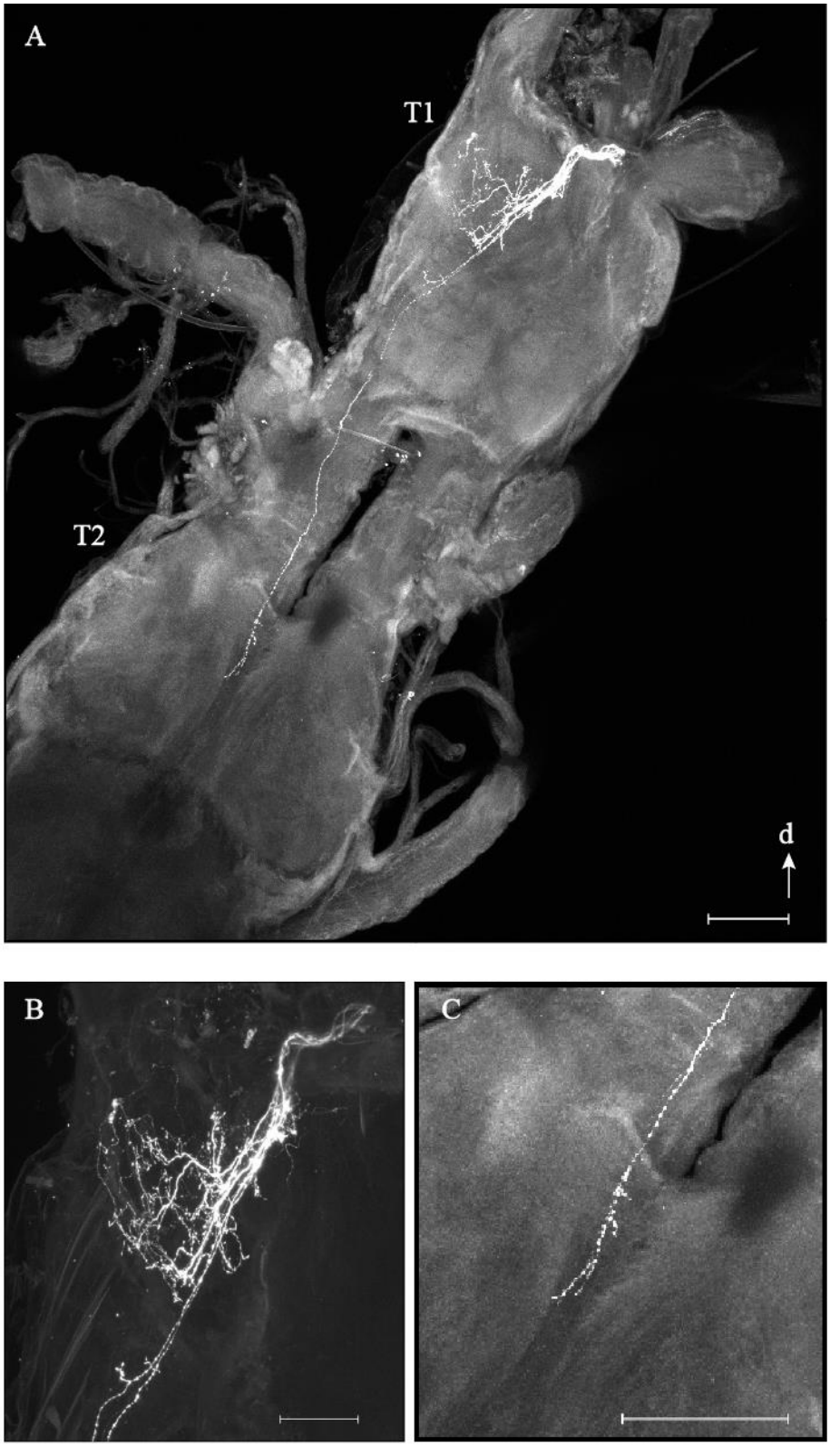

Figure 14. Labeling in the thoracic ganglia of the ventral nerve cord. Projection images of preparation no. 6. **A:** Overview of the relatively extensive labeling in the first thoracic ganglion (T1) and the modest labeling in the second thoracic ganglion (T2). 10x objective. **B:** Innervation pattern in T1. 20x objective. **C:** A few fibers terminate in T2. 10x objective. Dorsal d. Scale bars = 100 µm

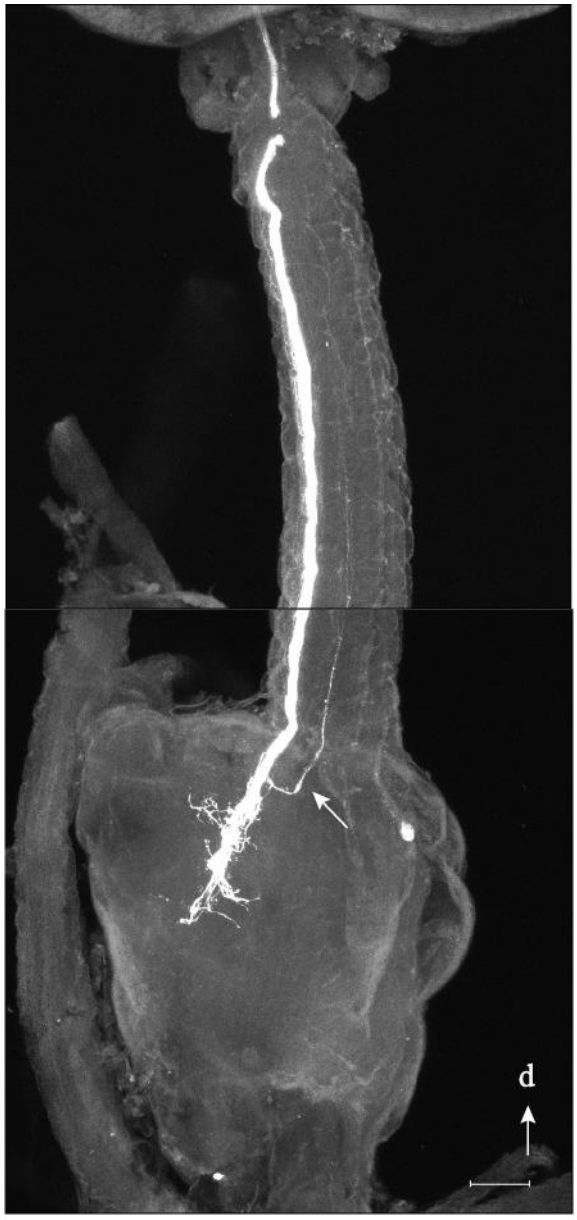

Figure 15. Overview of the labeling in the cervical connective and first thoracic ganglion. Projection images of preparation no. 27. The arrow indicates a possible contralateral projection, which terminate in the ipsilateral part of the ganglion, together with the other axons. 10x objective. Dorsal d. Scale bar = 100 µm

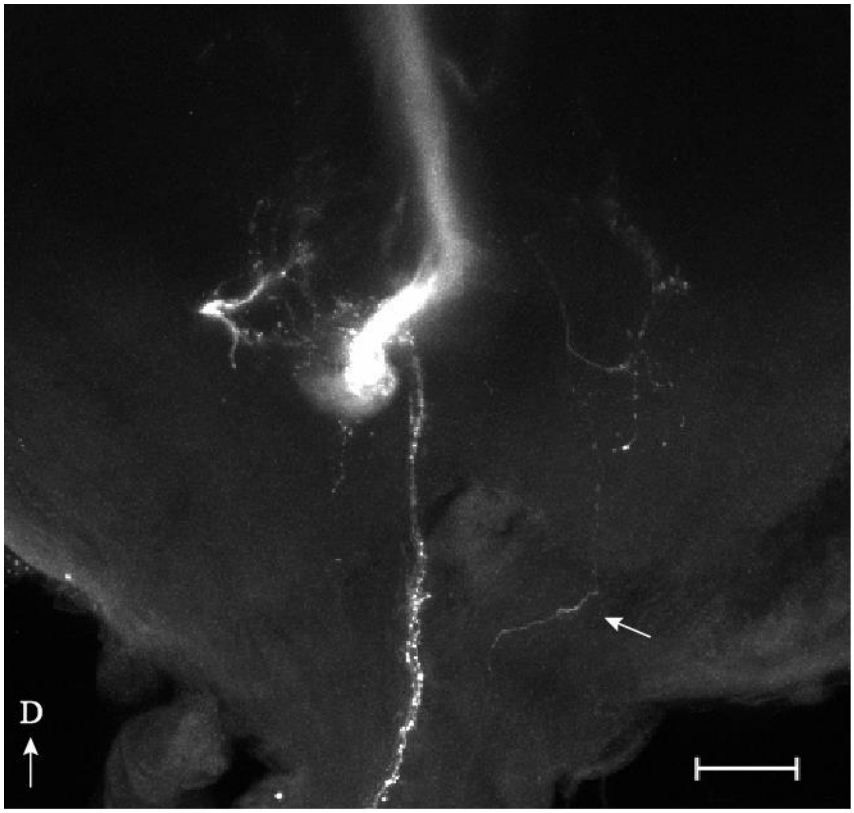

Figure 16. Close-up image of the ventral region of the gnathal ganglion showing one descending fiber projecting on the contralateral side (arrow). Confocal projection image of preparation no. 9. 20x objective. Dorsal d. Scale bar = 50 µm

Discussion

Summary of results

Descriptive anatomical studies of neural architecture are crucial for understanding the function of any neural system. The results presented here contribute to new insight in the anatomy of a particular olfactory sub-system in the heliothine moth, i.e. the CO_2 pathway. In spite of being a highly relevant olfactory cue for many insect species, this part of the chemosensory system has not been investigated at the same level as for example that devoted to pheromones. In the present study the central projections of sensory neurons tuned to CO_2 in the noctuid moth *H. armigera* were mapped by means of anterograde staining from the LPO combined with synapsin immunostaining of relevant neuropil regions. The successfully stained preparations being scanned via confocal microscopy gave rise to high-resolution images exploring the detailed projection pattern of the axon terminals. In full agreement with previous findings, both in *H. armigera* (Zhao et al., 2013a) and several other moth species (Kent et al., 1986; Lee & Altner 1986), the CO_2 sensitive neurons originating from the LPO projected to 1) the LPOG in each AL, 2) the SEZ, and 3) the ventral nerve cord. In the present study, new findings were reveled at all three levels. In particular, the present study is the first to map the distinct target regions of CO_2 sensory projections in the nerve cord. The existence of projections to the nerve cord was mentioned in previous literature but their actual innervation patterns were never investigated before.

Complex bilateral projections from the LPO to the AL

The AL is the main projection area of the CO_2 sensory neurons

As previously reported, the sensory neurons originating in the labial palps project to one large glomerulus in each AL, the LPOG (Kent et al., 1986). The fact that the main projection area of the CO_2 sensitive neurons is one AL glomerulus is still considered to be a noteworthy arrangement, as one might assume the these sensory axons should terminate in the labial neuromere of the GNG (Guerenstein et al., 2004). However, the direct connection with the AL allows for integration, via AL local interneurons, of CO_2 information with other olfactory signals already at the first processing level. The local interneurons are believed to be central in coupling, for example, the pheromone and the general olfactory system. Thus, a significant group of global multiglomerular local interneurons arborizing in both the macroglomerular complex and ordinary glomeruli has been found (Seki & Kanzaki, 2008). Possibly, this early integration based on input from distinct olfactory sub-systems is necessary for quick behavioral responses. For instance, Ian, Zhao, Lande, and Berg (2016) identified a

multiglomerular projection neuron that innervated one of the male specific pheromone glomeruli, one ordinary glomerulus and the LPOG. Perhaps this type of AL neuron helps the male to find a potential mate – a female, possibly sitting on a plant. In addition, the AL is established as a site of associative learning, independent of the mushroom bodies (Hammer & Menzel, 1998). Interestingly, plasticity of CO_2 responses has been found in *M. sexta* (Thom et al., 2004). Here, it was reported that the preference for plants that give off high levels of CO_2 (a cue for nectar) would disappear when no reward was given. It is reasonable to speculate that this learning might happen in the AL.

Putative function of the bilateral projections in the AL

Unlike olfactory sensory neurons on the antennae, the CO_2 sensory neurons from the labial palp project bilaterally already at the first level of processing. The functional significance of this arrangement might be related to the unique properties of the CO_2 sensitive neurons and a possible behavioral role of the stimulus. Most odors are made up of several compounds, released as plumes from a source. These plumes are extremely irregular, constantly changing with the movement of the air (Ache & Young, 2005). When insects try to find the source of an odor they fly in zig-zag, constantly striving to follow and stay within the odor plume. CO_2 is a curious odor cue as it is ever-present. The sensory neurons in the LPO allow for continuous monitoring of the ambient level of CO_2, while they at the same time are extremely sensitive to small differences in local gradients. Possibly, the CO_2 pathways of moths are able to measure the concentration gradient of CO_2 directly. Therefore, CO_2 might be an olfactory cue that is better suited for orientation than plant odors, which are brief plumes of intermixed scents.

Innervations in the SEZ

The sensory neurons from the LPO innervated two areas in the SEZ: the GNG and the AMMC. The GNG consist of the mandibular, maxillary, and labial neuromeres. The projections to this region might give input to motor neurons that control the mouthparts (the palps and the proboscis). Also, it is not unlikely that they are connected with some of the gustatory sensory neurons. Jørgensen et al. (2006) mapped the gustatory projections to the SEZ, including both those originating from the antennae and the proboscis. It might be expected that the innervations of CO_2 neurons in the GNG would overlap with this previously described gustatory center in the SEZ. Yet, this seems not to be the case as the CO_2 sensory neurons terminated more ventrally in the GNG than the gustatory projections. However, one

gustatory fiber from the antennae, found to project to the ventral GNG (Jørgensen et al., 2006), may have processes overlapping with axons terminals from the LPO. Based on previous studies on the role of CO_2 for nectar-feeding in *M. sexta*, this is not unlikely (Thom et al., 2004). Generally, stimulation of GRs on legs and antennae elicits the proboscis extension reflex (PER; Jørgensen et al., 2006; Zhang, van Loon & Wang, 2010). Actually, it would be interesting to investigate whether CO_2 has any effect on the PER.

Some of the stained axons from the LPO terminated in the AMMC. To the knowledge of the researcher, this has never been reported previously. The AMMC is an innervation area of mainly mechano sensitive antennal neurons, as well as motor neuron dendrites. Similar to the stained processes in the GNG these projections are likely connected to either motor neurons that control muscles in the antennae, and/or they provide information being integrated with sensory input from the antennae. Moths use the tip of their antennae, which is covered by both olfactory receptors and GRs, to investigate interesting substrates (Jørgensen et al. 2006). It is reasonable to believe that CO_2 cues can initiate exploration of a surface, by activating motor neurons in the antennae. Following this line of thought, CO_2 cues may be used as an approximate map of the surroundings to guide the moth towards desirable items, while the antennae and the general olfactory system give more specific information on what the moth encounters. As previously mentioned, mechano sensitive neurons on the moth antenna are involved in regulating flight stability (Sane et al., 2007). The possibility of an overlap between these neurons and CO_2 neurons in the AMMC strengthens the previous suggestion that CO_2 plays a role in orientation.

CO_2 sensory terminals in the first and second thoracic ganglion

In accordance with the objectives of the study, the previously unknown innervation pattern of CO_2-sensory axons in the ventral nerve cord was investigated. Most of the descending axons terminated ipsilaterally in the first thoracic ganglion. Here, the terminals formed an extensive branching pattern close to two nerve roots: 1N1 and 1N2. These nerves control muscles and sensory organs in the head and prothorax, including the forelegs, and carry sensory input from these sites (Yack & Homberg, 2003). Actually, both gustatory and mechano receptors are located on the tarsi of the moth legs (Zhang et al., 2010). Altogether, such a network may suggest that the CO_2 information that projects to the ventral nerve cord is integrated with mechanosensory and gustatory input from, for example, the forelegs. This kind of integrated signals may, in its turn, be transferred to the motoric system, eliciting walking behavior, for instance.

The population of CO_2 neurons comprise morphologically different types

Nobody has so far labeled one individual LPO neurons. We can therefore not say whether each neuron target all three main target regions or whether there are three main neuron types, each innervating one region. The results presented here, however, demonstrate that not all CO_2 sensory neurons innervating the same region, the LPOGs for instance share the same projection pattern. The numerous sub-branches forming the two thick bundles targeting these glomeruli indicate the presence of several neuron categories. In addition, the fact that the ipsilateral bundle is thicker than the contralateral suggests that there are morphologically distinct types of CO_2 neurons. Interestingly, three CO_2 GR genes were recently identified in *H. armigera* (Xu & Anderson, 2015). According to the 'one receptor one neuron' rule, this means that there are at least three CO_2 neuron types. In addition, two morphologically different sensillum types have previously been identified in the LPO of *H. armigera* (Zhao et al., 2013a). The morphologies of the sensory neurons housed in each of the two sensillum types, including target regions, may also be distinctive.

Together, this suggests that distinct sub-types of CO_2 sensitive neurons exist. Distinct categories based on projection pattern suggest a functional segregation of the CO_2 first order neurons. Tracing of single sensory neuron tuned to CO_2 is therefore an important task for further investigation.

Valence and modulation of the CO_2 olfactory cue

The behavioral role of CO_2 in moths is still poorly investigated, and what is known is based on a few studies. However, the neural space dedicated to CO_2 processing in the central nervous system of the moth proves that it is of particularly strong biological value (Ache & Young, 2005). Anatomically, it constitutes a complex system, distinct from – but at the same time closely connected to the olfactory system. In moths, CO_2 has been suggested to play a role in feeding and oviposition, both concerning their interactions with plants. This makes it likely that CO_2 information is highly integrated with both olfactory and gustatory sensory information.

Actually, CO_2 is a sensory cue that might not be very valuable on its own. This small molecule is a much more unspecific olfactory stimulus than say conspecific pheromone blends, therefore the CO_2 system might need to be more globally integrated with those of other senses. This kind of integration would allow for modulation of behavior (McMeinman, Corfas, Matthews, Ritchie, & Vosshall, 2014), which enables the previously mentioned

context dependent responses to CO_2 observed in *D. melanogaster* (Turner and Ray, 2009). The results presented here, demonstrate that CO_2 sensory neurons project to three main sites, each of which may be a region where CO_2 input is integrated with mainly olfactory, gustatory, and mechanosensory input, originating from the antennae, proboscis, and legs. In addition, CO_2 olfactory cues might be involved in motoric control of these sensory organs. In other words, it is likely that CO_2 olfactory cues participate in neural networks that facilitate multisensory integration and guide appropriate behavior.

Methodological considerations

The preparations obtained from the mass staining experiments showed considerable variability in their projection pattern, which implies that partly different populations of neurons were stained in each trial. This is most likely caused by deviations in the experimental procedure. Unfortunately, it is not possible to control that the lesion of the labial palp is in the exact same position in every experiment, especially not when the procedure is conducted manually. This uncertainty is a general disadvantage of the mass staining technique and means that each stained preparation will vary slightly from the others, as it is not possible to trace exactly the same neurons.

Nevertheless, the nervous system of insects is organized in a stereotypical manner, with little variation across individuals. Therefore, what is observed in one sample can be generalized to the species. Here, the most successfully stained preparations were compared to each other and used as templates for the analysis, as they hold the greatest amount of information. One can assume that the projection patterns observed in these preparations apply to other samples, but that it was not possible to observe because the staining was insufficient. Mass staining is an excellent approach for gaining an overview of the anatomy of a neuronal network. Combined with tracing of individual neurons, this method will provide detailed knowledge about the neural architecture characterizing any functional system. The moth brain is particularly well suited for neuroanatomical studies since it is small enough to be scanned as a whole in the confocal microscope. As demonstrated in the figures presented here, mass staining combined with confocal microscopy can give high quality images with excellent resolution.

Also, insects are exceptional good models to study the close connection between structure and function in the nervous system. Neurons are the basic functional units of the nervous system, and their individual structure and connectivity patterns are intimately linked to their function. This two-way relationship between structure and function is central to any

form of evolution and development; structures arise partly to solve a problem (function) and partly by coincidence, and new functions can arise for the structures that already exists. In the context of the current study, the description of the anatomy of the CO_2 pathways is useful as it indicates how this kind of information is processed in the moth nervous system, and ultimately the behavioral relevance of CO_2 for the animal.

As described in the materials and methods section, the immunostaining experiments were conducted in two different ways: 1) directly after dissection and fixation of the preparations and 2) after rehydration of previously scanned preparations. There seemed to be no difference in the staining results. However, the first procedure was preferred as it saved time and minimized risk of damage to the tissue. This form for double staining proved to be successful, and in general, mass staining in combination with immunostaining makes it possible to map specific pathways more exactly, as it permits visualization of central nerve structures. Here for instance, it allowed for better visualization of the LPOG in the AL (see Figure 7).

Conclusions

In the current thesis new details about the total assembly of axonal projections of the CO_2 pathways in the central nervous system of the noctuid moth *H. armigera* are presented and discussed. Based on this work several interesting conclusions can be drawn, as summarized below:

- The sensory neurons tuned to CO_2 in the noctuid moth *H. armigera* have axonal projections in three main regions of the central nervous system: 1) The LPOG of both ALs, 2) the SEZ, and 3) the ventral nerve cord.
- The LPOG of the AL is the main projection area of sensory neurons originating from the LPO. Unlike the olfactory sensory neurons from the antennae, the CO_2 neurons project bilaterally to the AL.
- The detailed projection pattern of CO_2 neurons in the SEZ, described for the first time, showed a dense network of thin terminals targeting the ipsilateral region of the GNG, close to the midline, plus a few processes ending in the AMMC.
- The projection pattern in the ventral nerve cord, described for the first time, demonstrated relatively extensive projections in the ipsilateral part of the first thoracic ganglion. In addition, a few axons innervated the second thoracic ganglion.
- CO_2 sensitive neurons from the LPO terminate close to the antennae, proboscis, and forelegs - areas of the central nervous system associated with olfactory, gustatory, and mechanosensory input.
- The results presented here indicate that CO_2 plays a significant role in the moth's life
- Further studies are necessary to reveal the morphology and physiology of individual neurons tuned to CO_2, and to explore how CO_2 information is integrated with olfactory cues and other sensory modalities.

Abbreviations

AL	Antennal lobe
AMMC	Antennal mechanosensory and motor center
CO_2	Carbon dioxide
GNG	Gnathal ganglion
GR	Gustatory receptor
LPOG	Labial pit organ glomerulus
LPO	Labial pit organ
ON3	Labial palp nerve
Prow	PRW
SEZ	Subesophageal zone
T1	First thoracic ganglion (prothoracic)
T2	Second thoracic ganglion (pterothoracic)

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