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Morphological Characterization of Descending Neurons and Determination of Output areas in the Brain of the Moth *Heliothis virescens*

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Siri Børø

SAMMENDRAG

Kunnskap om atferdsendringer hos nattsvermeren *Heliothis virescens* som respons på kjemiske substanser i omgivelsene kan optimalt sett benyttes i en biologisk bekjempelse av skadedyr. Koblingen mellom sensoriske prosesseringsystemer i hjernen og motorisk respons er antatt å være efferente (nedadgående) nevroner som formidler bearbeidet informasjon fra hjernen til motorkretser i lokale nervesamlinger (ganglier) langs den ventrale kroppsaksen hos insekter. I den foreliggende studien har tilsammen 88 hjerner av *H.virescens* blitt brukt for å systematisk studere lokaliseringen av disse efferente nevronene fra hjernen til gangliene ved at thoracalgangliet ble farget med et fluoriserende fargestoff. Ved massefarging ble alle projeksjonene mellom hjernen og thoracalgangliet farget og ved å benytte et konfokal mikroskop kunne man visualisere opprinnelsen eller projeksjonene til de efferente nevronene i hjernen. De kondenserte områdene av fargede fibre ble betraktet som hjernens «utputt-områder». I *H.virescens* kunne man finne to hovedområder der dendrittene til efferente nevroner projiserte svært kondensert. Dette var i et anterior-ventralt område av laterale protocerebrum og i en struktur kalt de laterale assesoriske lobene. Siden insekter i stor grad benytter seg av kjemosensorisk informasjon for å utføre spesifikk atferd, var det svært interessant å undersøke om dendrittene til efferente nevroner overlappet med et kjent, distinkt lukteområde i hjernen hos *H.virescens*. For å undersøke dette ble deres primære luktesenter, antenneloben, farget med ett fargestoff, mens et annet fargestoff ble applisert i thoracalgangliet i samme individ. Denne dobbelfargingen, samt sammenligning mellom to digitale hjerneatlas for *H.virescens* viste at det distinkte lukteområdet og dendrittene til efferente nevroner hadde ingen eller minimalt spatielt overlapp i laterale protocerebrum. Dette understreker behovet for en detaljert undersøkelse for å finne ut om sensorisk informasjon overføres til motoriske systemer via 3. ordens nevron som finnes lokalt i protocerebrum hos *H.virescens*.

ABSTRACT

Knowledge on how an insect's behavior changes in response to chemical substances in the environment can ultimately contribute to the development of biologically harmless pest controls. One of the links between sensory processing systems in the brain and motoric output is thought to be the neurons mediating the pre-processed information from the brain to act on motor circuits in the ganglia of insects. In the present study, the noctuid moth *Heliothis virescens* was the insect of study, and the thoracic ganglion of in total 88 preparations was stained with fluorescent dye to systematically study the descending neurons mediating information out of their brains. Performing mass-staining of the thoracic ganglion have contributed to knowledge on the areas where these neurons originates or projects. Visualizing the stained neurons in a confocal microscope showed condensed staining of dendrites of descending neurons in the anterior-ventral part of the lateral protocerebrum and in the lateral accessory lobes. Knowing that processing of chemosensory information might result in activities of behavioral relevance, it was especially interesting to see whether projection neurons making up the olfactory axis in the lateral protocerebrum overlapped spatially with dendrites of descending neurons eventually terminating in motor systems in the thoracic ganglia. Double staining of both the antennal lobe and the thoracic ganglion were therefore performed in the same individual, in 31 preparations. Three dimensional reconstruction and registration of descending neurons into the digital standard brain atlas (SBA) of *H.virescens* supported the spatial position of descending neurons in the anterior-ventral part of the lateral protocerebrum. The SBA containing the descending neurons was visually compared to a separate SBA containing the olfactory axis in the lateral protocerebrum, indicating no or minimal spatial overlap between the olfactory axis and the dendrites of descending neurons. This highlights the need for a detailed examination to discover if sensory information can be transferred to motor systems via 3rd order neurons local to the protocerebrum.

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1 INTRODUCTION

Preface

Carl Linnaeus (1707-1778), considered father of the modern taxonomy did one great mistake in his early research day; he claimed that one criterion of insects was their lack of brains! (Howse 1975) *Although the existence of an insects' intelligence is highly unlikely, biologist today know for sure that insects do possess brains and that they apparently do complex tasks where higher processing centers in the brain are involved.*

1.1 Introduction

The noctuid moth *Heliothis virescens* is considered an insect pest in the agricultural industry, inflicting large economic losses on the cotton industry, since the moth larvae eats buds of the cotton plant (Matthews 1999). Today's pest control is engaged in developing methods with the aim of only affecting the pest of concern, without using poisonous pesticides that might do damage to other species and humans. In this regard, discovering a link between sensory input and motoric output could be of ecological and economical importance. If knowledge on how an insect's behavior changes as a response to chemical substances, the idea is that biological methods instead of pesticides can be used in preventing insects from destroying crops.

Here, at the Neuroscience Unit at the Dept. of Biology, basic scientific research on the sensory system of *H.virescens* have contributed to knowledge on how their nervous system handle relevant chemosensory information that results in behavior related to food intake, reproduction and egg lying. Sharply tuned plant odor receptors in *H.virescens* have been found and from these studies both primary and secondary biological relevant odor substances have been identified (Mustaparta and Stranden 2005; Røstelien et al 2005). A principal question has been how olfaction- and taste information is processed in their neural system, senses that in evolutionary context are considered to be the oldest and crucial for all organisms (Jørgensen et al 2006). Physiological and morphological data on peripheral and central neurons have contributed to a growing knowledge on coding strategies and connectivity in these sensory systems while technical and instrumental development has made it possible to visualize stained neurons with a confocal microscope. Computer programs have in addition enabled reconstruction in three dimensions to determine neurons spatial relationship to each other. Unraveling the neuroanatomical logic on how sensory information in neural maps is relayed and integrated along its central pathways contributes to an increased understanding on how the sensory world is internally organized in the brain and how the brain translate the stimulus features to ultimately elicit a behavioral response (Marin et al 2002; Wong et

al 2002). By investigating morphological properties of the neurons passing information from the brain (descending neurons; DNs) to activate motor circuits in the thoracic ganglion, the link between sensory information and behavior might be revealed, and this knowledge can ultimately contribute in developing methods for pest control that is biologically harmless.

1.2 The insect as a model organism of the olfactory system

Organization of the olfactory system is largely conserved and shows striking similarities across species. Because of this, the use of model organisms for the study of molecular and neural mechanisms in the olfactory system in general has been of great importance and a good alternative to perform interventions on the vertebrate system. Nocturnal insects like *H.virescens* are fundamentally dependent on chemical communication and well suited model organisms for exploring the logic of chemosensory principles with their relative simple and compartmentalized nervous system (Mustaparta 2002). A final argument for using insects in this type of research is also the accessibility of their nervous system that allows a detailed investigation of the basic neural arrangement.

1.3 Anatomical organization of the insect olfactory system

The ability to detect and discriminate between the large repertoire of odorants present at all time in the environment, depend on a species specific number of olfactory receptor (OR) proteins. In insects, the olfactory receptor neurons (ORNs) are housed in sensilla that often have a hair like outer structure. They are located on the antennae and in some species also in the maxillary palps. Each ORN expresses only one receptor type, where each subtype expressing the same OR type projects in a specific glomerular structure in the primary olfactory center of the brain, the antennal lobe (AL) (Boeckh & Tolbert 1993; Hildebrand & Shepherd 1997; Mombaerts et al 1996; Strausfeld & Hildebrand 1999; Vosshall 2000; Vosshall & Stocker 2007). This is a principle called "the molecular logic of the sense of smell" (Axel 1995). The numbers of glomeruli are species-specific and vary from 43 in *Drosophila melanogaster*, 60- 70 in moth species and 160 in the honey bee, *Apis mellifera* (Flanagan & Mercer 1989; Rospars & Hildebrand 2000; Skiri et al 2005; Vosshall 2001). The AL of *H.virescens* contains 66 and 67 glomeruli, respectively in female and males (Løfaldli et al 2010). The male- specific macroglomerular complex (MGC) located at the entrance of the antennal nerve (dorso- laterally in the AL) contains four glomeruli and is responsible for processing information about sex pheromones produced by the con specific as well as sympatric females (Berg et al 1998). The remaining glomeruli are responsible for the processing of plant compounds and are present in both males and females.

The glomeruli are condensed synaptic structures processing the odor information. Receiving

information from one subtype of ORNs it is regarded as the functional unit of the AL, each glomerulus working as a local neuronal network between the axon terminals of ORNs and mainly two central interneurons (Boeckh & Tolbert 1993). The local interneurons (LNs) are mainly GABA-ergic inhibitory neurons, innervating most glomeruli. The projection neurons (PNs) receive information both directly from the ORNs and LNs, mediating this information to higher olfactory areas in the protocerebrum. These PNs may innervate single glomerulus (uni-glomerular) or several glomeruli (multi-glomerular) in the AL. The centrifugal neurons, having dendrites and somata outside the AL, also innervate glomeruli and have modulatory functions.

1.3.1 Antenno-protocerebral tracts connecting the antennal lobe with the protocerebrum

Olfactory PNs project to higher- order centers through three major protocerebral tracts, connecting the glomeruli in the AL with the protocerebrum. The higher processing areas in the protocerebrum to receive innervation from the tracts are the mushroom body calyces (MBCal), an anatomical structure of high relevance in learning and memory, the lateral protocerebrum (LP) which is considered to be a premotoric area housing dendrites of DNs (Menzel 2001), and the superior protocerebrum (SP), an area located dorsally of the MB output regions (Li & Strausfeld 1997; Rø et al 2007; Tanaka et al 2008). The nomenclature of the protocerebral tracts mentioned has differed between species and is in this study used as suggested by Galizia & Rössler (2010). In most insects studied, three major antenno- protocerebral tract (APTs) share a relative common position in the brains of these insects, but may not necessarily have the same function or homology. The largest tract is the medial APT (m-APT), followed by the medial- lateral APT (ml-APT) and finally the lateral APT (l-APT) (Galizia & Rossler 2010).

In *H.virescens,* mass staining of PNs revealed the three APTs (Rø et al 2007). The most prominent tract was the m-APT with PNs having morphological characteristics of dense arborizations in single ordinary glomeruli in the AL, and axons terminating first in the MBCal and the LP. The tract as a unity exits the AL dorso-medially and runs posteriorly in the protocerebrum and takes a lateral turn close to the lateral edge of the central body (CB) before it then reaches its main target; the MBCal, and then extending to the LP. Multiglomerular PNs coalesce to make the smaller ml-APT. It follows the m-APT as it exits the AL along the dorsomedial axis for a short distance before this tract also bends laterally at the level of the CB, and divides into several smaller branches terminating in slightly different parts of the LP (Rø et al 2007). The l-APT is made up of PNs showing both uni- and multiglomerular properties, and leaves the AL from a more ventral location than the two others and continues laterally and divides in branches where they end up mostly innervating the LP, though with some fibers turning dorso-medially in the LP to innervate the MBCal (Rø et al 2007). The l-

APT has been described as loosely fibered and the diameter of the fibers is smaller than those projecting in the other tracts (Homberg et al 1988; Rø et al 2007). Olfactory coding mechanisms have been studied in many insect species. It is reported that both spatial and temporal principles may contribute in coding of odor quality in the olfactory processing centers and several research groups are currently investigating this highly complex olfactory system (Galizia & Szyszka 2008; Laurent et al 1996; Lei et al 2004)

1.3.2 Odor processing in higher brain areas

In general, scarce knowledge exists on the functional organization of the higher olfactory centers in insects. The MB receives odor information from PNs in the m-APT and the l-APT in an opposite direction, where the PNs synapse on the major intrinsic neurons of the MB, the Kenyon cells (KC) (Heisenberg 2003; Keene & Waddell 2007; Menzel & Müller 1996). The MB system is closely connected to other brain areas through several types of MB extrinsic neurons (Homberg 1984; Mauelshagen 1993; Rybak & Menzel 1998; Tanaka et al 2008). Projections from brain areas like the SP and LP/LH have been shown to give afferent projections to the MB complex, while efferent extrinsic neurons of the MB have been shown to project from distinct part of the MB lobes, the output area of the MB. These projections are in the inferior medial protocerebrum, the SP, the LP/LH as well as recurrent to other part of the MB complex. The MB complex is central in olfactory learning and memory, and several MB extrinsic neurons have shown to change response activity after conditioning. This indicates that these efferent neurons may convey learned information from the MB complex to the termination areas, especially the SP and the LP (Mauelshagen 1993; Rybak & Menzel 1998). Thus, the olfactory pathway via the MB complex is regarded as an indirectly route to convey information from the AL to the LP, mediating associative or experience-dependent information (Heisenberg 2003; Keene & Waddell 2007).

This is in contrast to the more direct route of olfactory information from the AL to the LP/LH that is mediated parallel to the indirect route, via all the APTs. In *D. melanogaster*, three types of lateral horn neurons (LHNs) have been identified, showing dendritic arborizations in the LH and axonal projections in different parts of the brain, like SP and in more ventral areas of the LP (Jefferis et al 2007; Tanaka et al 2004). To what extent these neurons integrate different olfactory information in the LH is debated by the two groups. In the LP of *H.virescens*, intracellular coding and staining (Løfaldli et al 2010) showed olfactory PNs following the m-APT and the ml-APT towards the protocerebrum. Registration of these PNs into a digital standard brain atlas of *H.virescens* (SBA), revealed a stratified projection pattern of PNs along the dorso-ventral axis in the brain. m-APT PNs innervating the same glomerulus in the AL, had an intermingled and similar projection pattern,

whilst m-APT PNs innervating different glomeruli projected to partly overlapping areas along the mentioned dorso-ventral axis, which they named “the olfactory axis” (Løfaldli et al 2010). Similar findings have been shown in *D.melanogaster* (Jefferis et al 2007; Tanaka et al 2004; Wong et al 2002). Regarding the functional role, the direct pathway from the AL to the LH/LP has been suggested to represent a more naïve and experience-independent processing stream for olfactory information than the indirect route via the MB (Heisenberg 2003; Keene & Waddell 2007).
The third area in protocerebrum to receive olfactory information is the SP. It receives projections from the AL, MB complex and the LP, however the specific role of SP in processing olfactory information is uncertain (Kirschner et al 2006; Li & Strausfeld 1997; Løfaldli et al 2012; Rø et al 2007; Tanaka et al 2008)

How olfactory information is integrated in the three protocerebral areas and further conveyed to premotoric areas, is the ultimate question regarding the functionality of the olfactory system. To gain knowledge about this process, it is necessary to investigate neurons both physiologically and morphologically, as well as revealing the connectivity between the neurons. To spatially relate and visualize identified neurons from different preparations in a common framework, digital standard brain atlases (SBAs) for several species have been made (see Brandt et al 2005; Kurylas et al 2008; Rein et al 2002), including two moth species, *M.sexta* (El Jundi et al 2009) and *H.virescens* (Kvello et al 2009). The advantage with the *H.virescens* SBA is that the atlas of the AL have been included in the SBA, thus the projection input and output of PNs can be registered in the same framework (Løfaldli et al 2010). Identification of the spatial relationship between neurons have been especially important in the LH/LP, since this area contains no structural landmarks appeared by immunostainings. By collecting data of olfactory neurons from several brain preparations, Løfaldli et al (2012) have recently published a putative circuit on how information about a ten component plant odor mixture is handled in the brain of *H.virescens.* One single olfactory neuron, responding selectively to the multicomponent mixture of plant odorants was found to have dendrite arborizations ventral of the olfactory axis and its axon extending out of the brain via the ventral cord (Løfaldli et al 2012). Thus, by unraveling the neural connections presented in this putative circuit, one could possibly trace the odor signal all the way from the ORNs in the antennae, to dendrites of DNs in output areas of the brain. A similar circuit of how pheromone information is handled from sensory input to motoric output in *D. melanogaster* has been described by Ruta et al (2010). Whereas growing data of olfactory neurons located in the AL and the protocerebrum in *H.virescens* are seen, information on DNs has lagged behind. It is therefore of great interest to investigate the morphological and physiological properties of DNs that finally mediates the processed olfactory information out of the brain.

1.4 The nervous system of insects

"The nervous system of insects is an information processing and conducting system that ensures rapid functioning and coordination of effectors, producing and modifying an insect's response to input from peripheral sense organs" (Chapman 1998). An introduction to the insect nervous system is essential in understanding the neural connection between sensory- and motor systems. Insect belongs to the phyla *Arthropoda*, which have a characteristically exoskeleton, jointed appendages and a body built up of segments, the head, the thorax and the abdomen. Like the segmented exterior, the insects' interior is also generally built up of repeated segments, including their nervous system. It stretches as a chain of ganglia, along the anterior- posterior body axis, termed the ventral cord. The ganglia are joined by a pair of interganglionic connectives that have no somata or synapses, only glia and axons. The subesophageal ganglion (SOG) is regarded as the first ganglion in the chain and is in Lepidoptera fused with the brain (Jørgensen et al 2006; Raina 1993). The first ganglion is followed by typically three thoracic ganglia (pro-, meso- and metathoracic ganglion) and finally a varying number of abdominal ganglia. Each segmental ganglion contains the somata of motor neurons concerned with controlling the muscles of the same body segment, as well as somata of afferent sensory fibers from each body segment ascending to the insect brain and interneurons, probably modifying both the motoric output and the ascending sensory signals. (Chapman 1998). Thus, each ganglion contain a complex organized synaptic network of DNs, ascending neurons (ANs), local interneurons, as well as highly specialized motoric neurons (Mobbs 1985; Weevers 1985). It is important to realize the complexity of the neural organization in the ganglia in order to study the connections between the brain and the ganglia.

1.5 Descending neurons

The ganglia contain the motor neurons innervating the body muscles, and activation of these neurons will elicit a behavioral response (Mobbs 1985; Wessnitzer & Webb 2006; Weevers 1985). It has been discussed how DNs control certain behavioral routines and if they are "command" fibers that initiate fixed pattern of motor actions (Strausfeld et al 1984). It is actually assumed that few DNs indeed carry commands that override all other activity. A general feature seems to be that the majority of DNs innervate parts of the ganglion that contain minimal motor neurone arborizations (Strausfeld et al 1984). Possibly, the fundamental task of DNs is to synapse on interneurons in the ganglion to modulate the activity of thoracic interneuron- motor neuron circuits (Strausfeld et al 1984). For instance, the male giant sphinx moth, *Manuca sexta*, is known to perform a distinct behavior when a conspecific female releases a pheromone plume (Kanzaki et al 1991a). Studies have shown that processed pheromone olfactory information is carried from higher processing centers like the MB and the LP, to innervate a region lateral to the CB on each side of the

protocerebrum; the lateral accessory lobe (LAL). Thus, the LAL is thought to be an important region where the olfactory information from other regions of the protocerebrum converge (Kanzaki et al 1991a). Since the LAL is shown to be innervated by branches of neurons responding to pheromonal olfactory stimuli having axons descending in the ventral cord, these DNs have been a subject for investigation in some studies. It is thought that the activity of the neurons represent an integrated multimodal output from brain circuits that acts on thoracic motor circuits in the ganglia to affect behavior (Kanzaki 1991a). How these DNs affect motor circuits in the ganglion is debated, but the LAL as a "relay station" before activity is elicited is thought to play an activating role and perhaps regulate the motor outflow that results in an oriented flight behavior towards the female moth (Kanzaki 1991a).

Scarce data exists on DNs mediating information about plant odors from the brain to the ganglia. However, evidence that the ventral area in the LP of *H.virescens* houses a DN responding to a multicomponent mixture of plant compounds, implies that information about plant odors might lead to a motoric response by activating or inhibiting circuits in the ganglia (Løfaldli et al 2012).

1.6 Aim of the thesis

The aim of the present study was to identify output areas housing the dendrites of DNs, connecting the brain with the thoracic ganglion of *H.virescens*. This study is the first attempt to systematically investigate the output areas in *H.virescens*, trying to establish a structural connection between sensory information and motoric output. By staining the thoracic ganglion it is assumed that both ascending and descending fibers will be stained, and it will be necessary to clarify the distinction of these two pathways when interpreting the results.

The work consisted of three parts:

1. Establishing a method for staining the thoracic ganglion and the antennal lobe in the same preparation of *Heliothis virescens*.
2. Morphologically identifying output areas in the brain of *Heliothis virescens*, and visualize the descending neurons connecting the protocerebrum to the thoracic ganglia.
3. Identifying a possible spatial overlap between the area of DNs and the olfactory axis in the brain of *Heliothis virescens*.

1.7 Hypothesis

Based on the findings of an olfactory responding descending neuron ventral of the olfactory axis, hypothesis 1 is: One output area is present in the ventral part of the lateral protocerebrum in Heliothis virescens. Based on previous findings in Manduca sexta, hypothesis 2 is: A second output area is present in the lateral accessory lobes in Heliothis virescens.

Based on findings of the olfactory system in other species, hypothesis 3 is: There is no spatial overlap between the olfactory axis and dendrites of descending neurons in Heliothis virescens.

2 MATERIALS AND METHODS

2.1 The insects

The moths, *Heliothis virescens* (Heliothiane; Lepidoptera; Noctuidae) was imported as pupaes from a laboratory culture in Switzerland (Syngenta, Basel). After arrival, the pupae was sorted and separated according to gender and placed in square plexi glass containers with a perforated lid (height: 18 cm, width: 12 cm, depth: 17 cm). The containers were kept in a Refitherm 6 E incubator (Struers) with a reversed photo period (14-h light and 10-h dark), temperature around 22-24 °C and relative humidity of ~ 70 %. After hatching, the insects were transferred to cylindrical plexi glass containers (height: 20 cm, diameter: 10 cm), maximum 8 insects in each, with perforated lids. The containers were equipped with a paper towel for the insects to fly up on and they were given fresh sucrose solution (50 g/l) every day. The experiments were performed on the seemingly healthiest adult females, one to three days after they had hatched.

2.2 Preparation and staining

In trying to discover DNs and the output area in the brain of *H.virescens*, single staining of only the thoracic ganglion was performed. *H.virescens* has at least two thoracic ganglions, whereas the first in the chain was stained in the present study. This prothoracic ganglion will further only be mentioned as "the thoracic ganglion". Examining whether the DNs and the olfactory axis showed spatial overlap, double staining of both the thoracic ganglion and the antennal lobe was performed in the same insect.

2.2.1 Staining of the thoracic ganglion

Moths (83 female, 5 males) were placed dorsally, with their ventral side facing up (figure 1), on to a piece of plastelina clay. The wings and approximately 2/3 of the body, except the head and the first leg pair, was immobilized by dental wax (Kerr Corporation, Romulus, MI, USA). A pin was placed under the eye cuticle to stretch the neck area, and the first leg pair was removed by scissors. Removal of trachea and thoracic muscle tissue with forceps and micro scissors provided a full view of the thoracic ganglion and the ventral cord extending to the brain. The thoracic ganglion was stained with color crystals of micro-ruby (Molecular Probes®, ex/em: 555/580) and immediately covered with medical wipes (Kimberly- Clark® Professional) soaked in Ringer solution (NaCl: 150 mM, CaCl2: 3 mM, KCl: 3 mM, TES buffer: 10 mM, sucrose: 25 mM, pH 6, 9). They were further placed in refrigerator for 4-6 hours to let the dye be taken up by neurons innervating the ganglion.

2.2.2 Double- staining from the thoracic ganglion and the antennal lobe

Moths (31 females) were placed in plastic tubes, with their head immobilized by dental wax, after a

cooling period (5-15 minutes) at 4°C. Hairs on the insects head was removed with forceps. With the use of micro scissor and scalpel, the cuticle between the two antennae was removed. A membrane surrounding the brain was opened and the AL was exposed for staining. Color crystals of micro-emerald (Molecular probes ®, ex/em: 494/518) was applied directly into the antennal lobe. After staining, the "hole" in the cuticle was covered with a piece of medical wipe, soaked in Ringer solution. To ensure that no leakage of haemolymph could occur, dental wax was melted (soldering station, ERSA®, WM 1500 S, Germany) over the hole to create a watertight membrane. Further, the insects were released from the plastic tube and cooled in a refrigerator at 4°C.

The insects were held in the refrigerator for 10-12 hours to let the dye be taken up by the neurons innervating the AL. Then the thoracic ganglion of the same insect was stained (following the same procedure as mentioned in section 2.2.1.) After ganglion staining, the insects were placed in the refrigerator at 4°C, in 4-6 hours, to let the dye be taken up by neurons innervating the ganglion.

Custom made insect needles were used to apply the dyes in the AL and the ganglion. Color crystals were added to the needle which was manually maneuvered to these areas. Both sharpness and the diameter of the tip were optimized to pass through the membrane under the cuticle. The needle remained in the tissue for some seconds after application of the dye as a reassurance that the dye crystals could be taken up by the neurons.

2.3 Dissection and protocol

After both single- and double staining, and in total 4-18 hours in refrigerator at 4°C, the insects were decapitated, and their brains dissected in Ringer solution and fixed for >24 hours (Roti Histofix). Following fixation the brains were rinsed in a phosphate buffer solution (PBS; NaCl: 684 mM, KCl: 13 mM, Na2HPO4: 50, 7 mM, KH2PO4: 5mM, pH 7, 2) for ten minutes. This was followed by dehydration in an increasing series of ethanol (50%, 70%, 90%, 96% and 100 %; each for ten minutes). The brains were finally cleared with methyl salicylate and mounted as whole mounts in custom- made aluminum plates with double sided cover glass. This protocol was performed on the brains of both the ganglion- and the double staining. In some preparations the ventral cord was cut ventral of the thoracic ganglion to be included in the confocal scanning. This did not change the design of the protocol.

2.4 Visualization of stained neurons

Staining of the AL and the ganglion resulted in mass staining of the neurons innervating these areas, and provided an overview of the neural connections to the protocerebrum from the AL and the

thoracic ganglion.

2.4.1 Confocal laser-scanning microscopy

Visualization of the stained neurons was performed using a confocal laser-scanning microscope (Leica TCS SP5; Leica Microsystems CMS GmbH, Mannheim, Germany). Whole brain preparations were scanned, creating a stack of pictures representing the z-axis in either anterior-posterior direction or in a dorsal- ventral direction. This was done both with a 10 x 0, 4 dry objective and a 20 x 0, 7 dry objective. DPSS- laser (1-channel scanning) using 532 nm wavelength green laser pointer, to excite micro- ruby was used, and the pre-stored beam path settings was set to CY3. The resolution of the xy- plane was set to 1024 x 1024 pixels, and the interslice distance was calculated with a predefined formula by the Leica software, to fit each preparation, ranging between 1,5 µm- 3,10 µm.

The same microscope settings as mentioned was used to visualize the micro- ruby in the double stained preparations, but in addition a 2-channel scan was performed to visualize the micro-emerald in the same brain preparation. The argon laser using 488 nm wavelength laser pointer to excite micro-emerald was used and the pre-stored beam path settings was set to FITC (fluorescin isothhiocyanate). 2-channel scanning "in between frames" was chosen. Overview scans were scanned with a speed of 400 Hz, while some especially good preparations and areas of special interest were scanned with a speed of 100-200 Hz. The resulting scans were stored as Leica Image File (.lif) and converted to Amira Mesh format (.am) in Amira 4.2 (Mercury Computer Systems, San Diego, CA, USA).

2.4.2 Three- dimensional reconstruction of stained neurons and transformation into the standard brain atlas

Two brain preparations were reconstructed; brain preparation 43 (partly reconstructed, data not shown) and brain preparation 24 (figure 6). Brain preparation 24 was chosen as template for 3D-reconstruction based on a remarkably good preparation in regards of neuron staining and visibility of brain neuropils. To create a three-dimensional model, raw data from the confocal microscope was used as a template and the neurons were manually reconstructed in segmentation editor of Amira. Due to the refraction in methyl salicylate, a scaling factor of 1.6 was multiplied to the z- axis, to compensate for the refraction index-difference between the preparations in the solution and the air objective. The whole of protocerebrum and central body was reconstructed as label images and the mass- stained neurons were reconstructed in the same way. Bundles of DNs in the posterior-most part of the LP were treated as a unity when reconstructed. Before registration into the standard brain atlas (SBA) the label images of protocerebrum and central body were affine- and elastically registered to corresponding label images of the standard brain, so that the label images of the

preparations were given the same coordinates as those of the standard brain. Since the DNs were reconstructed as label images they had to be transformed into a skeleton file in Autoskeleton in Amira before they could be integrated in the SBA. The coordinates and parameters obtained after neuropil registration in the SBA was applied to the reconstructed neurons when it was affine- and elastically registered into the SBA. The registration of neurons in the SBA follows a similar procedure as described by Brandt et al (2005).

2.5 Evaluation of the preparations

Evaluation and making a representative selection of the preparation was based on subjective interpretations and the "best" brains are presented in the result section of this study. The main focus areas were the lateral LP and the LAL, and preparations showing adequate and interesting staining in these areas were selected for further examination. Projection views were made in the Amira software where editing alternatives rendered it possible to digitally clean the pictures, by removing distracting "noise", apply smoothing filters as well as adjusting light intensity to enhance the image of the stained neurons

2.6 Location

All lab work was performed at the laboratory of the Neuroscience Unit, Department of Biology, located in the Medical Technical Research Center (MTFS), NTNU, Norway, providing all necessary equipment's. Scanning in the confocal microscope was performed at the Department of Physics at Realfagsbygget, NTNU, Norway

Figure 1: The position of the moth in order to stain the thoracic ganglion. The ventral side is facing up, with the body covered with dental wax. The blue color inside the thorax is the color Janus Green, applied to enhance the view of the ganglion. Photo: Per H. Olsen

3 RESULTS

Injection of dye into the thoracic ganglion revealed the dendrites of descending neurons, as well as projections of ascending neurons in the brain of the moth *Heliothis virescens*. As shown to be the major output areas of the brain, the focus was on the lateral protocerebrum and the lateral accessory lobes, which showed heavily staining in the preparations presented. As for anatomical and morphological differences in output areas between males and females, the results gave an indication of a possible sexual dimorphism in the LAL. To answer the question whether DNs overlap with antennal lobe projection neurons terminating in the "olfactory axis" of the moth brain, double staining from the thoracic ganglion and the AL was performed. These results showed that DNs coincide to form a descending neuron- output tract, which are co-localized with the l-APT, coming from the AL. However, the majority of PN in all the three APTs seem to terminate in other areas of the lateral LP than the DN dendrites, suggesting minimal direct overlap between olfactory PNs and the dendrites of DNs. However, the possibility of direct overlap of medial fibers could not be excluded.

The results are presented in two parts:

3.1 The results of the single staining from the thoracic ganglion in female and male moths.

3.2 The results of the double staining from both the thoracic ganglion and the AL in female moths.

3.1 General description of descending neurons after staining of the thoracic ganglion

The thoracic ganglion of 52 female and 5 male moths was injected with dye, followed by the procedure resulting in visualization of the neurons in the confocal microscope. The results presented in this section are based on data from in total 14 selected brain preparations that showed the best staining. 11 of the preparations are included in the main result section, while 3 preparations are presented only in appendix I (figure A1, A2 and A3).

3.1.1 Directly connecting fibers between the thoracic ganglion and the antennae

Among the prominent results was a bundle of fibers, making direct contact from the thoracic ganglion to both antennae, extending into the antennal nerve (figure 2). The stained fibers bypassed posterio-laterally of the AL, extending to the ventral part of the suboesophageal ganglion and eventually proceeding to the thoracic ganglion. Another important finding was the heavily stained

area of the antennal mechanosensory and motor center (AMMC). The AMMC seemed to be innervated especially by at least one pair of fibers with extraordinary large axon diameter. These fibers were given the name “giant fibers”. The giant fibers seemed to follow a bilateral symmetrical organization with neurons entering the anterior SOG extending from the midline towards each lateral side (figure 2 and 9A-C).

3.1.2 Number and distribution of somata of descending neurons

Somata were seen in different locations in the brain of the moth but an accurate number was not possible to give, because of methodical limitations. A rough estimate based on analysis of the confocal z- stack scan, suggested more than 50 somatas of DNs. Groups of somata were seen in the periphery of the SOG, dorso- ventrally and dorso- medially around the mushroom body calyces, postero-ventrally of the LALs (figure A1, appendix I) and superior- peripheral of the central body. Because of massive staining, separation of the somata into anatomically organized clusters was not performed, although some clusters were more distinct that others. In the most posterior part of the brain, clusters of cell somata appeared around the MBCal dorso- medially and dorso- ventrally (figure 3; 4B; 11A, C and A2, appendix I). Moving anterior more cell bodies were seen, with relative large, separated soma lying superior and peripheral to the CB (figure 3A). One particular well stained cell cluster of 5-10 somata was located most peripherally in the medial- posterior part of the two protocerebral hemispheres (figure 5A-C; 7A-C and A3, appendix I). From the somata, their remarkably fiber bundle extended in a posterior direction in the SP, before bending anterior, continuing ventrally as their fibers intermingles with other DN fibers surrounding the LAL neuropil.

Figure 10C shows stained somata in the SOG, lying extremely superficial in the most ventral part, with their dendrites extending in an anterior-posterior direction to the middle of the SOG before leaving the SOG to enter the ventral cord. The further path in the ventral cord is depicted in figure 10A and B. In spite of dye- saturation, the ventral cord clearly appear as divided in two parts, where each part containing fibers from the ipsilateral protocerebral hemispheres, projecting to the thoracic ganglion (also seen in figure 7A-D).

3.1.3 The lateral protocerebrum

Figure 2D depicts the anterior-most part of the output area in LP (small dashed circles), located almost peripheral in the anterior-ventral LP. The output area stretches in an anterior-posterior direction, terminating in the mid- ventral LP. Figure 6 depicts what is regarded as the posterior-most part of the main output area in the LP of *H.virescens*. The two symmetrically stained DN- output-tracts was positioned medial of the output area, entering the SOG before it is assumed that the DNs

extend in the ventral cord. The left side of the brain in the mid-ventral part of LP, was reconstructed in three dimensions and registered into the standard brain atlas of *H.virescens*, to reveal spatial location of the neural branching, the DN- output tract and neuropil structures (protocerebrum and CB) in relation to each other (figure 6B-E). Of information, the brain preparation in figure 6 is scanned in a dorso-frontal position. Figure 6C shows an overview of the neural branches in the mid-ventral part of LP, with the eye lobes included. The DNs have a few fine branches extending in a dorsal direction from the majority of the neuropil, though still holding a relatively lateral position in the protocerebrum. Figure 6D is a section of the ramifications in the mid-ventral LP. The lateral view in figure 6E shows the DN- output tract, stretching in a posterior-anterior direction before extending ventrally to the SOG and in the ventral cord (not depict). The output area in the LP seems roughly to be positioned in the anterior half of the brain, with minimal neural arborization any posterior than the CB.

3.1.4 The lateral accessory lobes

The LAL neuropil showed heavily staining, and is therefore regarded as a major output area together with the anterior- ventral area in the LP. The LAL in *H.virescens* is two ellipsoid structures located anterior and ventro-laterally of the CB. Figure 7A-D shows four projection views of LAL, taken at two different positions in the female (7A-B) and the male (7C-D) moth. In figure 7A, an arrow highlights the LAL commissure that connects the LAL of the two hemispheres. The view of the whole commissure appeared as the most anterior part of the LALs, positioned anterior of the central body. The commissure makes a slight curvation in posterior direction between the two LAL, housing fiber branches. The same results were obtained in the male preparation, and figure 7C-D depicts the same areas as previously mentioned for the female. Visually comparing the neural arborization of DNs in the female and male, revealed that the male LAL are more heavily innervated by DN dendrites than the female.

3.1.5 Directly connecting fibers between the thoracic ganglion and the eye lobe

Evidence of a neural connection between the thoracic ganglion and the eye lobe is evident in figure 8, where two fibers from the eye lobe intermingles with DNs in the output- area of the protocerebrum

3.2 General description of the double staining from the thoracic ganglion and the antennal lobe

The thoracic ganglion and the AL of 31 female moths was injected with two different dyes (micro-ruby in the thoracic ganglion and micro- emerald in the AL), followed by the procedure resulting in

visualization of the neurons in the confocal microscope. 3 of the preparations are included in the main result section, while 1 preparation is presented only in appendix I, figure A4.

3.2.1 Co-localization of the l-APT and the descending neuron output tract

As in other preparations, staining of the thoracic ganglion revealed the majority of stained neurons to be located in the anterior- ventral part of the LP and the LALs. The red color shows the staining from the thoracic ganglion and the green color shows the projection from the AL (figure 11; 12; 13). Cell clusters of somata superior and lateral for the central body is especially well- stained, as seen in the picture with both dyes (figure 11A) and the picture showing only the staining from the thoracic ganglion (figure 11C). The m-APT, ml- APT and the l- APT are marked with arrows (figure 11B). The l-APT from the AL and the DN- output tract follow the same path, though the DN- output tract holds a more ventral position than the l-APT in the protocerebrum. It seems like the DN- output tract stretches in a posterior-anterior direction, with its most posterior location being the mid-ventral LP, the posterior-most termination area of DNs (see also figure 6). Figure 12 demonstrates the same findings as in figure 11, but lets us follow the projections of the three APTs in the protocerebrum better, and supports that the DN- output tract holds a ventral position in the pathway. Neurons entering the DN- output tract come from a ventral part of the LP, as figure 12D describes. These results indicate that the l-APT and the DN- output tract separates at one point, where the l- APT continues laterally probably towards the lateral horn, and in a posterior direction to innervate the MBcal (not shown in the pictures). The DN- output tract takes a soft antero-ventral curvation in the mid-ventral LP (figure 11; 12; 13).

3.2.2 Staining in the ganglion

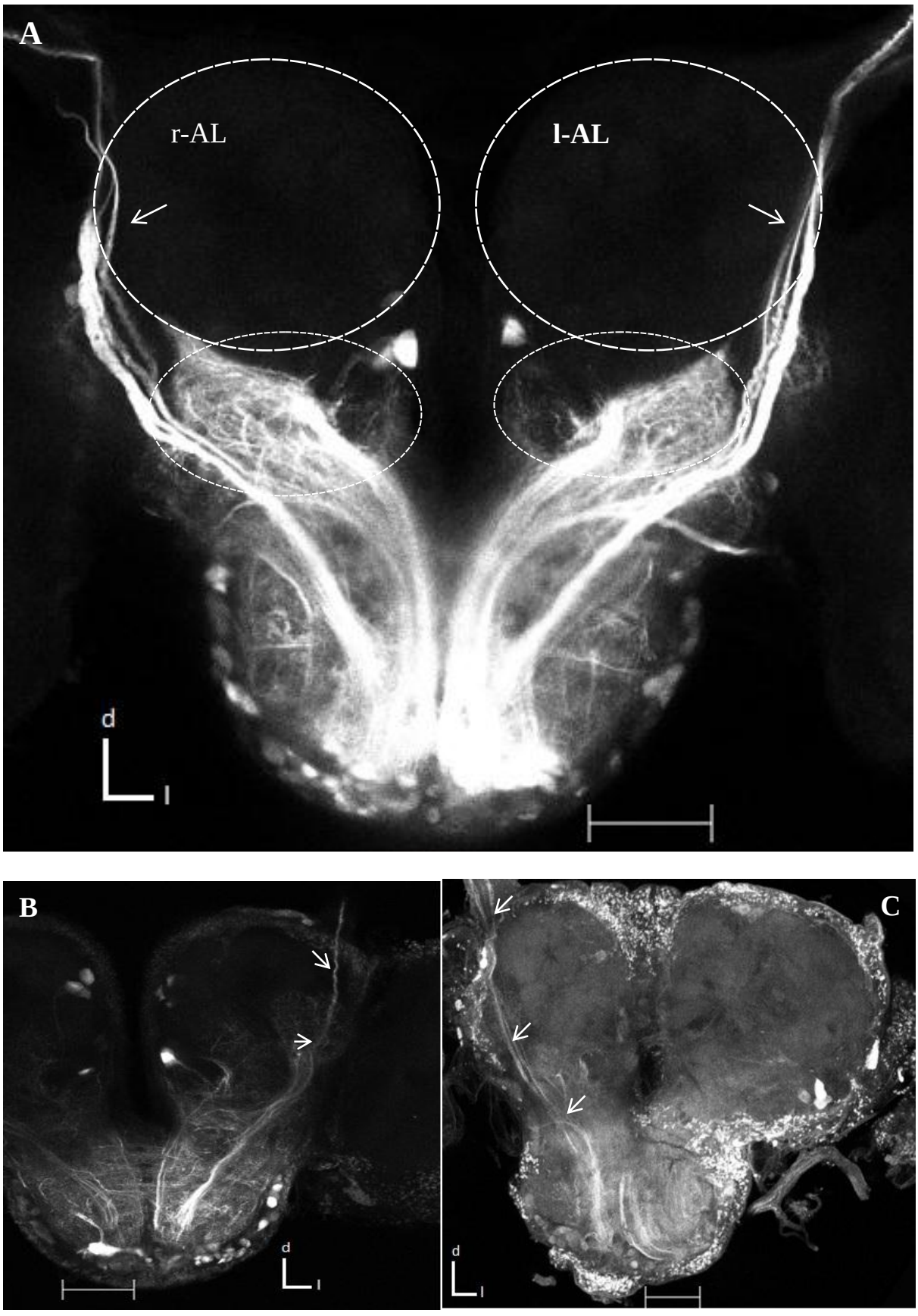
Figure A4, appendix I, shows cell somata located superficially in the ventral part of the ganglion. Applying dye in the thoracic ganglion is an invasive operation and destruction of the ganglion will happen. However, the figure is still included because of the informational value. Fibers extending from the thoracic ganglion, continuing "downstream" of the ganglia are also stained and visible in the figure.

3.3 Comparing two Standard Brain Atlases

DNs was reconstructed from brain preparation 24 and inserted in the SBA of *H.virescens* (figure 6). The position of the reconstructed DNs in the SBA was compared with the position of the olfactory axis, registered in a separate SBA (Løfaldli et al 2010). Visually comparing their spatial localization indicated minimal spatial overlap between the majority of fibers from PNs following the m-APT and the ml-APT with dendrites of DNs. However, spatial overlap in the medial areas could not be

excluded.

Analyzing the z-stack scan of the preparations revealed that PNs following the l-APT seemed to terminate in other areas of the LP, than the DN dendrites, indicating no or minimal overlap between DNs and any kind of PN from the AL.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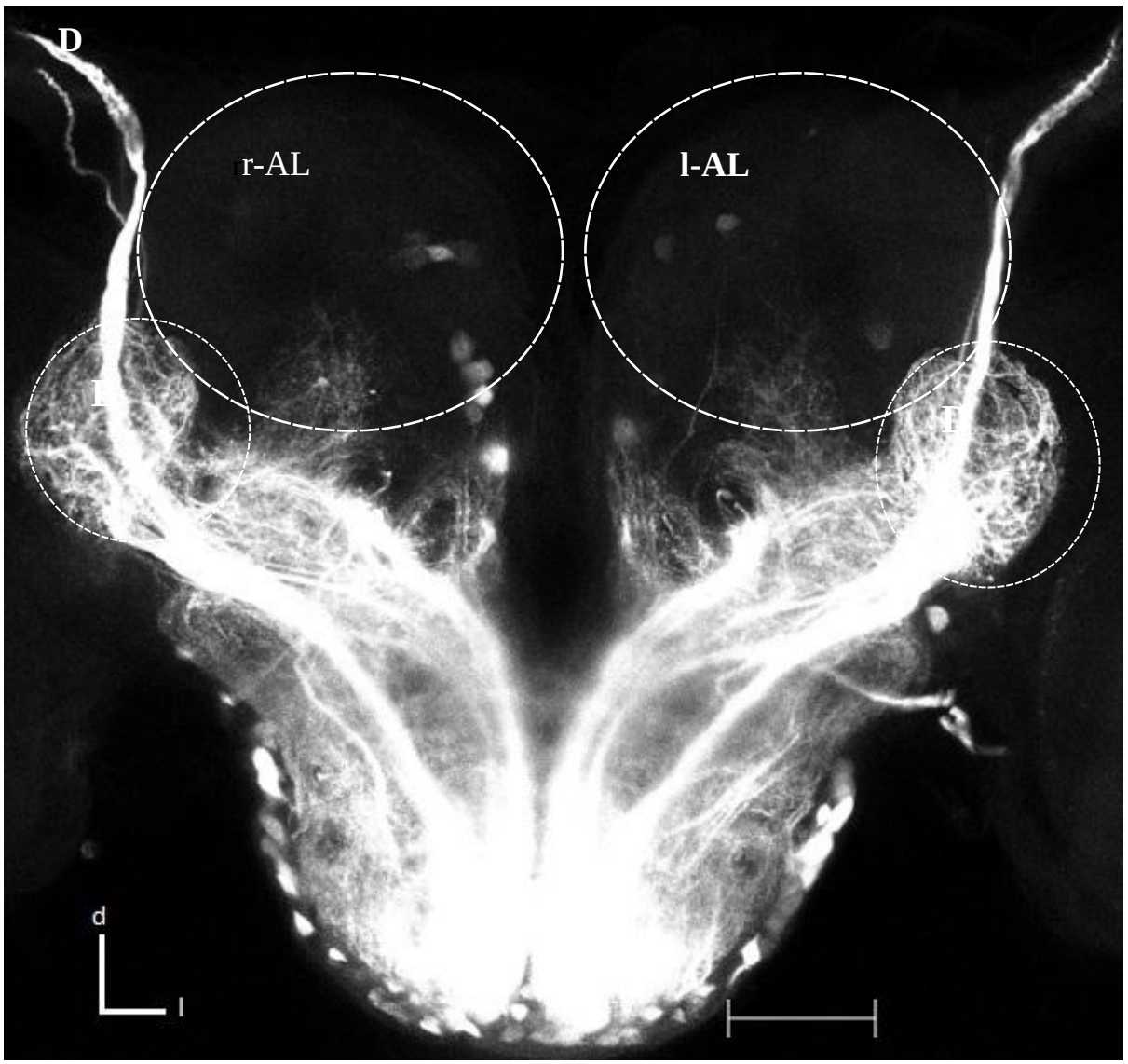


Figure 2: Confocal images (projection views) of fibers from the antenna, bypassing posterior of the antennal lobe, directly fusing with the giant fibers in SOG, continuing down the ventral cord (not depict), in three different brain preparations. **A**: Overview of the projection from the antennae (indicated by arrows), in brain preparation 53 (male). The AMMC; antennal mechanosensory and motor centers, is stained and indicated with small dashed circles. **B**: Brain preparation 39 (female), with the arrows indicating the axon from the antennal nerve bypassing posterior of the antennal lobe. **C:** Illustrates the same as A and B, from brain preparation 35 (female), arrow indicating the projection of the axons. **D**: Brain preparation 53 (male), showing the same antennal projections as in A, but included is the anterior-most part of the output area in the anterior- ventral part of the LP (outlined with dashed circles). The left and right antennal lobes (r-AL,l-AL) are circled in A and D for orientation, because of dark background. A-D is frontal oriented and scanned with a 20x objective. Scalebars: 100 µm.

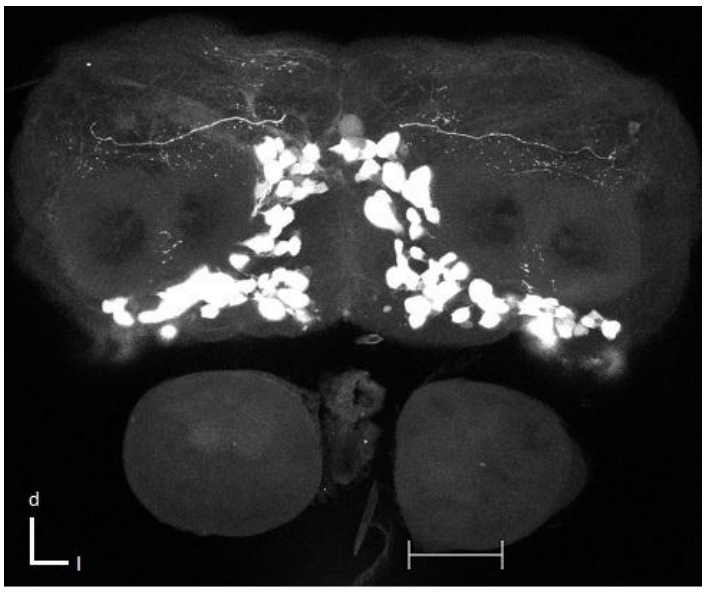


Figure 3: Confocal image (projection view) of cell clusters of somata dorso-medially and dorso-ventrally around the mushroom body calyces in brain preparation 53 (male).

The preparation is frontal oriented, and scanned with a 20x objective. Scale bar: 100 µm.

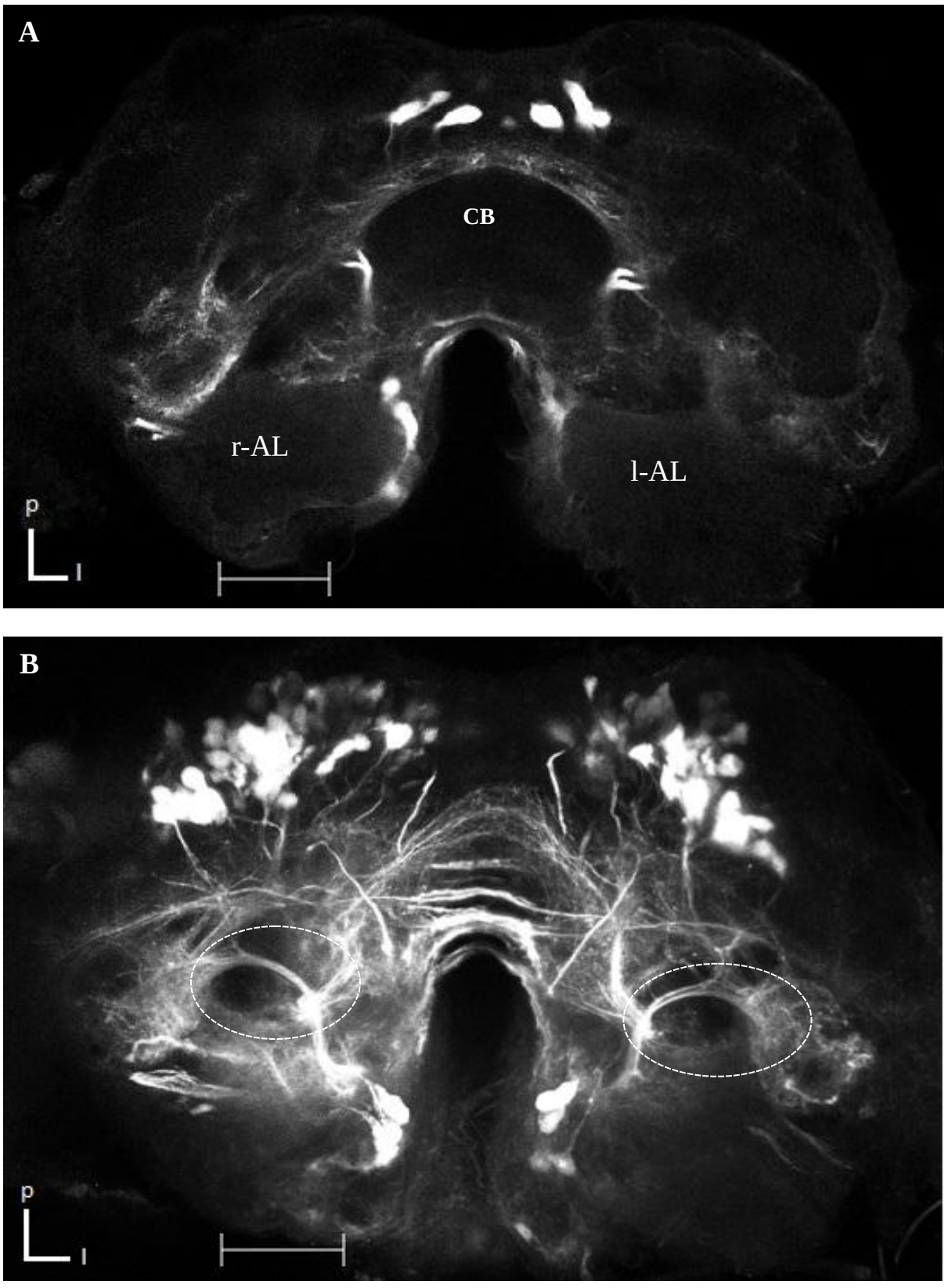


Figure 4: Confocal images (projection views) of brain preparation 43 (female) as an overview of projections visualized in the brain, stained from the thoracic ganglion. **A**: Cluster of somata located superior of the central body. Left- and right antennal lobes (l-AL, r-AL) and central body (CB) are named as landmarks. **B**: A section showing cell somatas, axons and the DN- output tract (in dashed circles). A-B is dorsal oriented and scanned with a 20x objective. Scale bars: 100 µm.

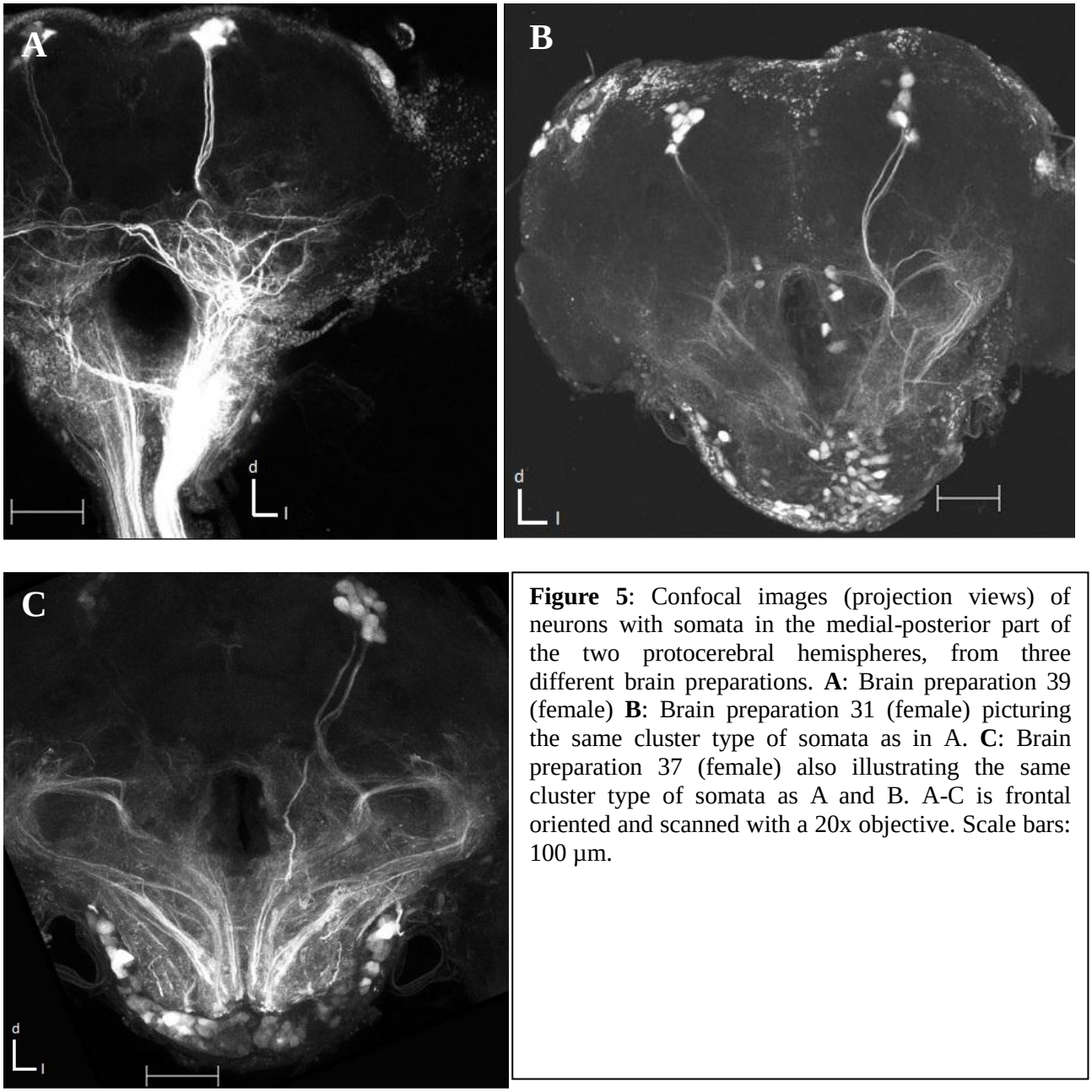
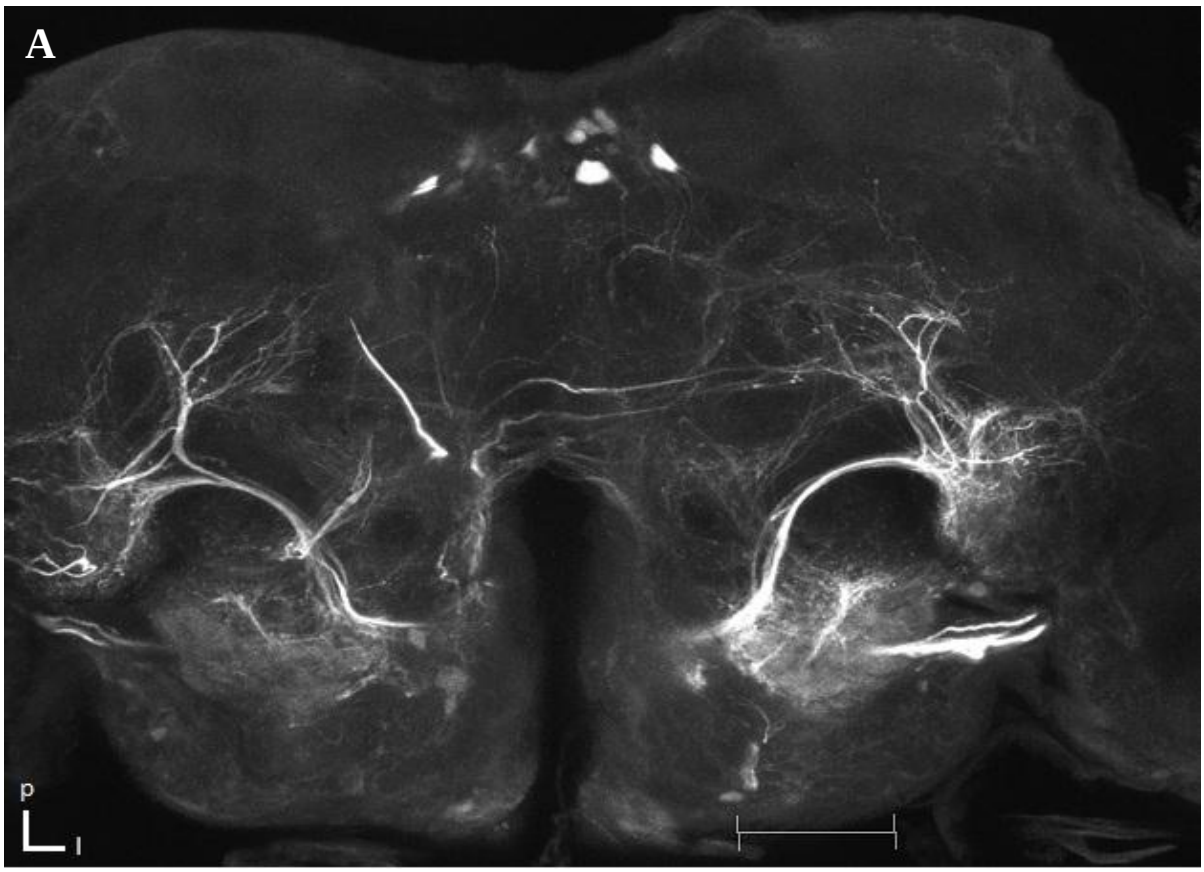


Figure 5: Confocal images (projection views) of neurons with somata in the medial-posterior part of the two protocerebral hemispheres, from three different brain preparations. **A**: Brain preparation 39 (female) **B**: Brain preparation 31 (female) picturing the same cluster type of somata as in A. **C**: Brain preparation 37 (female) also illustrating the same cluster type of somata as A and B. A-C is frontal oriented and scanned with a 20x objective. Scale bars: 100 µm.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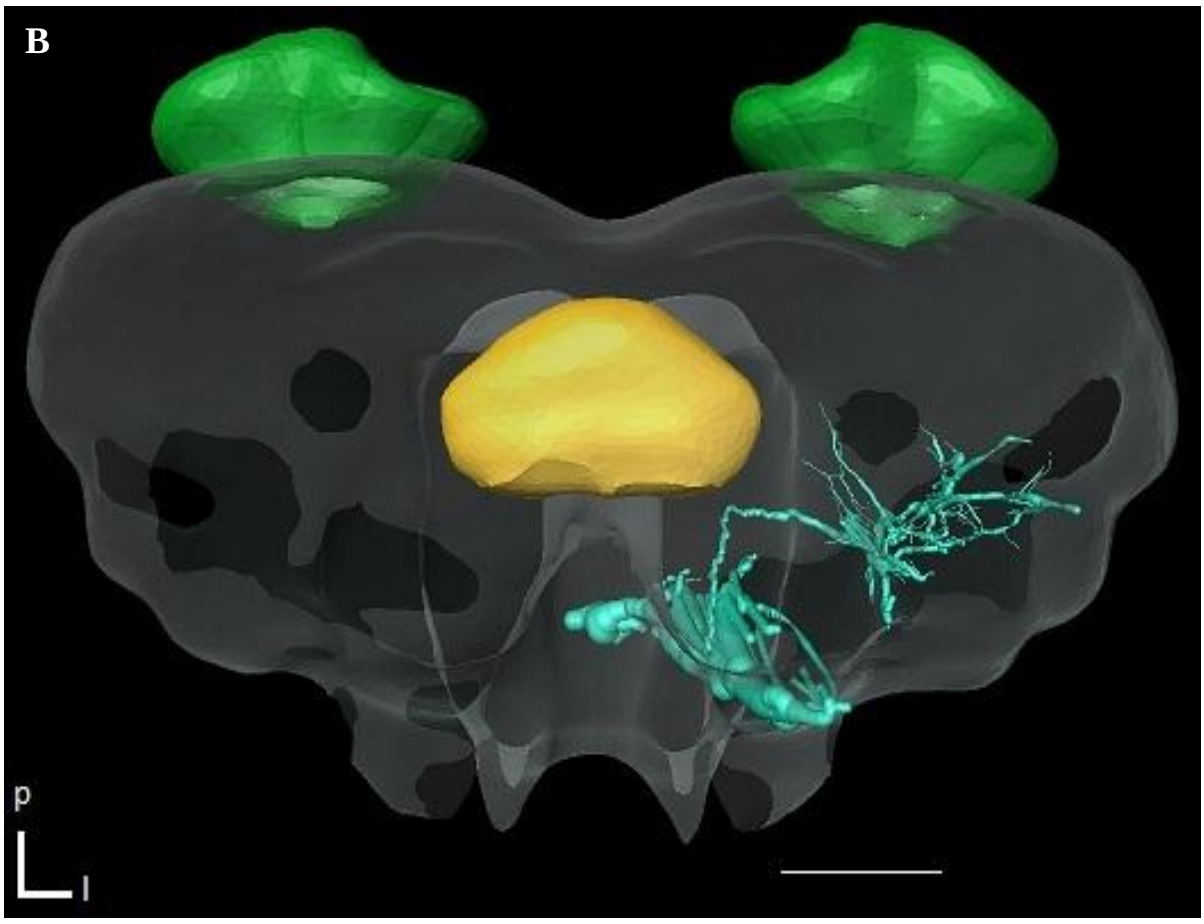
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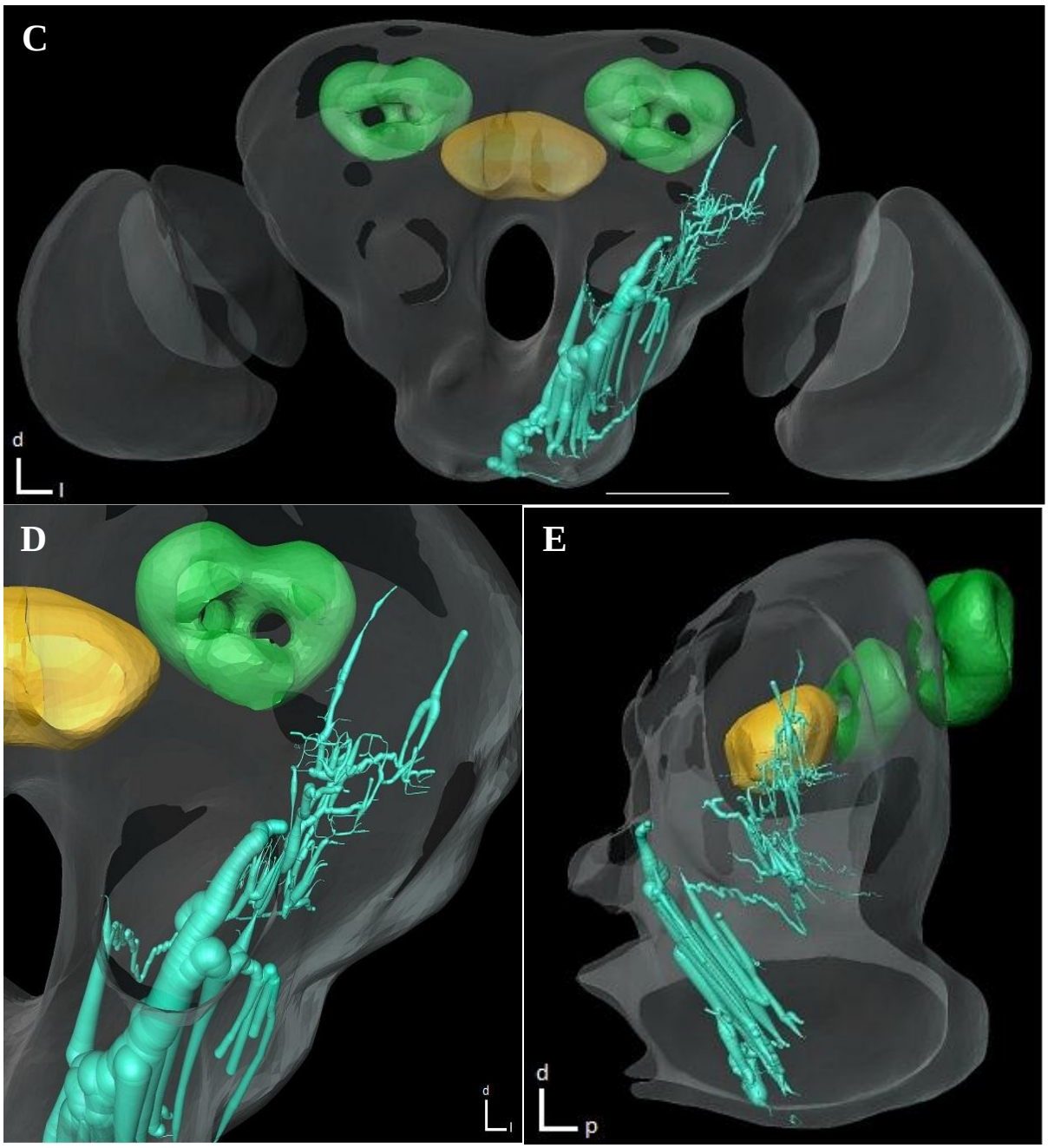


Figure 6: **A**: Confocal image (projection view) from brain preparation 24, stained from the thoracic ganglion with micro- ruby (frontal-dorsal view). Showing the DN- output tract, and projections in the mid-ventral part of LP **B**: Reconstructed DNs (turquoise) from brain preparation 24, registered in the SBA of *H.virescens* **C**: Frontal oriented SBA, gives an overview of the spatial projections of the DNs. Eyelobes are included as landmarks. **D:** Frontal oriented SBA, a section from the LP and the DN projections. **E:** Ventral view SBA, projection pattern of the DNs. (B-D are reconstructed models). Central body in yellow and the mushroom body calyces in green are included in B-D as landmarks. Several other brain- structures are removed for a better visualization of the output areas, including the antennal lobes. Scale bars: 100 µm.

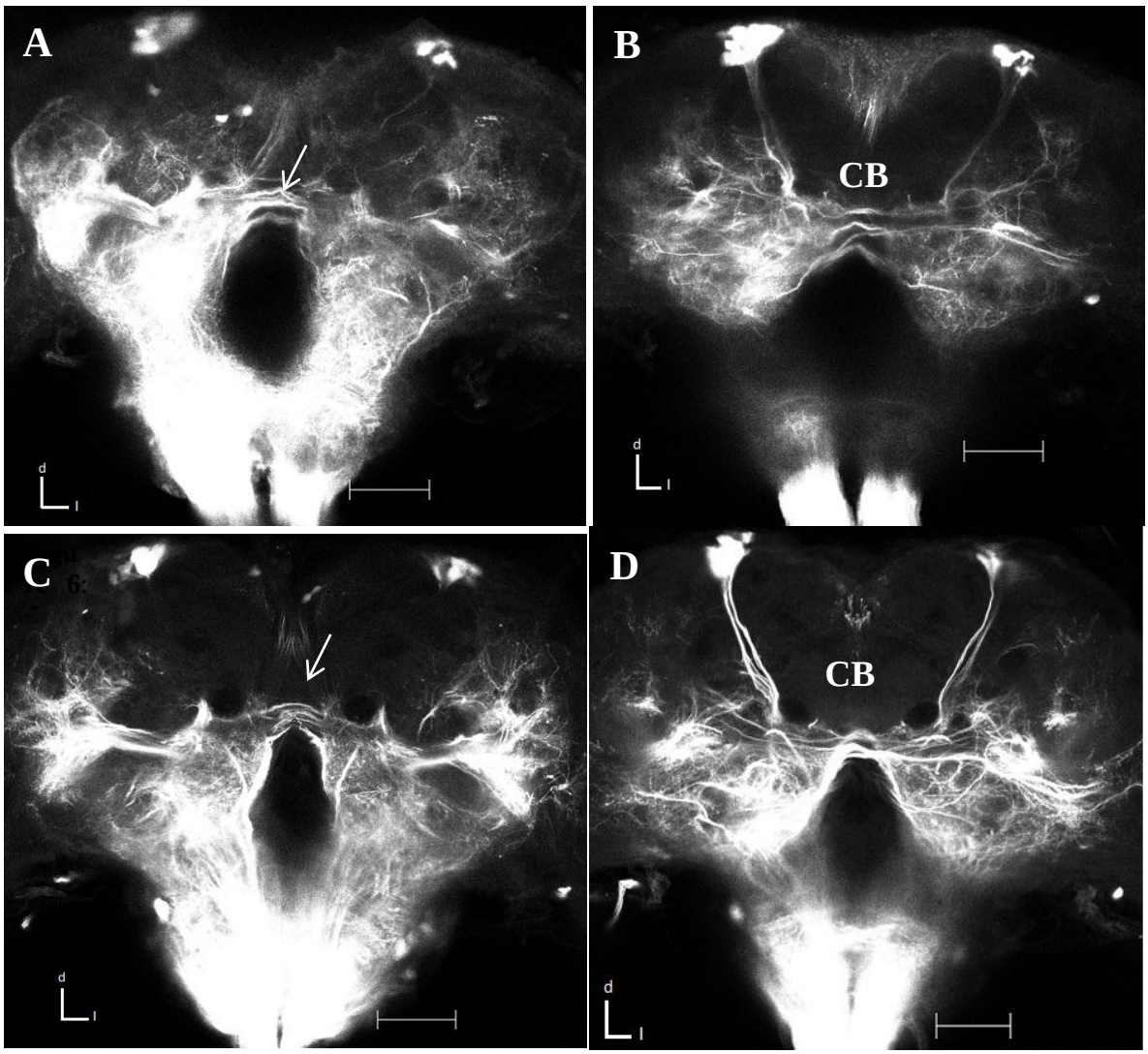


Figure 7: Confocal images (projection views) of stained fibres innervating the lateral accessory lobes (LAL) in respectively female and male. **A**: Brain preparation 49 (female), arrow indicates the LAL- commisure. **B**: The same brain preparation as A, and shows the LAL at its biggest. **C**: Brain preparation 53 (male), arrow indicates the LAL- commisure. **D**: The same brain preparation as C, and showing LAL at its biggest. The cell somata located medial-posterior in the two brain hemispheres is also stained in both preparations. Central body (CB) is named as a landmark. A-D is frontal oriented and scanned with a 20x objective. Scale bars: 100 µm.

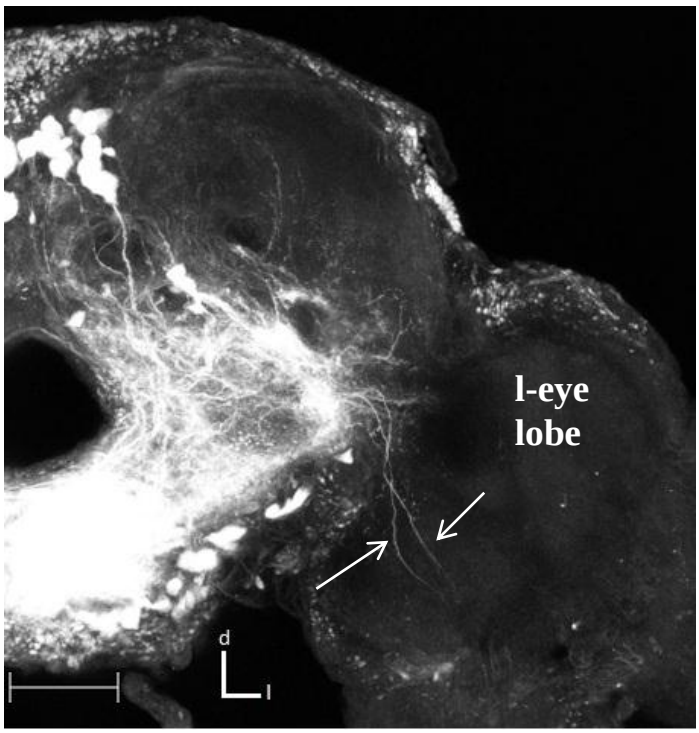


Figure 8: Confocal image (projection view) of two stained fibers (indicated by arrows) from the eyelobe, entering the LP in brain preparation 38 (female). Left eyelobe (l-eye lobe) is marked. The preparation is frontal oriented and scanned with a 20x objective. Scale bar: 100 µm.

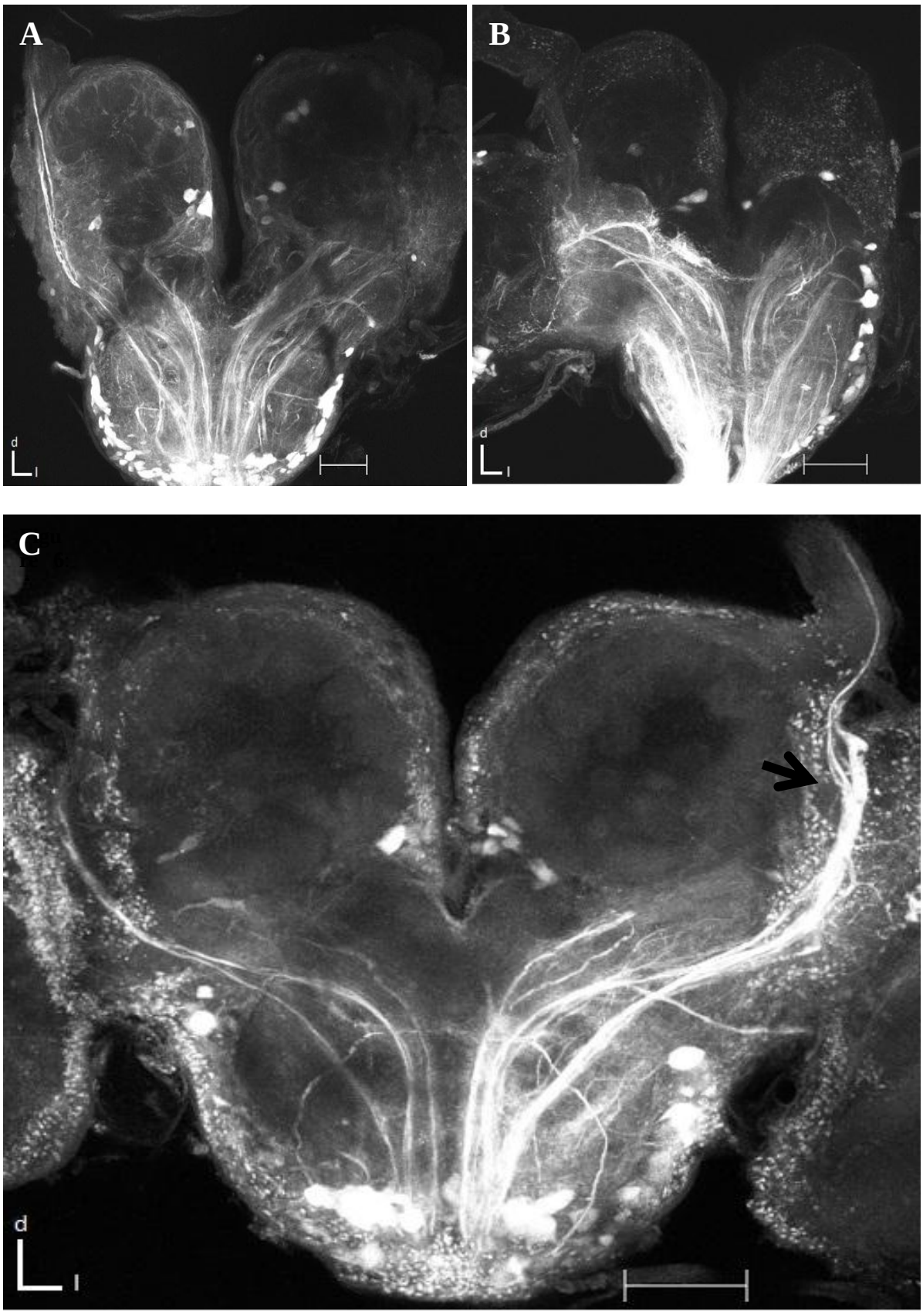


Figure 9: Confocal images (projection views) of so called "giant fibers" (white bundles of axons), projecting from the brain, entering the anterior SOG extending in the ventral cord (not depict) in three different brain preparations. **A**: Brain preparation 22 (female). **B**: Brain preparation 30 (female). **C**: Brain preparation 42 (female). Fibers from an antennal neuron evidently fusing with the giant fibers (black arrow in figure C) can also be seen. A-C is frontal oriented and scanned with a 20x objective. Scale bars: 100 µm.

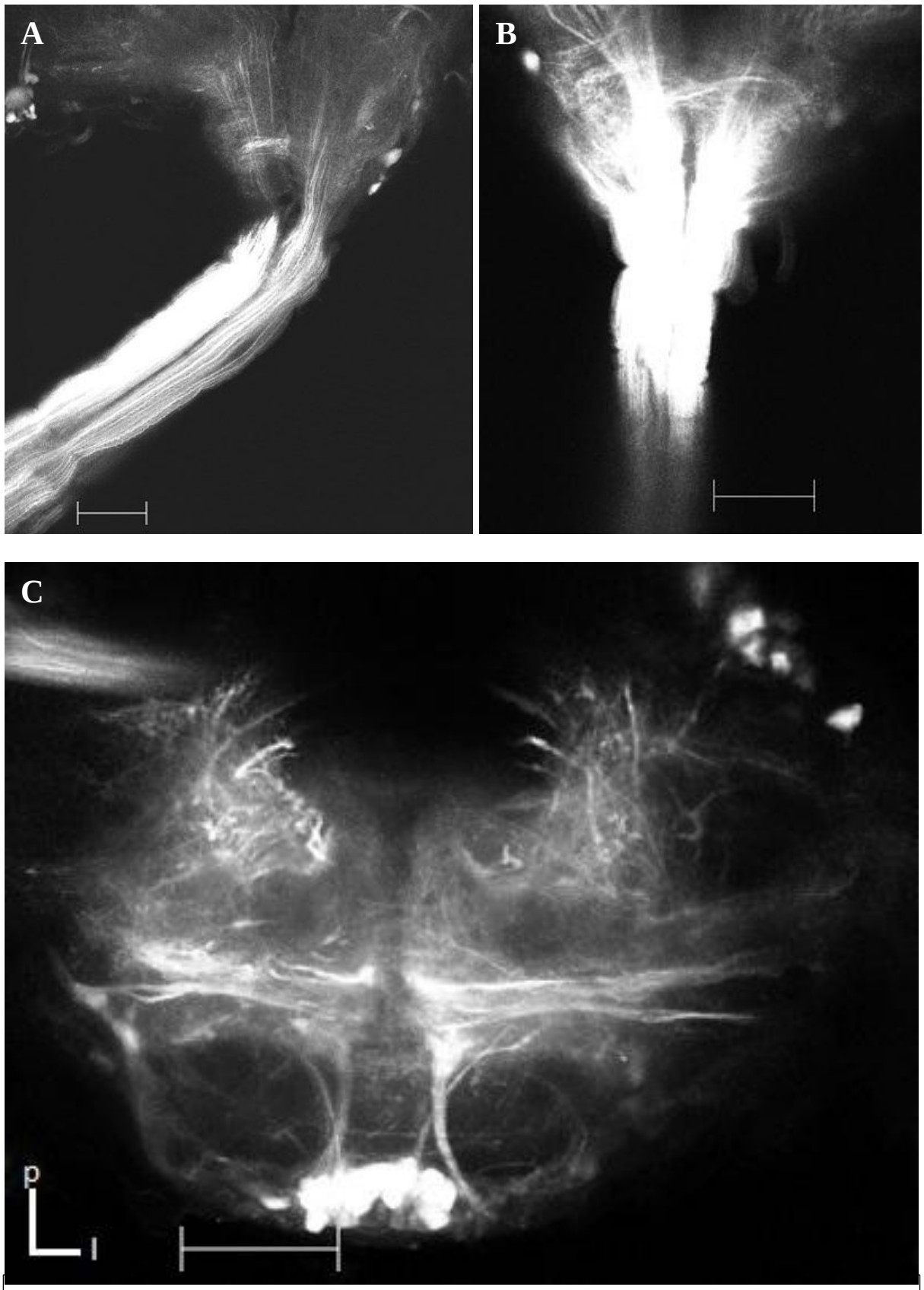


Figure 10: Confocal images (projection views) from two different brain preparations, showing the organization of stained fibres in the ventral cord (VC) and the SOG. **A**: Brain preparation 39 (female) shows the organisation of neurons, projecting through SOG in the VC. **B**: Brain preparation 53 (male) shows the same structural organization of fibre through the SOG and in the VC as A. **C**: Brain preparation 43 (female) shows the organization of stained fibres in the ventral part of SOG. A and B is scanned with ventral side up. C is dorsally oriented and A-C is scanned with a 20x objective. Scale bars: 100 µm.

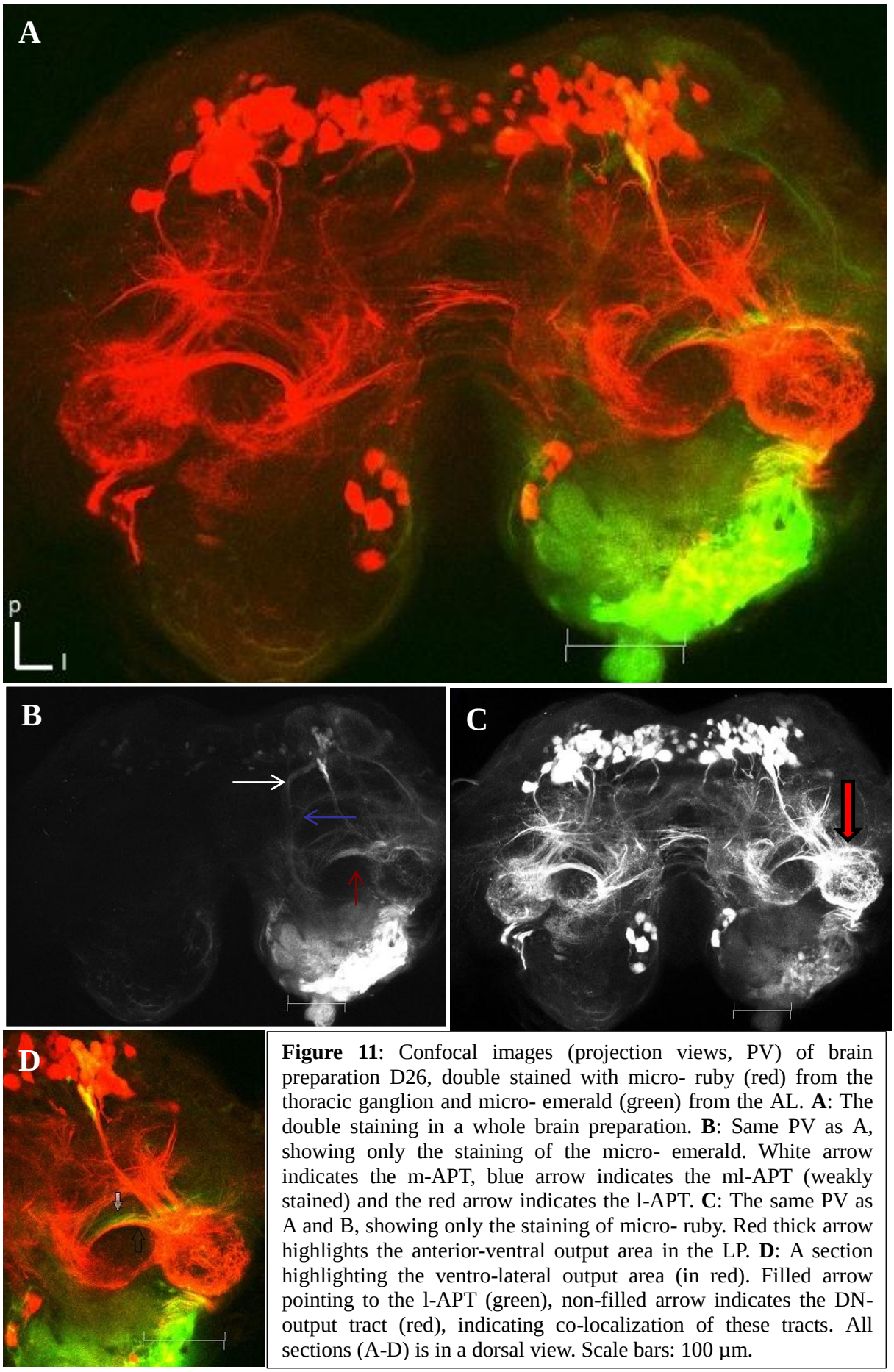


Figure 11: Confocal images (projection views, PV) of brain preparation D26, double stained with micro- ruby (red) from the thoracic ganglion and micro- emerald (green) from the AL. **A**: The double staining in a whole brain preparation. **B**: Same PV as A, showing only the staining of the micro- emerald. White arrow indicates the m-APT, blue arrow indicates the ml-APT (weakly stained) and the red arrow indicates the l-APT. **C**: The same PV as A and B, showing only the staining of micro- ruby. Red thick arrow highlights the anterior-ventral output area in the LP. **D**: A section highlighting the ventro-lateral output area (in red). Filled arrow pointing to the l-APT (green), non-filled arrow indicates the DN-output tract (red), indicating co-localization of these tracts. All sections (A-D) is in a dorsal view. Scale bars: 100 µm.

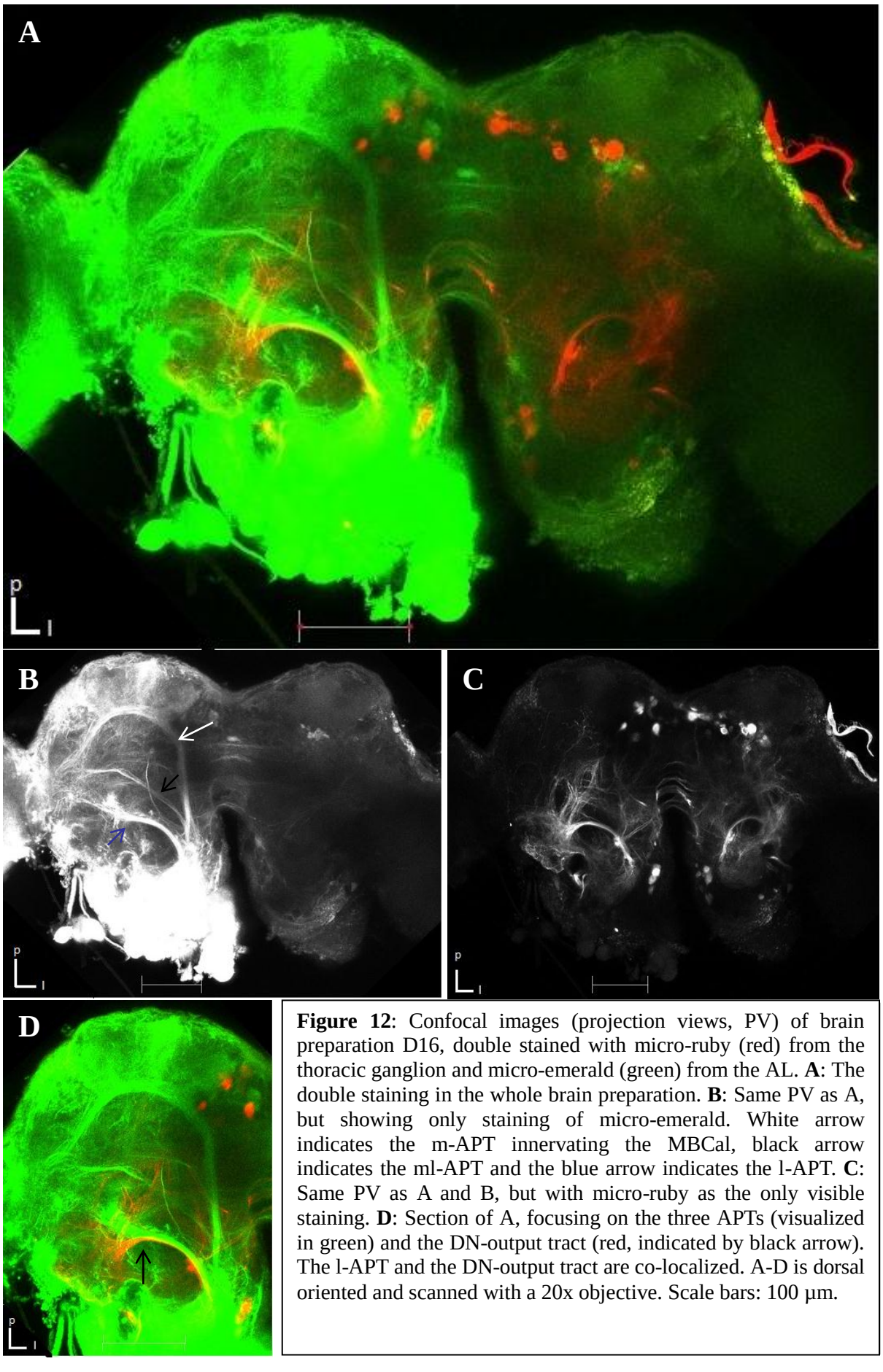
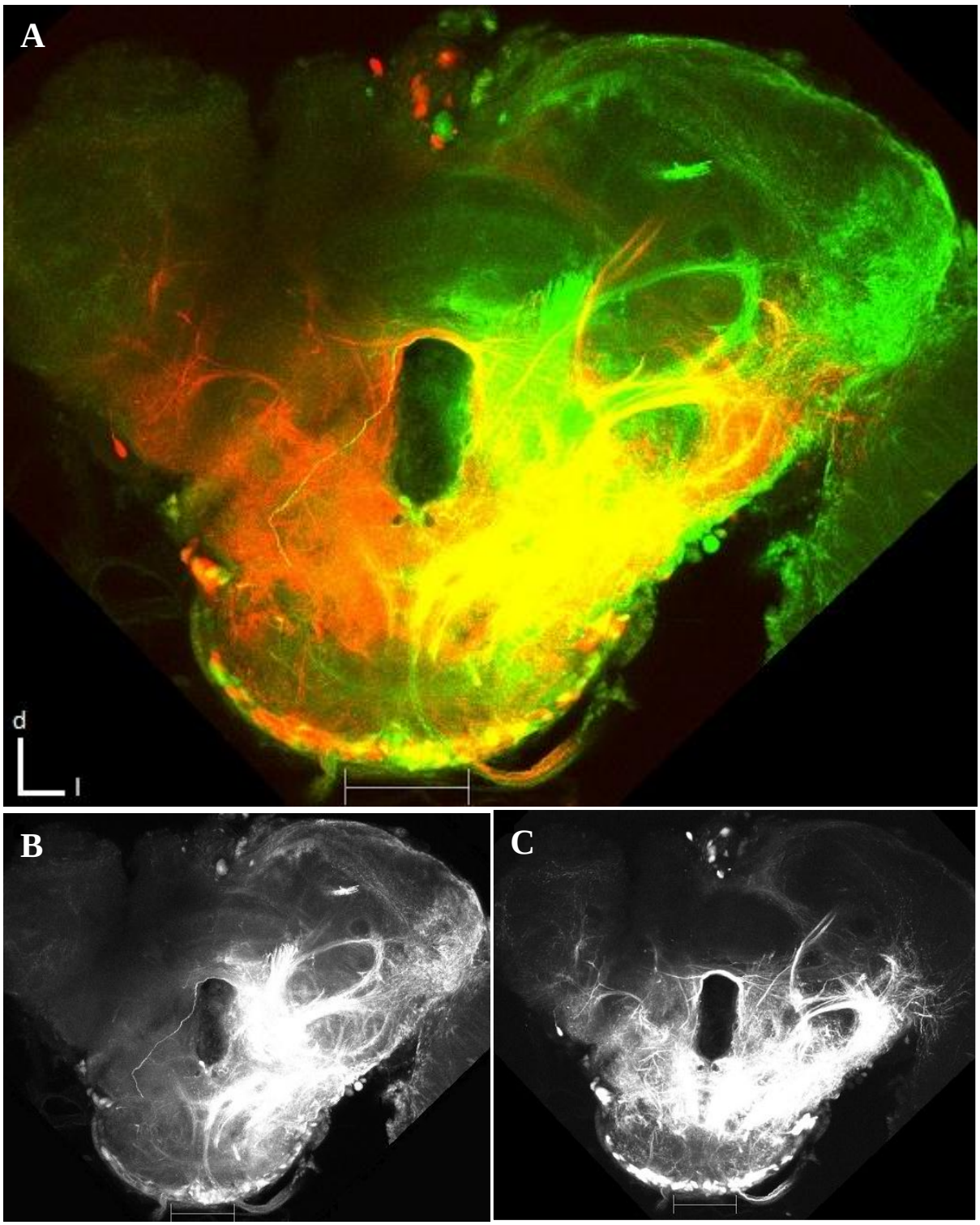


Figure 12: Confocal images (projection views, PV) of brain preparation D16, double stained with micro-ruby (red) from the thoracic ganglion and micro-emerald (green) from the AL. **A**: The double staining in the whole brain preparation. **B**: Same PV as A, but showing only staining of micro-emerald. White arrow indicates the m-APT innervating the MBCal, black arrow indicates the ml-APT and the blue arrow indicates the l-APT. **C**: Same PV as A and B, but with micro-ruby as the only visible staining. **D**: Section of A, focusing on the three APTs (visualized in green) and the DN-output tract (red, indicated by black arrow). The l-APT and the DN-output tract are co-localized. A-D is dorsal oriented and scanned with a 20x objective. Scale bars: 100 µm.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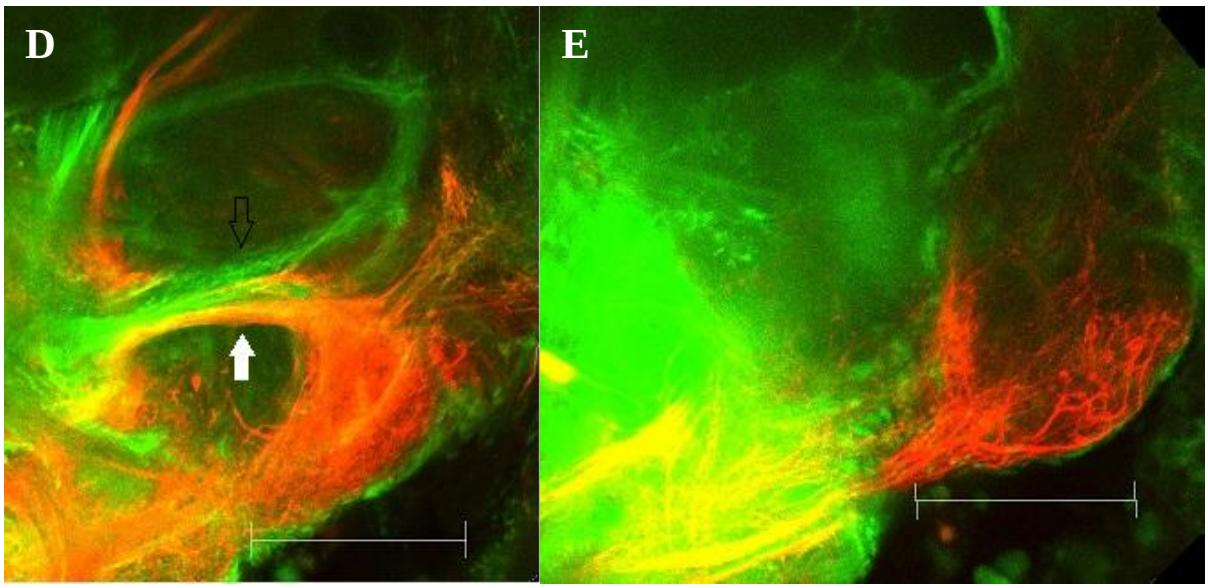

Figure 13: Confocal images (projection views, PV) of brain preparation D28, double stained with micro-ruby (red) from the thoracic ganglion and micro-emerald (green) from the AL. **A**: The double staining in the whole preparation. **B**: Same PV as A, showing only the staining of the micro-emerald. **C**: Same PV as A and B, showing only the staining of the micro-ruby. In addition, a section of the ventral part of LP is shown in **D**, where the white arrow indicates the path of the DN-output tract and non-filled arrow marks the path of the l-APT from the AL. **E**: PV of a section of the anterior-most part of the output area in the anterior-ventral LP. All sections A-E are in a dorsal view. Scale bars: 100 µm

4 DISCUSSION

The present study is the first systematic investigation searching for neurons mediating information out of the brain in *Heliothis virescens* and the results contribute to knowledge on the so called "output areas" in their brain. Applying dye in the thoracic ganglion revealed condensed areas of stained neural fibers in the anterior-ventral part of the lateral protocerebrum and in the lateral accessory lobes. Visualizing areas containing stained neurons with the confocal microscope led to reconstruction and registration of these neuron fibers into the standard brain atlas of *H.virescens*, for determining their relative spatial relationships. The discovery of the dorso-ventral olfactory axis in the LP, in addition to the identification of a descending neuron responding selectively to a multicomponent mixture of odorants by Løfaldli et at (2010; 2012), was the basis for performing double staining from the antennal lobe and the thoracic ganglion, to find out whether there was an overlap between this sensory area and the ganglia. The present study providing no physiological data, obviously do not inform about connectivity and coding of neural information. Though, it provides morphological data regarding the location of output areas in the brain of *H.virescens*, findings that are important particularly since limited knowledge exists on this topic in general.

4.1 General distribution and morphology of descending neurons

The main goal of this study was to determine the output area housing the dendrites of DNs in the brain of *H.virescens.* By injecting fluorescence dye in the thoracic ganglion, neurons connecting the brain with the thoracic ganglia were visualized using the confocal microscope. The results showed condensed arborizations in the LP and LALs, in addition to extensive staining of fibers extending towards the antennae, as well as scattered stainings in the suboesophageal ganglia and other areas in the ventral protocerebrum. Obviously, the neurons stained in the thoracic ganglion contained efferent as well as afferent fibers, as sensory fibers from each body segment projects to their corresponding ganglia and further to the brain (Chapman 1998).

In order to decide which areas in the insect brain mediate premotoric information to the thoracic ganglia, findings and assumptions from previous studies of the central nervous system of *H.virescens* and other insect species have been important to validate whether the areas presented here in fact can be considered output areas. In spite of scarce knowledge on distinct anatomically output areas, neuroanatomical descriptions of DNs in some species are available, but they often stem from dye injections of intracellularly recorded cells in the brain showing only the single neuron output area. Consequently, detailed knowledge about whole brain areas from which populations of DNs originates or projects is sparse. To my knowledge, there are few previous

studies particularly on mass staining from the thoracic ganglion or the cervical connectives. One of the contributions to this topic has been given by Okada et al (2003) who examined the distribution of somata and dendrites of DNs in the brains of cockroaches (*Periplaneta amaricana*) by filling the axons from their cervical connectives. They highlighted that dendrites of DN are hard to distinguish from axons of the ascending neurons. Though, by high- magnification light microscopy they observed single DNs and ANs, where the dendritic arborizations of DNs very often were rich in spines and the terminal arborizations of ANs had more varicose- rich neuritis. This is a micro structure distinction often used to distinguish between axon terminals and dendrites of insect neurons (Strausfeld & Camposortega 1977). In the present work, specific input and output structures of the terminals was not visible.

4.1.1 Number of stained somata in the brain of *H.virescens*

DNs could have been ascribed to the numerous, clearly stained somata in protocerebrum and the SOG (figure 3; 4; 5; 11). Unfortunately it was difficult, if not impossible to resolve which fibers belonged to each soma, since the connection between their dendrites and axons could not be resolved. Thus, in massively stained preparations, determination of the exact number of neurons is hard. The number of stained somata in *H.virescens* was quite low (counted to about 50) compared to what is found in cockroaches and crickets. Okada et al (2003) gives a number of 235 pairs of DNs in the cockroach, a number in the same range as Staudacher (1998), presenting approximately 200 pair of neurons descending from the brain to influence motor circuits in the thoracic ganglion in crickets. The reason for this discrepancy may be due to species-specific differences, but could also be a result of the difficulty of separating one soma from another in the present study. The number of DNs presented in *H.virescens* may therefore be an underestimate. Species-specific differences seem to be present concerning the distribution of DN somata. Whereas somata were found only in the dorsal and ventral part of the cricket brain, they were in *H.virescens* spread from central positions (e.g. around the mushroom body calyces) to the periphery of the SOG. However, the presence of somata as mirror-image pairs, stated by Staudacher (1998) for the crickets, was also found in *H.virescens* (all figures). Both location of somata and the neural arborizations seem to be bilateral-symmetrical. Evidence that the ventral cord is divided in two parts suggests that the mirror- image pairs in the two hemispheres descends in their separate half of the ventral cord (figure 7; 10).

4.1.2 Other stained neurons in the brain of *H.virescens*

In addition to the condensed stained areas in the LP and the LAL of *H.virescens*, scattered stained fibers were seen in other areas of mainly the ventral LP. In regards of this, an interesting observation was the occurrence of no stained fibers in any of the distinct neuropil like the central body, the mushroom bodies or the AL, an observation in accordance with the study by Staudacher

(1998), who backfilled cervical connectives of crickets and discovered that their DNs only arborized in the nonglomerular neuropils of the brain. The same organization of DN arborizations has also been showed in cockroaches, by staining from the ventral cord (Okada et al 2003).

4.1.3 The lateral protocerebrum

The LP is considered a higher olfactory center, receiving olfactory information from projection neurons of the AL via antenno protocerebral tracts (Galizia & Rossler 2010). In addition it has been considered a premotoric area from where DNs mediate the output information and therefore being one of the main output regions of the insect brain (Hammer & Menzel 1995; Strausfeld et al 1984). Although the quality of the staining varied between brain preparations in this study, the majority showed a distinct and condensed arborization pattern of stained fibers in the anterior- ventral part of the LP. In fact, this area corresponds to the anterior-ventral area of LP containing the dendrite arborizations of the plant odor mixture responding DN, identified in the *H.virescens* female brain (Løfaldli et al 2012). Thus, the present results support the notion that LP contain the output region of insect brains, and that this region is specifically located in the anterior-ventral area, as suggested by Løfaldli et al (2012). The results of the present study showed that the output area in the LP of *H.virescens* extended in an anterior-posterior direction, almost from the periphery of the anterior-ventral LP to the mid-ventral LP. This mid-ventral position was validated by the three dimensional reconstruction of stained fibers and the subsequent registration into the standard brain atlas of *H.virescens* (figure 6). Analyzing the confocal z-stack scan of several preparations, revealed what seems to be a common pathway or tract for DN axons from the brain to the thoracic ganglion. This descending neuron- output tract was positioned medially to the dendritic branching area in the ventral LP, extending anterior before running ventrally through the SOG and into the ventral cord. No other central descending pathways/tract could be seen in the protocerebrum, suggesting that this DN-output tract contain all output fibers from the LP and the LAL to the thoracic ganglion.

Identification of the olfactory axis, made up by projection neurons in the m-APT and the ml-APT (Løfaldli et al 2010), raised the question whether information from these sensory pathways directly transmit information to DNs in the LP. If this was the case, one might find DN dendrite arborizations in the olfactory axis. By injecting two different dyes in the AL and in the thoracic ganglion respectively, the sensory projections and the DN dendrite arborizations was visualized as different colors in the confocal microscope. Although the projections of single PNs and DNs could not be resolved, the result showed the position of all AL- projections relative to the dendrite arborizations of the DNs. These results indicated no or minimal overlap between the olfactory axis and dendrites of DNs. Three dimensional reconstruction of the posterior-most part of the output area

in the LP was registered into the SBA of *H.virescens* and visually compared with the position of the olfactory axis registered in the SBA by Løfaldli et al (2010). This supported the hypothesis that the two areas are spatially separated, perhaps with exceptions of some overlapping medial fibers. Registration of both areas into the same SBA would have given a definite answer to this. Though, this result suggests that the information from olfactory PNs following the m-APT and the ml-APT to DNs are not directly transmitted to DNs, but are probably mediated by local interneurons in the protocerebrum, so called 3rd order neurons. This is in accordance with findings by Okada et al (2003) who discovered that DNs, although having widespread arborizations in the cockroach brain, did not arborize in the postero-ventral part of the lateral horn, the termination area of major types of olfactory PNs from the AL.

Establishing that PNs from the m-APT and the ml-APT from the AL did not overlap with dendrites of DNs, it was interesting to see whether PNs in the l-APT showed the same pattern. Several studies have indicated that the l-APT is a somewhat diffuse tract with fibers terminating at different locations along the pathway possessing both uni-and multi- glomerular properties, and that the functional organization is unclear (Homberg et al 1988; Rø et al 2007). Staining of the AL in the double- stained preparations revealed diffuse projection of fibers following the l-APT with fibers seemingly terminating along their path at different locations in the LP of *H.virescens*. However, several studies are needed to reveal whether single PNs in the l-APT do terminate along the tract and whether some of them could be in direct contact with DNs.

An evident finding in the present study was that the DN-output tract and the l-APT from the AL were partly co-localized in the LP (figure 11; 12; 13). As shown by figure 12D and 13D, the two tracts follow the same path, although with the DN- output tract holding a more ventro-lateral position than the l-APT coming from the AL. In spite of the co-localization, the present findings showed that the majority of l-APT PNs terminated in other areas of the LP than the anterior- ventral LP, housing the DN dendrites. Co-location of tracts mediating afferent and efferent information is not an unusual organization and may probably save space in the small brain of insects. It will be interesting to find out whether a similar co-localization exists in other insect species.

4.1.4 Lateral accessory lobes

Injecting dye in the thoracic ganglion also gave a remarkably staining pattern in two spherical structures, located anterior and ventro-lateral of the CB in the brain of *H.virescens.* These distinct structures, termed the lateral accessory lobes, are in this study for the first time identified in *H.virescens,* as the position corresponded to the anatomical description of LALs in other species,

including moths (Kanzaki et al 1991b). The LALs are considered as accessory structures to the central complex to which they are well connected. It is generally accepted that LALs and their ventral adjacent LP are innervated by branches of odor responsive DNs with axons in the ventral cord innervating motor neurons in the thoracic ganglion (De Belle & Kanzaki 2010; Kanzaki et al 1991a; b).

The present work was mainly performed on female moths, because the intracellular recordings and the SBA of *H.virescens* are based on the female brain. However, at a later stage male brain preparations were included because of the following reasons. The staining of the LALs in the female brain was relatively weak, suggesting the LAL to be less innervated by DNs in females. In addition, was the awareness that LAL is involved in sex-specific pheromone information processing and behavior as shown in moth species, including *H.virescens*.

When a conspecific female moth releases a sex pheromone blend, the male respond with arousal and an upwind flight toward the female (Kanzaki et al 1991a). This characteristic behavior is thought to be reflected in information processing about the pheromone blend in brain neurons. Following the synaptic processes from the AL to the protocerebrum, the authors discovered that many olfactory protocerebral neurons innervated a neuropil region lateral to the central body in each protocerebral hemisphere (LAL), possibly being the location where integrated information about the pheromone-blend takes place. DNs overlapping with the LAL and the arborizations of olfactory protocerebral neurons are thought to be multimodal and integrate information about pheromones and other sensory modalities. DNs innervating the LAL were found to exhibit a long lasting excitation (LLE) when a mixture of pheromones was applied to the antennae. However, a direct relationship between LLE in DNs and the activation of motor neurons remains to be established in *M.sexta*, partly because the projections of the DNs in the ganglia could not be resolved (Kanzaki et al 1991a). Irrespective of how the DNs activate motor circuits in the ganglia, the results in *M.sexta* reveals that DNs overlapping with the LAL may have a specific role in mediating behavioral relevant information. It was therefore interesting to include the male brain of *H.virescens* in the present study, to find out whether they were more extensively innervated by DNs than the female LALs. Figure 7 depicts the LALs in one female and one male brain, showing that the male LAL has indications of more DN arborizations, especially in the medial parts, than the female moth. The increased numbers of DNs in this area indicate that the male LAL have a higher potential for signal transmission to DNs than female LALs. This also reflects the importance of LAL in processing especially pheromone information in the male. However, these results need to be validated by more experiments, to sort out whether the difference is really sex- specific or due to

individual or methodological variations.

4.1.5 The antennal mechanosensory and motor center (AMMC)

A direct connection between the thoracic ganglion and the antennae was shown as massive stained fibers extending from the thoracic ganglion to the antennal nerve. These neurons seemed to project from the ventral cord, entering the ventral SOG, bypassing the AL postero-laterally before extending in the antennal nerve. Staining the thoracic ganglion also revealed clear staining of fibers in the AMMC. The position of AMMC described by Jørgensen et al (2006) as being located posterior-ventral of the AL was in accordance with the findings in the present study. Jørgensen and coworkers stained contact chemosensilla on the antennae of *H.virescens* and the results showed a group of stained axons following the antennal nerve, bypassing the AL postero-laterally, with some having fan-shaped projections in the AMMC. In addition, other stained fibers projected in the dorsal SOG from where single fibers extended into the ventral cord, suggesting projections into the thoracic ganglion. These results fully correspond with the massive stained fibers between the thoracic ganglion and the antennal nerve shown in the present study. Not knowing which of the fibers is ascending or descending, it might be possible that some of the stained fibers seen in the brain in the present study are sensory fibers from the tarsi or ovipositor extending to the brain. To my knowledge, the AMMC has never been regarded as a main "output area" in the brain of insects. However DNs in flies and other species have shown to have arborizations in the AMMC (Homberg et al 1989). In fact, Bacon & Strausfeld (1986) identified a pair of extraordinary giant diameter descending neurons (GDNs) in the brains of *Musca domestica* and *Calliphora erythrocephala*, which seemed to directly receive input from antennal mechanosensory fibers. In accordance with these findings, the present study also revealed a group of characteristically large DNs projecting towards the AMMC and antennae in several preparations (figure 2 and 9). The neurons were termed "giant fibers" according to the large axon diameter, described in Diptera. (Strausfeld et al 1984; Strausfeld & Bacon 1983). Whether the large axon-neuron shown in this study can be considered as corresponding with the giant descending neuron- system described by Strausfeld and coworkers cannot yet be concluded. However, they described the dendrite arborizations of the GDNs in Diptera to project to anterior parts of the SOG like the giant neurons in the present study did. The present giant fibers were bi-lateral with at least one pair of fibers extending from the mid-line in the SOG towards each lateral side. Regarding the functional role of GDNs, it has been suggested that they possess important functions in activating specific motor circuits in the ganglion as well as being essential for flight initiation (Bacon & Strausfeld 1986). Although staining of the thoracic ganglion resulted in visualization of both ascending and descending fibers in the present study, it seems likely that the giant fibers are descending neurons.

4.2 Structural organization can have behavioral implications

Although the results in the present study is gained from morphological investigations of DNs, the data can contribute to an increased understanding of the structural and functional connectivity of the sensory neural pathways to motoric output in the brain of *H.virescens*, and will briefly be acknowledged in the following section.

4.2.1 Local protocerebral neurons

Understanding the spatial connectivity between neurons mediating chemosensory information to motor neurons leads us one step closer to decipher the pathway from sensory input to motoric output. This study has investigated whether olfactory information could give directly input to DNs and the results suggested that this were not evident. However, information can be conveyed between neurons in other ways than by direct transmission with spatial overlap. Løfaldli et al (2012) presented a putative circuit that suggested how the brain of *H.virescens* handles information about a ten component plant odor mixture. Their results have shown that information about this blend is parallel processed and mediated to the LP, the MB complex and the superior protocerebrum. A putative 3rd order neuron responding to the ten component blend had dendrites in the olfactory axis of the LP and axonal projections in the SP, implying that this neuron might be positioned to integrate information about this blend from the APTs and convey this information to the SP (Løfaldli et al 2012). In addition, efferent MB extrinsic neurons responding to the same plant odor mixture showed axonal projections in different areas, suggesting their capability of conveying information about the same compound from the associative networks of MB to higher olfactory areas like LP and SP in *H.virescens* (Løfaldli et al 2012). The findings by Løfaldli et al (2012) suggest that there exist local interneurons in the LP that might modify and integrate olfactory stimuli in a highly complex matter before neural activation of DNs. 3rd order lateral horn neurons have been found by genetic labeling in *D.melanogaster*, revealing what is believed to be LHNs receiving input in the LH, sending axonal projections to widespread areas of the brain, like the superior medial and superior lateral protocerebrum, the ventro-lateral and ventro- medial protocerebrum, in addition to areas of the AMMC, supporting the assumption that local interneurons have the possibility of integrating sensory stimuli before information is transferred to DNs (Jefferis et al 2007; Tanaka et al 2004).

4.2.2 The lateral protocerebrum receives information from several modalities

Although the LP of *H.virescens* and the LH in other species receives major olfactory input from the AL via the APTs, relatively little is known about both function and connection of neurons in this area, other than the consideration that the LP represent a naïve or inexperienced odor processing

route from AL to motor control (Hammer & Menzel 1995; Heimbeck et al 2001; Heisenberg et al 1985; Homberg et al 1988; Galizia & Rössler 2010; Kirschner et al 2006; Løfaldli et al 2010,2012; Rø et al 2007). However, what is known is that the LP in *H.virescens* has been shown to receive input from several modalities, not only olfactory information along the dorso-ventral axis. Ventral areas of the LP are found to receive gustatory information (Kvello et al 2009; Løfaldli et al 2010). In addition to olfactory and gustatory input, the LP also receives auditory information. Intracellular recordings have shown neurons in the anterior-ventral part of LP, responding to auditory stimuli in three species of Lepidoptera; *Helicoverpa armigera*, *Helicoverpa assulta and H.virescens* (not published). The similar location and arborization pattern of these auditory neurons and the DN dendrites presented in this study suggest the possibility of spatial overlap between them. This location might imply the importance of auditory stimuli in eliciting motor responses mediated by DNs (Gerit Phühl, personal communication). In fact, the ear of the noctuid moth appear to serve one single purpose; detection of the ultrasonic cries of bats, emphasizing the importance of auditory information in the escape from predators (Surlykke 1984). The ear of the moth is located in close proximity of the thoracic ganglion, where sensory fibers from the ear synapse on interneurons in the ganglion (Bente Berg, personal communication). The projection pattern of neurons from the ear to the brain in *H.virescens* is not known and needs to be established in order to separate between the arborizations of ascending auditory fibers and DNs in the anterior-ventral part of LP.

Regarding visual input to the LP, neurons mediating visual information from the lobula and the lobula plate in the optic lobes to the lateral midbrain have been described in Dipterous insects (Nässel & Strausfeld 1982). The exact localization of these neurons in the lateral midbrain is not described in their study. Thus, it is not possible to say with certainty whether their axonal arborizations is in close proximity to the other sensory modalities or DNs that terminate in the LP. However, a unique neuron has been seen to project directly from the lobula complex of the optic lobe to the thoracic ganglia of Diptera (Nässel & Strausfeld 1982). The authors named this single neuron lobula descending neuron (LDN), where its dendrites were seen in the lobula and their axonal branches projected to the lateral midbrain, before extending into the contralateral part of the ventral cord, towards the ganglion (Nässel & Strausfeld 1982). A similar morphological neuron was found in the present study (figure 8). Although the projection in the mid-LP was impossible to separate from other stained neurons, the thin dendrites in the optic lobe were clearly seen. Findings of a DN in the optic lobe are in contrast to Staudacher (1998), who stated that DNs never arborized in the optic lobes of crickets. Thus, it is necessary to perform more studies to investigate specifically whether the optic lobe-DN in the present study is similar to the LDN described in Diptera by Nässel & Staudacher (1982).

4.3 Concluding remarks

The results presented in this study revealed the positions of DNs thought to mediate information out of the brain in *H.virescens*. The major condensed areas of DN dendrites were located in the anterior-ventral LP as well as in the LAL, but scattered projections of DNs in other areas of the ventral LP were also seen after staining the thoracic ganglion. It is believed that DNs are mediators of a multimodal signal, giving input either directly or indirectly to motor circuits in the thoracic ganglion (Bacon & Strausfeld 1986; Kanzaki et al 1991a; Okada et al 2003; Staudacher 1998; Strausfeld et al 1984). The positions of DNs shown in the present study as well as in other studies, clearly suggest that they might have potential to receive information from different sensory modalities that terminates in the protocerebrum, as well as preprocessed information by local protocerebral neurons. The results presented here suggest that axon terminals of olfactory PNs do not spatially overlap with DNs, whereas findings of apparently specialized lobula-DNs seem to imply the opportunity for direct transfer of visual information to the ganglia. It could be a common strategy that different types of DNs receives sensory information either directly by spatial overlap, or indirectly mediated by local protocerebral neurons (Strausfeld et al 1984). In either way, the number of DNs in the brain of insects is approximately 0, 3 – 1, 5 per thousand neurons of the brain in total (Strausfeld 1976), suggesting an enormous degree of preprocessing and convergence onto DNs (Staudacher 1998). Studies have also suggested that some DNs have the properties of integration with each other as well (Strausfeld & Bassemir 1983). This highlights the necessity of gathering physiological data of more DNs since morphologically identified positions in close proximity, does not automatically mean that there exists a functional connection between the neurons of concern. As mentioned introductory, an aim at our research group is to reveal the link between sensory input and motoric output. Although no direct connection between the olfactory sensory system and the DN dendrites was discovered, the described output areas in the anterior ventral LP and LAL can be central in further investigating the occurrence of local protocerebral interneurons potentially transmitting preprocessed input to the dendrites of DNs before a motoric response is elicited.

4.4 Methodological considerations of the study

Since all DNs terminate in the thoracic ganglion or projects through to the next ganglion in the chain, it was thought than the chance of visualizing all these fibers was present if the ganglion was stained. However, the dye was placed in the center of the ganglion, and if there are fibers entering the ganglion extremely peripheral, it might be a chance that these fibers have not been stained. Cutting the ventral cord dorsal of the ganglion and staining separate parts might resolve this potential bias.

Because the preparations showed massively stained fibers, it was sometimes impossible to separate between each stained fiber in the LP. This was the reason why several DNs were treated as a unity during the three-dimensional reconstruction into the SBA (figure 6). This might have contributed to the inscription of wrong morphology to some of the neurons reconstructed.

5 CONCLUSION

The main goal of this study was to morphologically identify the output areas in the brain of *H.virescens*. By placing fluorescent dye in the thoracic ganglion, condensed stained fibers were seen in the anterior ventral part of the lateral protocerebrum and in the lateral accessory lobes. The majority of preparations used in this study showed this particular innervation and together with previous findings of single neuron output, the present results suggest that these areas are the main output areas in the brain of *Heliothis virescens*. Thus, these findings have provided answers to hypothesis 1 and 2 presented introductory. To answer hypothesis 3 whether olfactory projection neurons in the olfactory axis overlapped with the dendrites of descending neurons, double staining from the antennal lobe and the thoracic ganglion was performed in the same individual. Visually comparing the reconstructed position of the anterior-ventral output area in the standard brain atlas of *H.virescens*, with the position of the olfactory axis registered in a separate standard brain atlas, indicated no or minimal spatial overlap between these areas. In addition, analyses of the z-stack scans from the confocal microscope suggest that neither projection neurons in the l-APT overlapped with dendrites of descending neurons. Thus, no direct connection between the olfactory sensory system and descending neurons were seen, suggesting that local protocerebral interneurons potentially might transmit integrated information to descending neurons before a motoric response is elicited in the thoracic ganglia.

The lateral protocerebrum is frequently mentioned as a premotoric area. However, if a criterion to be regarded as such an area is innervation by DNs, the present findings advocate for the necessity to be area-specific when mentioning the lateral protocerebrum as a premotoric area.

6 ABBRIEVATIONS

OR ~ Olfactory receptor

ORNs ~ Olfactory receptor neurons

AL ~ Antennal lobe

MGC ~ Macroglomerular Complex

PNs ~ Projection neurons

LNs ~ Local interneurons

LP ~ Lateral protocerebrum

LH ~ Lateral horn

LHNs ~ Lateral horn neurons

SOG ~ Suboesophageal ganglion

DNs ~ Descending neurons

ANs ~ Ascending neurons

l-APT ~ lateral antenno protocerebral tract

ml- APT ~ medio-lateral antenno protocerebral tract

m-APT ~ medial antenno protocerebral tract

AMMC ~ Antenno mechanosensory and motor center

CB ~ Central body

MbCal ~ Mushroom Body Calyces

VC ~ Ventral cord

SBA ~ Standard brain atlas

KC ~ Kenyon Cell

GABA ~ Gamma amino butyric acid

LDN ~ Lobula descending neuron

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Per Harald Olsen, NTNU has provided me with the photography for the front page cover.

APPENDIX I

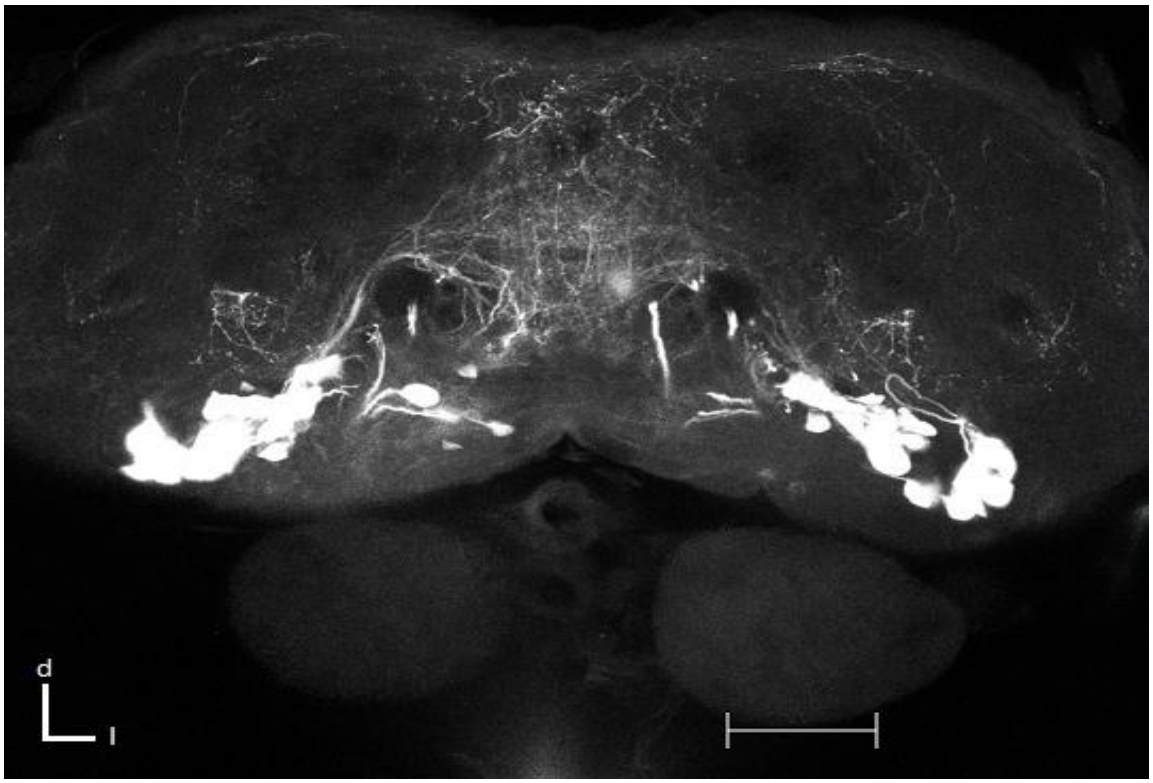

Figure A1: Confocal image (projection view) from brain preparation 53 (male) Staining in the thoracic ganglion revealed somata postero-ventrally of the LAL. The section is in a frontal view. Scale bar: 100 µm

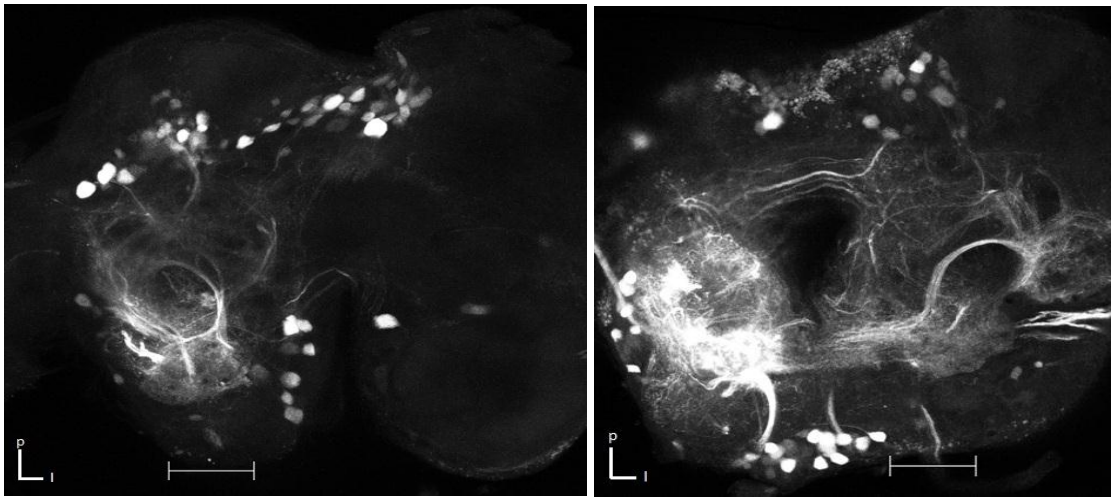

Figure A2: Confocal images (projection views) from brain preparation 41 (female). Staining of the thoracic ganglion revealed somata in different locations in the brain. Both sections are in a dorsal view. Scale bar: 100 µm

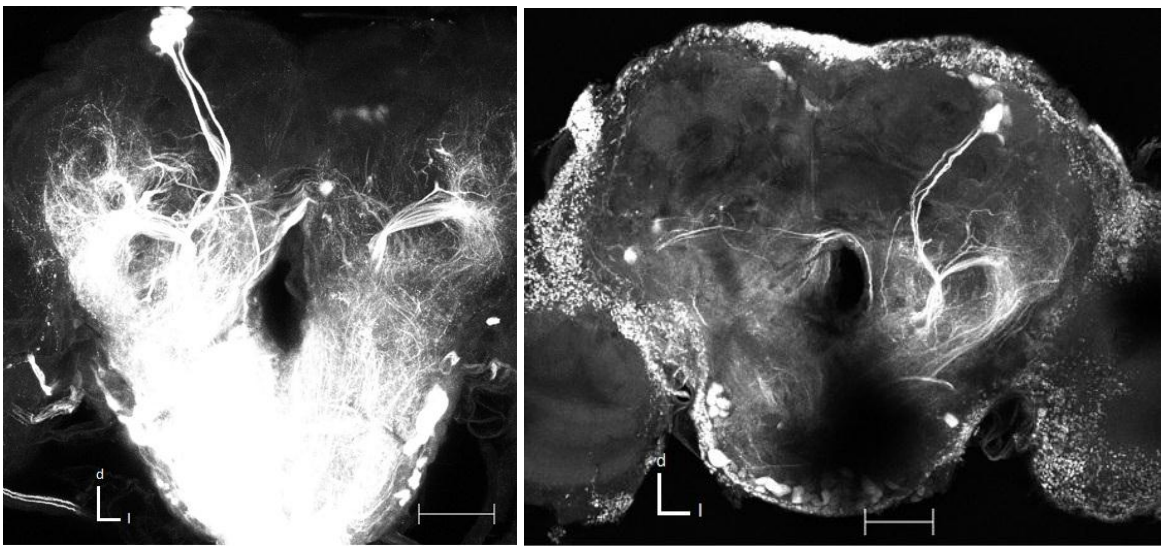

Figure A3: Confocal images (projection views) from preparation 51 and 34 respectively (females). Staining of the thoracic ganglion revealed the particularly well stained cluster of 5-10 somata, located peripherally in the medial-posterior part of the two protocerebral hemispheres. Sections are in a frontal view. Scale bar: 100 µm

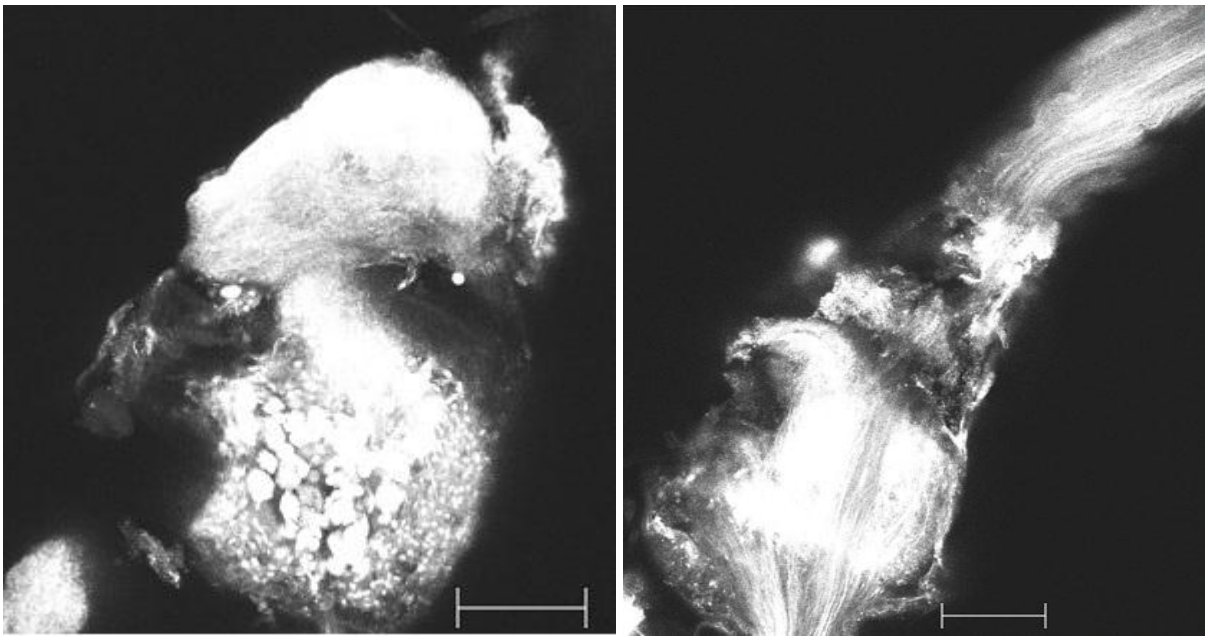

Figure A4: Confocal images (projection view) from the ganglion in preparation 30 (female). Parts of the ganglion have been destroyed because of dye application. Small somata can be seen, as well as projections both entering from the brain and leaving the ganglion ventrally. Scale bars: 100 µm

APPENDIX II

The material and methods presented in the main section was the final methods used after a long trial of optimization procedures. In the present section, all dyes, different ways of applying it and all protocols tested, will be presented.

Dissection:

Before using special made insect needles to stain both the AL and ganglion, glass electrodes was used, pulled with a Flaming- Brown horizontal puller (P97; Sutter Instruments, Novato, CA, USA). The tip of the glass electrode was broken and color crystals of the dye were applied onto it. The electrode was manually maneuvered and remained in the thoracic ganglion for several seconds to allow the dye crystals to be taken up by the neurons.

Five different dyes were tested for optimally performing double-staining experiments.

Dyes:

Micro- Ruby: Dextran, tetramethylrhodamine and biotin (Invitrogen, Molecular Probes, Eugene, Oregon, USA). Ex/em: 555/580

Micro- Emerald: Dextran, fluorescein and biotin (Invitrogen, Molecular Probes, Eugene, Oregon, USA). Ex/em: 494/518

Lucifer- Yellow: Dextran, Lucifer yellow (Invitrogen, Molecular Probes, Eugene, Oregon, USA). Ex/em: 425/528

Alexa- Fluor 633: Dextran, Alexa- Fluor (Invitrogen, Molecular Probes, Eugene, Oregon, USA). Ex/em: 632/647

Alexa- Fluor 488: Dextran, Alexa- Fluor (Invitrogen, Molecular Probes, Eugene, Oregon, USA). Ex/em: 495/519

Five different combinations of dyes and locations were tried:

Combination 1: Micro- Ruby in ganglion + Micro Emerald in AL, 19 preparations.

Combination 2: Micro- Ruby in AL + Micro Emerald in ganglion, 2 preparations.

Combination 3: Micro- Ruby in ganglion + Lucifer Yellow in AL, 3 preparations

Combination 4: Micro- Ruby in AL + Alexa- Fluor 633 in ganglion, 5 preparations

Combination 5: Micro-Ruby in AL + Alexa-Fluor 488 in ganlion, 2 preparations

In addition to micro- ruby, neurobiotin- tracer (SP-1120, Vector Laboratories, Inc.) was tested to stain the thoracic ganglion.

In total three different protocols was performed in the optimizing stage to see whether one over the other gave a better result. The protocol in the main section was sufficient to produce the results presented in this study, but in addition these two protocols was tested:

Protocol 2

Moths (8 females) were subjected for staining their ganglion with neurobiotin tracer. After staining, the insects were kept in the dark in room temperature for 3-5 hours followed by approximately 24-36 hours in refrigerator (4°C) to allow diffusion of the dye. The moths were decapitated, and their brains dissected in Ringer solution and fixed for >24 hours (Roti Histofix). The fixed brains were rinsed in PBS (0,1M) 3 x 10 minutes, before being washed with PBS (0,1 M) containing 0,5 % triton X, 6 x 1 hour in room temperature. The brains were further incubated overnight (4ºC) in PBS (0,1M) containing 0, 05 % triton X and the avidin- fluorescein conjugate (A-821, molecular probes,dilution 1:200). Following incubation, the brains were rinsed in PBS (0,1M) 3 x 10 minutes, before dehydration in an increasing series of ethanol (50%, 70%, 90%, 96% and 100%). Finally, they were cleared with methyl salicylate and mounted as whole mounts in custom- made aluminum plates with double sided cover glass, before the preparations was visualized in the confocal microscope.

The reason for testing neurobiotin tracer was the lower molecular weight of neurobiotin and the notion that smaller diameter dendrites could be stained. However, because of time limitations there was not enough time to optimize the composition of mixtures (neurobiotin tracer and avidin fluorescein conjugate), and nothing more was done after the first trial of visualization in the confocal microscope failed to show any staining at all.

Protocol 3

To check if results would be even better regarding the spatial location of the neurons, some of the brains were submitted to a new protocol after first being scanned in the confocal microscope. The protocol started with a decreasing rehydration series of ethanol (100%, 96%, 90%, 70% and 50%; each for ten minutes). The brains were rinsed with PBS for ten minutes followed by a dehydration series of ethanol (same as mentioned earlier). The brains were put in xylol (5-10 minutes) so that membranes and other fatty structures could easily be penetrated by the substances following in the protocol. The same decreasing rehydration series of ethanol as earlier was performed, followed by 30 minutes in collagenase (36°C, 5 mg pr ml PBS). Washing once again with PBS was followed by preincubation in a solution of PBStx (PBS with 0, 1 % Triton X, which is a detergent) and NGS (10%, Natural Goat Serum, Sigma, St.Louis, MO, USA) for 30 minutes. The monoclonal antibody

against synapsin; SYNORF was diluted in PBStx (0.1 %) and NGS (10%, 1:10), and the brains was incubated in this medium for 48 hours at 4°C. This was evidently followed by PBS wash (6 x 20 minutes, before the second antibody; anti-mouse- CY5 (Jackson Immunoresearch) diluted 1:500 in PBStx- solution, was introduced to the brain which was incubated for 48 hours in the refrigerator at 4°C. A second PBS wash (6 x 20 minutes) was performed before an increasing dehydration series of ethanol as described earlier, making the brains ready for clearing with methyl salicylate, and finally mounted on aluminum plates for visualization in the confocal microscope.

The reason for testing protocol 3 was to see whether background staining of brain neuropils would enhance the location of the stained neurons, making it possible to easier visualize their spatial relation to other brain structures. This protocol was performed on two brain preparations, but it failed to give new insight into where the stained neurons projected. The reason for this is only speculation, but with such massive original mass- staining, adding several antibodies could potentially cause “noise” into a already well- stained preparation. The preparations had already gone through one scanning in the confocal microscope and the stained neurons had potentially been bleached in the first place.