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Identification of different antennal-lobe neuron categories;

a morphological and physiological study of second order neurons in the primary olfactory brain centre of the moth *Heliothis virescens*

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Abstract

Olfaction is the sense of smell, first mediated by specialized sensory cells encapsulated in sensilla hairs covering the moth antennae. The sensory neurons project directly to the olfactory the primary olfactory centre of the brain, the antennal lobe. This first synaptic level, the secondary neuronal fibers, the projection neurons, follow one of the three antennal lobe tracts to higher olfactory neuropils called the calyces and the lateral horn. The antennal lobe is also innervated by local interneurons and centrifugal neurons. Moths are preferred objects of olfactory research due to their highly sensitive and species-specific olfactory systems. Their ability to process and compute plant odour information is still fairly unknown, this despite the many odour specific olfactory sensory neurons classified. The present study aimed to identify individual antennal-lobe neurons physiologically and morphologically in the moth *Heliothis virescens*. Insects were stimulated with identified primary plant odorants and multicomponent blends, in addition to known pheromone components during intracellular recordings from second order neurons in the antennal lobe. Then, staining of the neurons was attempted and successfully stained neurons were characterized. The categories of antennal lobe neurons being identified in insects are identified in this thesis. The current results show a varied antennal lobe neuron population concerning both the morphology and physiology of the individual neurons within each category. All in all, the presented data confirm the known morphologic characteristics of antennal lobe neurons, but also reveal exceptions previously not described in *H. virescens* specially or in moth in general. Also, no differences were found between the physiological properties of neurons following the three antennal lobe tracts. Of special interest is the centrifugal neuron presented in this thesis, previously not described in *Heliothis virescens*.

Sammendrag

Lukt er sammen med smak en kjemisk sans. De ulike odorantene blir først detektert av spesialiserte sanseceller i sensillehårene som dekker nattflyantennen. De sensoriske nevronene projiserer direkte til det primære luktesenteret i hjernen - antenneloben. Fra dette første synaptiske nivået, følger de sekundære nervefibrene, en av tre luktebaner til de høyere ordens luktesentre, kalt calyces og det laterale horn. På grunn av deres svært sensitive og artsspesifikke feromonsystem, er nattfly ofte benyttet til forskning på luktesystemet. Deres evne til å bearbeide planteduftinformasjon fremdeles relativt lite forsket på, dette til tross for de mange luktspesifikke sensoriske nevronene som er klassifisert. Dette studiet hadde som mål å identifisere individuelle antennelobe-nevroner fysiologisk og morfologisk i nattflyarten *Heliothis virescens*. Under intracellulære registreringer fra individuelle antennelobe-nevroner, ble insektene stimulert med kjente primære planteduft er og blandinger bestående av flere planteduftkomponenter, i tillegg til kjente feromonkomponenter, samt blandinger disse. Etter testing av ulik duftstimuli, ble nervecellene farget og klassifisert i henhold til eksisterende litteratur. Organiseringen av antenneloben og nevronenes morfologi er ganske lik for flere insektfamilier og består gjerne av de to hovedkategorier av antennelobenevroner: projeksjonsnevroner og lokale internevroner - i tillegg til de mindre tallrike sentrifugalnevronene. Disse tre har en variert morfologi og fysiologi, og alle tre klassene er presentert i dette studiet. Resultatene viser noe av det store mangfoldet av antennelobenevroner, både når det gjelder morfologi og fysiologi. I denne studien blir det også beskrevet flere særegenheter som tidligere ikke er rapportert i *Heliothis virescens* spesielt, men også i nattfly generelt. Videre ble det ikke funnet fysiologiske forskjeller mellom projelsjonsnevroner som følger de ulike luktetrakter. Imidlertid viste de ulike lokale internevronene spesifikke fysiologiske egenskaper. Av spesiell interesse er sentrifugalnervronet som er presentert i dette studiet. Det har ikke tidligere blitt identifisert i *Heliothis Virescens,* og funnet inkluderer interessante fysiologiske og morfologiske data.

Preface

This Master's thesis was written at the Department of Biology, at the Norwegian University of Science and Technology (NTNU), and the laboratory work at the Chemosensory Laboratory (NTNU) under the guidance of main academic supervisor Professor Bente Gunnveig Berg and co-supervisor Associate Professor Tor Jørgen Almaas. All finances were covered by the Department of Psychology and Professor Bergs` operational budget.

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This master thesis would not have been possible without the help and support of a lot of people. First and foremost, I would like to thank Professor Berg for being both an academic and personal role model, her knowledge and overview combined with her strong social awareness, always including the people surrounding her and standing up for what she believes is right, makes her truly inspiring. Thank you for all your help and guidance.

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Trondheim, June 13th 2014,

Ingrid Vaagland Stav

Abbreviations

5HT – 5-hydroxytryptamine, Serotonin

A – Anterior

AL – Antennal lobe

An – Antennal nerve

B10 – Blend 10

B. mori – Bombyx mori

CeB – Central body

CN – Centrifugal neurons

CNS – Central nervous system

CO_2 – Carbon dioxide

Cu – Cumulus of the MGC

D. melanogaster – Drosophila Melanogaster

GABA – γ-aminobutyric acid

H. virescens – Heliothis virescens

IP_3 - Inositol trisphosphate

KC – Kenyon cells

L – Lateral

LAL – Lateral accessory lobe

lALT – Lateral antennal lobe tract

latLFG – lateral large female glomeruli

LFG – Large female glomeruli

LH – Lateral horn

LN – Local interneurons

LP – Lateral protocerebrum

mALT – Medial antennal lobe tract

M. sexta– Manduca sexta

MB – Mushroom body

medLFG – medial large female glomeruli

M – Medial

MGC – Macroglomerular complex

mlALT – Mediolateral antennal lobe tract

OL – Optic lobe

OR – Odorant receptors

OSN – Olfactory sensory neurons

pI - pVII – Preparation number (one to seven)

P – Posterior

PC – Protocerebrum

Pe – Pedunculus of the MB

PLC – Phospholipase C

PN – Projection neuron

SI-neuron – Serotonin-immunoreactive neuron

S. littoralis – Spodoptera littoralis

Soma – Cell body

Somata – Cell bodies

T. ni – Tricholusia ni

Z9-14:AL – *cis*-9-tetradecenal

Z11-16:OH – *cis*-11-hex-adecenol

Z11-16:AL – *cis*-11-hexadecenal

Z11-16:AC – *cis*-11-hexadecenyl acetate

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1 Introduction

A well-known idea called the "Proustian phenomenon" proposes that distinctive smells are more powerful than any other stimulus in recalling distant memories. This theory is named after the French writer Marcel Proust, who in his novel *À la recherche du temps perdu* (In search of lost time) describes a character vividly recalling long-forgotten memories from his childhood after smelling a tea-soaked madeleine biscuit (Chu and Downes, 2002). It is well known that a particular scent can bring forth a rush of vivid memories and powerful responses almost instantaneously. Recently it was shown for the first time that a particular region in the mammalian brain involved in odour object recognition, the entorhinal cortex, communicates with the hippocampus, which is the formation seat of episodic memories during learning of an odour discrimination task (Igarashi *et al.*, 2014). The sense of smell is the most ancient of all senses during animal evolution, and it can be found in all phyla throughout the animal kingdom. The olfactory pathway is in-fact the best conserved sensory system throughout evolution, and metazoan, which includes all animals except sponges which do not have a nervous system (Sakarya *et al.*, 2007), all have in common that they can detect a variety of organic molecules using chemosensory neurons using cilia or microvilli that contact the external environment (Mustaparta, 2002).

1.1 The Olfactory system of insects

Insects are the most diverse and abundant animal group, representing over 70 % of all known species (Menini, 2010). It is commonly known that insects have a keen sense of smell, just think of the strong attraction over-ripe fruit in the kitchen holds for fruit flies (Soowon *et al.*, 2008). The olfactory organs detect small volatile molecules from the environment. This detection is crucial for the survival of most animals, insects included. The chemical senses; taste and smell, and their respective sensory organs communicate information about the access of food, predators, pathogens, related individuals or species among other things (Soowon *et al.*, 2008; Kaupp, 2010; Sachse and Krieger, 2011) The odorants comprise a variety of molecules, and it is their biological function that classifies them as general odorants or pheromones. Specific for the pheromones is that they are used for communication between individuals of the same species (intraspesific interaction), and the first known pheromone (bombykol) was isolated from the silkmoth *Bombyx mori* (Karlson and Lüscher, 1959).

1.1.1 Odour detection in the peripheral structures

Almost all organisms seem to have developed a peripheral detection system for smell that comprises a vast variety of olfactory receptor proteins (ORs). Individual insect ORs can detect large numbers of odorants and this broad recognition of odorants by “generalist” receptors has later been debated. The “specialist” receptor, however, detects a small number of odorants, sometimes just one. (Mustaparta,

2002; Soowon *et al.*, 2008). Every olfactory sensory neurons (OSN) expresses only one type of OR, and by coupling gas chromatography to OSNs it has been shown that plant odour responding OSNs are specialized for just one substance, named primary odorants (Røstelien *et al.*, 2000). Even so, the OSNs can respond to several odorants with structural similarities. Obviously, it is advantageous to have a large repertoire of receptors for recognizing relevant biological stimuli (Mustaparta, 2002). Large families of transmembrane receptors mediate the initial events of odour detection with specific expression patterns in the peripheral sensory neurons. ORs were first found in vertebrates (*Rattus norvegicus*) in 1991 (Buck and Axel, 1991), and in 1999 the first insect ORs were found in invertebrates (*Drosophila melanogaster*) following the breakthrough of the sequencing of the fruit fly genome (Clyne *et al.*, 1999). In heliothine moth ORs were first identified in *H. virescens* including a group of odorant receptors (Krieger *et al.*, 2002; Krieger *et al.*, 2004). ORs can be selective and also convey information about special odours like carbon dioxide (CO_2) (Stange, 1997).

The peripheral olfactory systems of mammals and insects are surprisingly similar in terms of architecture. Despite the less complex arrangement in insects, e.g. containing a lower number of olfactory sensory neurons (OSNs) than that of mammals, the insect system is by no means inferior regarding sensitivity to specific odorants (Sachse and Krieger, 2011). The insect OSN is a bipolar neurone embedded in aqueous lymph inside a cuticular, hair-like structure called a sensillum. The sensilla are perforated with pores allowing hydrophobic odour molecules to enter the lymph which contains binding proteins providing transportation of the odorant to an OR on the OSN dendritic surface. (Almaas and Mustaparta, 1990; Sachse and Krieger, 2011). So far, around 20 types of plant odour sensitive OSNs have been identified in *H. virescens* (Røstelien *et al.*, 2000; Stranden *et al.*, 2003; Skiri *et al.*, 2004). Generally, there seems to be a lower responsiveness of plant odour OSNs in males than in females, which may be explained by the considerably higher number of plant odour-sensitive sensilla on the female antennae (Almaas and Mustaparta, 1990; Skiri *et al.*, 2004). One antenna can have approximately 1200 OSN and their somata (cell bodies) are located in the antennae. The process of transducing the chemical signal into an electrical takes place in the OSN (Mustaparta, 1996), and molecular studies suggest that the chemo-electrical signal transduction process in antennal OSN is mediated via the G protein-controlled Phospholipase C/ Inositol triphosphate (PLC/IP_3)-pathway (Krieger and Breer, 1999; Krieger *et al.*, 2002; Sato *et al.*, 2008).

1.1.2 First order relay

The antennal lobe (AL) is the primary olfactory centre in the insect brain (Hansson, 1995; Fukushima and Kanzaki, 2009), and the corresponding brain structure in crustaceans and other invertebrates has functional and structural properties that are analogous to the olfactory bulbs of mammals, fish, and amphibians. A characteristic feature characterizing the primary olfactory centre in all species is the assembly of spherical neuropil structures termed glomeruli (Shepherd, 1974). Even though most of

the AL neurons process olfactory information, some of them are multimodal and can respond to mechanosensory stimuli temperature, humidity, and acoustic stimuli (Galizia *et al.*, 2000; Zhao *et al.*, 2013).

1.1.2.1 Antennal lobe circuitry

A glomerulus is a spheroidal synaptic condensation, forming a neuronal network between the axon terminals of the OSNs, first order neurons, and the dendrites of second order neurons, e.g. the projection neurons (PNs). The PNs follow specific tracts to higher olfactory processing areas in the brain, as will be described later. The glomeruli are also innervated by local interneurons (LNs) and centrifugal neurons (CNs) (Homberg *et al.*, 1988; Boeckh and Tolbert, 1993; Sun *et al.*, 1993). All the OSNs that express the same OR type project to the same glomerulus in the ipsilateral AL (Menini, 2010; Fişek and Wilson, 2014). The number of glomeruli is both gender and species specific (Matsumoto and Hildebrand, 1981).

Local interneurons

The different glomeruli interact through lateral innervation by a population of LNs. The morphology of this neuron group is diverse. However, two main subgroups include the multiglomerular (or pangloglomerular) neurons, which extend to most of the AL glomeruli and represents most of the LNs and the oligoglomerular neurons, which connect to a smaller number of specific glomeruli (Hoskins *et al.*, 1986; Christensen *et al.*, 1993; Seki and Kanzaki, 2008; Reisenman *et al.*, 2011). All LNs have their cell-body located in the prominent lateral cell cluster (LC), situated adjacent to the optic lobe on the lateral side of the AL (Homberg *et al.*, 1988). The majority of the moths LNs are γ-aminobutyric acid (GABA)-ergic, providing inhibitory input in the antennal lobe. (Hoskins *et al.*, 1986; Seki and Kanzaki, 2008; Berg *et al.*, 2009). However, the LNs can also contain other neurotransmitters and neuropeptides, the latter often as co-transmitters (Nässel and Homberg, 2006; Berg *et al.*, 2007; Reisenman *et al.*, 2011). Some LNs are reported to be excitatory possibly contributing to a broadening of the AL output signals (Olsen *et al.*, 2007).

LNs mediate a variety of functions that include lateral inhibition (for the GABA-ergic neurons), synchronisation of the PNs, refinement of temporal response properties and global gain control of the AL. In other words the LNs shape and modulate the output of the AL (Reisenman *et al.*, 2011). To understand how olfactory information is being processed in the AL it is fundamental to know how signals from different glomeruli are combined. The connections between glomeruli in the ALs have thus far only been demonstrated for the fruit fly, where they contributed to a broadening of the AL output (Olsen *et al.*, 2007; Fişek and Wilson, 2014). The physiological characteristics of LNs are reported to be remarkably diverse, including various types of odour responses and different intrinsic

electrophysiological properties (Christensen *et al.*, 1993; Reisenman *et al.*, 2004; Reisenman *et al.*, 2005; Reisenman *et al.*, 2011; Heinbockel *et al.*, 2013).

1.1.2.2 Sexually dimorphic glomeruli

The moth *H. virescens* has 62 ordinary glomeruli in total (Berg *et al.*, 2002; Løfaldli *et al.*, 2010). In addition, the male has a macroglomerular complex (MGC) consisting of 4 glomeruli exclusively processing information about female-produced signals (Berg *et al.*, 1998; Vickers *et al.*, 1998). The four MGC-compartments comprise two large units, the cumulus, and the dorso-medial unit, plus two smaller glomeruli located ventrally. The cumulus and the dorso-medial compartment receive pheromone information, which leads to attraction and sexual behaviour, whereas the two ventral compartments receive information about the presence of sympatric species, that disrupts the attraction (Christensen and Hildebrand, 1987; Hansson, 1995; Berg *et al.*, 1998; Galizia *et al.*, 2000; Kanzaki *et al.*, 2003; Vickers, 2006). In *H. virescens*, two female-produced pheromone components being necessary and sufficient for eliciting the male sexual response have been identified; the major pheromone component *cis*-11-hexadecenal (Z11- 16:AL) and the second pheromone component *cis*-9-tetradecenal (Z9-14:AL). Two other compounds, *cis*-11-hexadecenyl acetate (Z11-16:AC) and *cis*-11-hex-adecenol (Z11-16:OH), not produced by the con-specific female, are shown to disrupt the pheromone attraction of males, thus preventing cross-attraction and mating mistakes. Four functionally different types of OSNs have been identified in the *H. virescens* male, each tuned to one of these insect-produced compounds (Christensen and Hildebrand, 1987; Almaas and Mustaparta, 1990; Christensen *et al.*, 1991; Berg *et al.*, 1995; Hansson, 1995; Berg *et al.*, 1998; Hillier *et al.*, 2006).

Much is known about the pheromone system in moth compared to the complex plant odour system. Many behavioural studies have proven that plant odours play an important role in the life history of adult moths, females especially, and several studies have recognized the importance of host plant odours in female oviposition site choice (Bernays and Chapman, 1994; Hansson, 1995). This close interaction includes, for instance, the use of host-plant products as pheromone precursors (Reddy and Guerrero, 2004). In the *H. virescens`* AL, some of the primary host odorants are specifically represented in single glomeruli, whereas others are represented by activity in two or three glomeruli (Galizia *et al.*, 2000; Skiri *et al.*, 2004). In different moth species, two female specific glomeruli called the large female glomeruli (LFGs) including one medial LFG and one lateral, both being positioned at the base of the antennal nerve, have been described (Rospars, 1983; Christensen *et al.*, 1991; King *et al.*, 2000; Berg *et al.*, 2002). In the *Manduca sexta* female, attraction towards host-plants and other odours is shown to be similar to the male attraction towards pheromone plumes (Willis and Arbas, 1991) implying the same underlying mechanism (Menini, 2010). The ability of the olfactory system to encode information about the spatio-temporal properties of the odour stimulus is

therefore not limited to males or to pheromone information processing (Hansson and Christensen, 1999), suggesting that the two LFGs are homologous to the two main glomeruli of the MGC (Rospars and Hildebrand, 1992).

1.1.2.3 Antennal lobe projection neurons

From the AL, the olfactory information is carried to higher brain centres via PNs. The axon of each PN follows one of the antennal lobe tracts (ALTs) targeting two main brain regions in the protocerebrum, the calyces of the mushroom bodies (MB) and the lateral horn (LH) in the lateral protocerebrum (LP). The somata of the PNs are located in three cell clusters positioned on the surface of the AL (Berg *et al.*, 2002; Heisenberg, 2003; Ito *et al.*, 2014)[1]. Thus the prominent lateral cell cluster, which houses all somata of LNs, also including somata of PNs. The smaller medial cell cluster being positioned dorsally in the AL towards the midline of the brain, and the tiny anterior cell cluster positioned at the most anterior site of the AL, contain somata of PNs exclusively (Homberg *et al.*, 1988). Of the three cell clusters, the medial cell cluster shows sexual dimorphism by having unique numbers of somata in males and females (Homberg *et al.*, 1988; Homberg *et al.*, 1989; Hansson, 1995). The master thesis of Berg (2013) revealed a small fourth AL cell cluster, located slightly dorsally of the anterior cell cluster, previously not described.

PNs are essential neuronal populations that transmit information about odour quality, odour intensity, and the spatiotemporal nature of the odour plume (Namiki and Kanzaki, 2011). Most PNs have uniglomerular arborisations in the AL, and a large part of the population use acetylcholine as its neurotransmitter. Other PNs are multiglomerular and many of these are GABAergic (Albrecht, 1953; Mustaparta, 2002; Rø *et al.*, 2007; Chapman *et al.*, 2013). The response properties of the PNs are mainly influenced by which glomeruli they innervate, and therefore, by implication, what receptor-type the glomerulus represents. Therefore, the output information from the PNs will in summation reflect the many different stimuli they receive. In addition, an underlying regular 20–30 Hz oscillation in the membrane potential occurs synchronously across the entire population of PNs (Chapman *et al.*, 2013). The spatial and temporal features of PNs are suggested to be important components for perception and memory of the odour identity (Menzel, 2001; Mustaparta, 2002).

1.1.3 The antennal lobe tracts

There are three main nerve pathways that project from the AL to the protocerebrum (PC); the medial antennal lobe tract (mALT), the mediolateral antennal lobe tract (mlALT), and the lateral antennal lobe tract (lALT) (Homberg *et al.*, 1988; Rø *et al.*, 2007). These tracts were formerly known as the

[1] This area is called the cell body rind, which is the accumulation of cell bodies that cover the surface of the different neuropils in the insect brain, formerly referred to as the cortex. HEISENBERG, M. 2003. Mushroom body memoir: from maps to models. *Nature reviews. Neuroscience,* 4**,** 266-275.

inner, medial and outer antennocerebral tracts respectively (Homberg *et al.*, 1989; Almaas and Mustaparta, 1990; Ito *et al.*, 2014). The different tracts are described in Figure 1A.

mALT

As in other insects, the most prominent tract in heliothine species is the mALT. It exits from the AL dorso-medially and projects posteriorly in the protocerebrum, passing close to the lateral edge of the central body. Then it turns laterally and continues along the anterior surface of the ipsilateral calyces. From here, the mALT sends branches innervating the calyces. From the calyces, the mALT continues anteriorly towards the LH in the LP. The mALT consists primarily of uniglomerular projection neurons, which most often arborize in the calyces and in the LH. Both in *M. sexta* and *H. virescens,* the mALT is described as projecting from the AL by the means of two main sub-unit branches, or so-called roots. The axons of PNs having somata in the lateral cell cluster make up the ventral root whereas PNs having somata in the medial and anterior cell cluster make up the dorsal root (Homberg *et al.*, 1988; Rø *et al.*, 2007; Berg, 2013)

mlALT

The considerably smaller mlALT follows the mALT for a short distance before it bends laterally at the level of the central body. Then this tract divides into several smaller branches, each innervating slightly different parts of the LP. The mlALT contains mainly multiglomerular PNs (Homberg *et al.*, 1988; Rø *et al.*, 2007). Also, a second mlALT (2^{nd} mlALT) is described in *H. virescens* (Corneliussen, 2013). Corneliussen (2013) determined these neurons to mainly be connected to ordinary glomeruli. In the recent master project by Dahl (2013), however, a partly similar projection pattern as that characterizing the 2^{nd} mlALT was seen, suggesting that the 2^{nd} mlALT also might be involved in processing information about CO_2 in the environment.

lALT

The lALT exits the AL more ventrally as compared to the mALT and the mlALT, and then turns in a lateral direction. Ventral to the pedunculus, some of the fibres make a dorsal turn away from the common tract, ending laterally to the lobes of the MB. The remaining fibres in the lALT continue in the common tract terminating in the LH. From the LH, some fibres in the lALT turn dorso-medially and some of the PNs also send terminals into the calyces. The l-ALT contains both uniglomerular and multiglomerular PNs (Albrecht, 1953; Homberg *et al.*, 1989; Rø *et al.*, 2007).

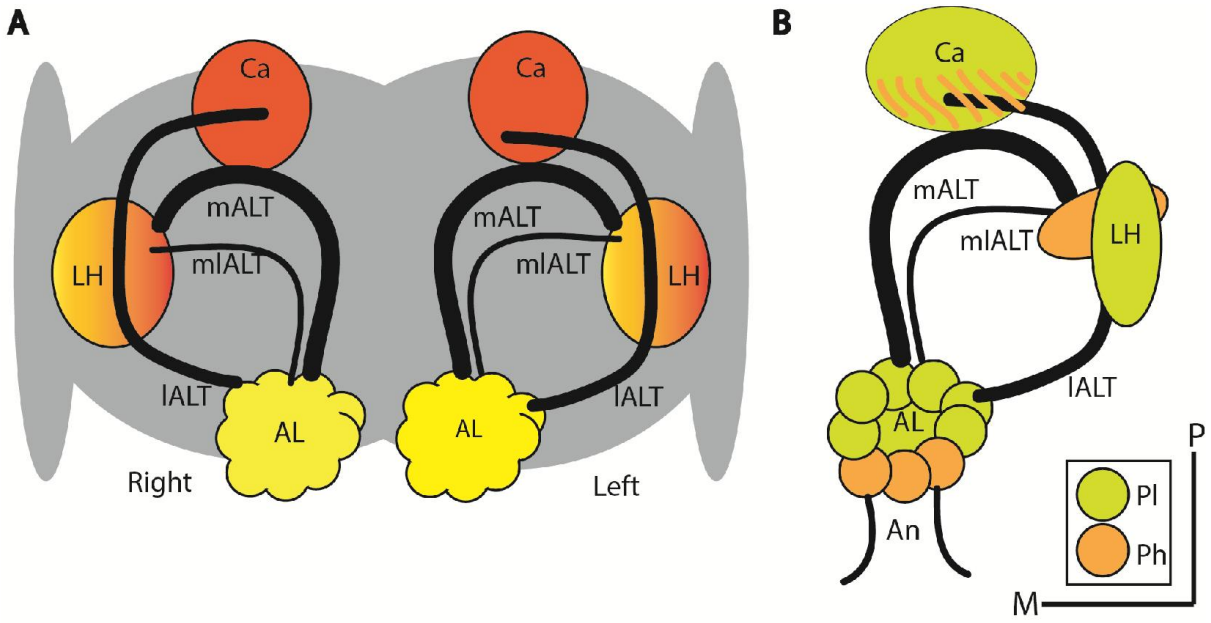

Figure 1: The schematic drawings are seen from a dorsal view. **A)** The three antennal lobe tracts (ALTs) and the main olfactory neuropils in the moth brain, the lateral horn (LH) and the calyces (Ca). **B)** Schematic overview of how the different odour qualities, pheromones (Ph) in orange and plant odours (Pl) in green, innervate the different higher brain areas in the Lepidopteran brain model. The antennal lobe (AL) of male moths is typically subdivided into a main AL and a macroglomerular complex (MGC) area consisting of pheromone-responsive glomeruli. Uniglomerular projection neurons (PNs) in the main AL project through the mALT and arborize throughout the calyces and the LH, while uniglomerular PNs from the MGC arborize chiefly in a smaller region of the calyces and in the medial part of the LH (modified from Martin *et al.* 2011). Abbreviations; An – antennal nerve; lALT – lateral antennal lobe tract; mALT – medial antennal lobe tract; mlALT – mediolateral antennal lobe tract.

1.1.4 Centrifugal neurons

Neuromodulators are released in the vicinity of the synapse, and are important mediators of neural plasticity. Their actions serve to rearrange neuronal circuits by altering the intrinsic firing probabilities of neurons and modify their synaptic connectivity. In this way they are allowing defined networks to switch between tasks and thus adapt to a changing environment (Kloppenburg and Hildebrand, 1995). Serotonin (5-hydroxytryptamine, 5HT) is the best-characterized neuromodulator in the insect brain, and is also proven to be important for vertebrates. Serotonin serves a function in spike synchronization, and increased serotonin levels are reported to potentially enhance the sensitivity of PNs to different olfactory stimuli. The serotonin level in the AL is fluctuating significantly over a 24-hour period, being low during the day and high during the night, especially around dusk and dawn when activity levels in noctuid moth are at a peak (Nässel, 1988; Dacks *et al.*, 2006; Chapman *et al.*, 2013). In *B. mori*, pheromone-induced behaviour was reported to peak around noon, right at the time when the levels of serotonin in the brain are highest (Hill *et al.*, 2002).

Both ALs are innervated by various centrifugal modulatory neurons, affecting the function of the AL according to circadian, appetitive, or associative information from other brain areas (Nässel, 1988; Dacks *et al.*, 2006; Martin *et al.*, 2011). One of them is the serotonin-immunoreactive (SI) neurons,

innervating both hemispheres extensively (Figure 2). All holometabolous insects studied, except hymenopterans, have a pair of SI AL neurons with a similar branching pattern. Hemimetabolous insects also have a pair of SI-neurons, though with ipsilateral arborisations in the antennal lobe. Generally, the arborizations of the SI-neuron are known to remain though all postembryonic stages in the holometabolous insects (Dacks *et al.*, 2006).

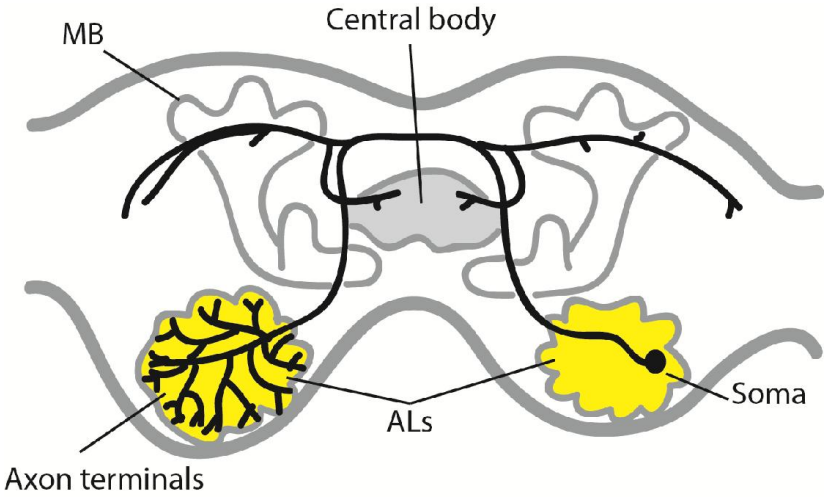

Figure 2: A serotonergic centrifugal neuron (CN) – the serotonin immunoreactive (SI) neuron, with terminals in all glomeruli and extensive branches throughout the other parts of the brain. An unbranched neurite projects from the large soma to the calyces following the mALT, then the axon crosses the midline and extends through the corresponding mALT to the contralateral antennal lobe (AL) where it innervates all the glomeruli, including the male-specific glomeruli. The SI-neuron also extends several fine branches in bilateral protocerebral regions anterior to the mushroom body (MB) calyces, particularly on the contralateral side (Chapman *et al.*, 2013). Two additional branches described project towards the central body from both sides. The branches are sent off from the SI neuron just before and just after the neurite crosses over the midline (After Kent *et al*. 1987).

1.1.5 Higher olfactory centres

As in other moths, the parallel antennal-lobe pathways of *H. virescens* connect the AL to two main olfactory centers in the PC, the MB calyces and the LH (Rø *et al.*, 2007). Both these centers are insect equivalents to the olfactory cortex in vertebrates (Fişek and Wilson, 2014).

The mushroom body calyces

The MB calyces are regarded as a higher order olfactory integration centre, and are thought to process and integrate olfactory information to form multimodal associative learning (Heisenberg, 2003; Rø *et al.*, 2007). The MBs are also involved in visual learning in some insects (Ito *et al.*, 1998). For instance, in *M. sexta* neithe the floral odour nor the visual aspect of the host-plant alone can attract the moth, but the association of visual and olfactory stimuli is required for it to actually visit the flowers (Raguso and Willis, 2002). The MB consists of a cap of neuropile, including the calyces, the accessory calyces, and the lobes. The MBs are given their name because of the shape formed by their interneurons called Kenyon cells (KC). These cells have their somata in the somata rind above the calyces. The KC-axons project from the calyces to form a stalk called the pedunculus, which runs

anteriorly and slightly ventro-laterally through the protocerebral neuropils. The pedunculus branches most anteriorly into three main lobes, the α-, β-, and γ-lobes. In these lobes, the KCs synapse on the third order extrinsic neurons, which in turn connect the MB system to other higher order brain areas such as the superior protocerebrum and the LP. The function of this circuit still is largely unknown. The calyces receive olfactory information from PNs running in the mALT and the lALT, in opposite directions (Christensen *et al.*, 1995; Hansson, 1995; Ito *et al.*, 1998; Fahrbach, 2006).

The lateral horn

It has been proposed that the LH computes naïve, or inexperienced odour signals from the AL for motor control (Homberg *et al.*, 1988; Keene and Waddell, 2007; Rø *et al.*, 2007). This motor control is what forms for instance the innate olfactory avoidance responses (Rø *et al.*, 2007; Soowon *et al.*, 2008). Segregation of inputs in the LH is largely correlated with the ALT through which each of the antennal-lobe PN axons project, or with the behavioural significance of the signal including especially food derived versus social or pheromonal stimuli (Martin *et al.*, 2011). Uniglomerular PN axons following the mALT from the ordinary glomeruli arborize throughout the calyces and the posterior-lateral part of the LH, while uniglomerular PNs from the MGC arborize chiefly in a smaller region of the calyces and in the antero-medial region of the LH (Figure 1B, Kanzaki *et al.*, 2003; Martin *et al.*, 2011; Zhao *et al.*, 2014 submitted article).

1.2 The heliothine in research

Insects can be a hazard against human health and a range of cultivated plants (Soowon *et al.*, 2008). Their unique adaptation to host plants, in which the chemical senses are essential, makes the insect olfaction a very interesting target of research (Mustaparta, 2002). It is also interesting to find alternative strategies to defend a crop against the insect pests, other than the major usage of toxins as is currently used around the world. Hence, pure research on insect olfaction is of substantial significance and scope (Sachse and Krieger, 2011). To understand how the information of smell is coded in the central nervous system (CNS) it is essential to know how the neurons convey the information through the nervous network. Actually, in the fruit fly *D. melanogaster*, the complete pathway from sensory input to descending neurons has been described for pheromone information (Ruta *et al.*, 2010). Invertebrates often have a simple nervous system and a stereotypic behaviour when they respond to olfactory stimuli, making them useful for understanding the neural representation of odour and behaviour.

Moths offers a suitable study design as there is few and relatively easy identifiable neurons in an easy accessible CNS (Matsumoto and Hildebrand, 1981; Mustaparta, 2002; Soowon *et al.*, 2008; Sachse and Krieger, 2011). The Heliothinae is a large subfamily of approximately 80 noctuid moths. This subfamily also encloses the *Heliothis* species including some of the most formidable crop eaters in the

world. Together, the earthworm complex, *Helicoverpa,* and the tobacco budworm, *H. virescens,* are called "the major-pest linkage". The bollworm *Helicoverpa armigera* is also often mentioned in this context *(Soowon et al., 2008)*. The usage of the heliothine moth, e.g. *H. virescens,* as a model organism has its advantages as there exists extensive detailed knowledge from previous studies (Homberg *et al.*, 1989; Almaas and Mustaparta, 1990; Mustaparta, 1996; Berg *et al.*, 1998; Mustaparta, 2002; Skiri *et al.*, 2004; Røstelien *et al.*, 2005; Rø *et al.*, 2007; Fukushima and Kanzaki, 2009; Zhao and Berg, 2010; Løfaldli *et al.*, 2012). Here, the pheromone system has been studied in detail, especially on the level of the AL. In adversative, the mechanisms that process plant odour information in this insect are almost unknown territory (Rø *et al.*, 2007).

1.3 Aims of the Master project

Main aim: To identify individual antennal-lobe neurons physiologically and morphologically.

Specific goals:

1. To describe the morphology of projection neurons passing in the various antennal-lobe tracts.
2. To describe physiological properties of projection neurons associated with the distinct antennal-lobe tracts.
3. To describe physiological and morphological properties of local interneurons.

2 Materials and Methods

2.1 The insects

Experimental studies were performed on the moth species *Autographa gamma, H. armigera* and *H. virescens,* however; data from *H. virescens* only was included in the results. A local culture of *H. virescens* (Heliothinae; Lepidoptera; Noctuidae) was bred in the lab with the original culture coming from Bayer CropScience, Germany. The eggs and larvae were bred in air humidity of 66 % and at a temperature of 28°C. Male and female pupae were separated and kept in distinct climate chambers. After hatching the adult insects were transferred to a cylindrical Plexiglas container, with perforated lids for air circulation. No more than eight insects were placed in each container. The insects were fed a sucrose solution (0,1 M), which was replaced every day. The pupae and adult insects were kept in climate chambers (Refritherm 200, Struers-Kebolab, Albertslund, Denmark) at 22-23°C, 70 % air humidity, and a phase-shifted photoperiod cycle (14 h light: 10 h dark) with reversed night and day. The insects were used for experiments one to ten days after hatching, but three to six days old insects were preferred if available.

2.2 Preparation of test substances

The odours were diluted in hexane (10^{-5} M, 10 ng/100 µl), and then 100 µl of each solution was fumed on to a filter paper (approximately two cm in diameter). Before putting the filterpaper into a glass tube the hexane was removed by evaporation via a nitrogen flow. For the pheromone-plant odour blends: 10 ng of the pheromone + 10 x 100 ng of the plant odour was applied on the filter paper. The pheromone blend was made by applying Z11-16:AL and Z9-14:AL at a ratio of 1:16 so that the total amount was 10 ng. A clean filter paper prepared in the same manner as the odour-bearing stimuli was used as a control. The samples were kept in the freezer (-18 °C) and renewed once a month. The odorants chosen were: a ten blend mixture (B10) (Løfaldli *et al.*, 2012), (+)-linalool, (-)-germacrene D, 2-phenylethanol, (3Z)-hexanyl acetate (from now on termed linalool, germacrene D, phenylethanol and hexanyl acetate), ocimene, farnesene and ylang-ylang (Table 6, Appendix 1). All of these were chosen because of their known effect on receptor neurons in *H. virescens* (Røstelien *et al.*, 2005). If the plant odorants did not give a response and the contact with the neuron was stable the pheromone compounds were also tested. The pheromone mix, the major component (Z)-11-hexadecenal (Z11-16:AL) and second component (Z)-9-tetradecenal (Z9-14:AL), before each of the two substances. Then, the interspecific components (Z)-11-hexadecenol (Z11-16:OH) and (Z)-11-tetra-decenyl acetate (Z11-16:AC) were tested. Finally, the mixes of Z11-16:AL+ Linalool and Z11-16:AL + B10 were tested. During the experiments, the various odours were applied in the same order as that listed above.

2.3 Preparation for intracellular recordings

The moth was kept in the refrigerator (4°C) for 10-15 minutes before preparation, in order to calm and sedate the insect. Then it was mounted in a plastic tube by means of dental wax with the head exposed (Kerr Corporation, Romulus, MI, USA). The antennae were fixed with tungsten clamps and the cephalic hairs removed with a toothpick or forceps to expose the cuticula. Then the cuticle between the antennae was cut off by means of a razor blade and the underlying muscles and main trachea removed in order to expose the brain including the frontally positioned ALs, see figure 3. Also, a part of the brain sheath was removed with a fine forceps to enable penetration of the recording electrode into the neural tissue without breaking. Ringer solution containing sucrose (all in mM: 150 NaCl, 3 $CaCl_2$, 3 KCl, 25 $C_{12}H_{22}O_{11}$, 10 TES buffer, pH 6.9) was applied in order to add nutrition and humidity.

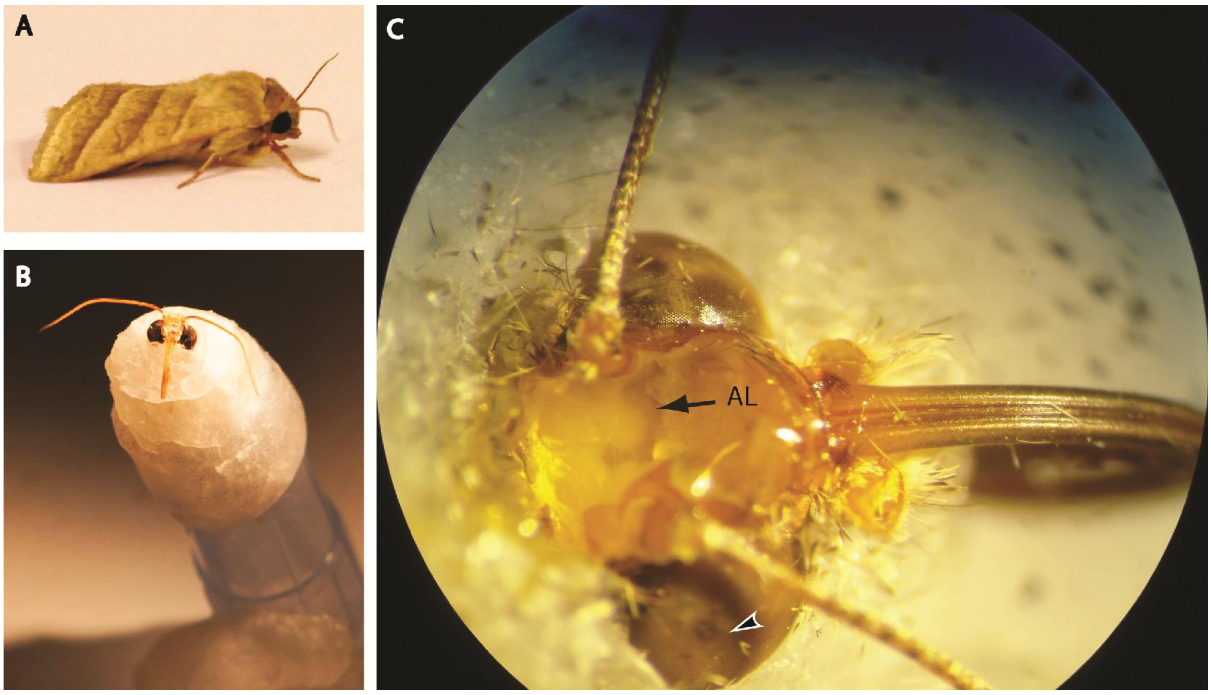

Figure 3: A) The moth species used in this thesis, *Heliothis virescens* (photo: Ragnhild Hannaas). **B)** The moth mounted in dental wax in a pipette tube (1000 µl) (photo: Jon Peter Stav). **C)** The cuticle between the antennae and towards the maxillary palps are removed together with underlying muscle and main trachea to get a good view of the antennal lobes (AL), indicated by an arrow, and the adjacent antennal nerves. The indifferent electrode was placed into the right compound eye (arrowhead) to be in contact with the hemolymph.

2.4 Intracellular recordings

The moth was placed in a faraday cage to avoid electric interference from the external environment. The preparation was positioned so that the antennal lobes were facing upwards. A silver nitrate coated silver wire placed into the hemolymph of the right compound eye was used as the indifferent electrode. A borosilicate glass microelectrode, pulled with a Flaming-Brown horizontal puller (P97; Sutter instruments, Novato, CA, USA, using the parameters; heat 330, pull 80, velocity 60, time 250), filled with the fluorescent dye tetramethylrhodamine-biotin dextran (Micro-Ruby, Invitrogen,

Germany), and back-filled with a solution of potassium acetate (0.2 M) was used as the recording electrode. The electrode, which had a resistance between 150 and 600 MΩ was connected to a preamplifier (Axonprobe-1A, multipurpose microelectrode amplifier, Molecular Devices, CA, USA) through a chloride coated silver wire inserted into the glass capillary. An oscilloscope displayed the change in voltage between the indifferent electrode and the glass electrode. This signal was digitalized by a data acquisition unit CED (Micro 1401 mk II, Cambridge Electronic Design Limited, Cambridge, UK). The software Spike2 (version 7, CED) was used for recording data and as an analysing tool. A custom written script running in Spike2 contained stimuli codes and these commands controlled the timing of odour stimulation. A micromanipulator (Leica) was used to inject the electrode into the left antennal lobe, aiming for the ordinary glomeruli.

During this procedure the left antennae was exposed to a continuous airstream at 400 ml/min delivered via a Teflon tubing. After obtaining contact with a neuron, either from hearing the activity in the load-speaker connected to the amplifier, or by observing activity in the oscilloscope, the test protocol was initiated. The glass tube containing the odour-bearing filter paper, being connected to a Teflon-tubing placed in parallel with that delivering the continuous air stream, pointed towards the antenna at a distance of 2 cm. During stimulation, the airflow was switched from the continuous stream to the odour-bearing cartridge via a valve system. The air puff ensuring odour stimulation was directed to the left antenna at 100 ml/min and had a duration of 400 ms. The time-delay from the opening of the valve to the odorant reaches the antenna is calculated to be 200 ms, and this is compensated for in the script (Høydal, 2012). Stimulation with different odours was given at least ten seconds apart to ensure that the neuron could reset back to its spontaneous activity.

2.5 Stimuli and test protocols

The first to be tested was always the control tube containing only hexane. This was to exclude a mechanosensory response. Each odorant was tested twice, if possible, to ensure repeatability of the response, and with ten seconds apart. The odorants were applied as described in subsection 2.2. Given the typically unstable connection characterizing these kinds of recordings it was not always possible to test all the substances in each sequence, and if the contact seemed to fade staining of the neuron was prioritized. After odour stimulation, the neuron was iontophoretically stained by passing a depolarizing current pulse (0.5-2.0 nA, 2 Hz) through the recording electrode. The depolarization pulses were given as long as the contact was intact, usually 10 to 15 minutes.

2.6 Fixation, enhancement and dehydration [2]

After the staining protocol was complete the insect was left over night (4□ C) to allow axsoplasmic transport. Then the brain was dissected out and fixated in 4% paraformaldehyde (24 hours, 4□C) to

[2] Room temperature if no temperature is specified.

prevent degradation of the neuronal tissue. Furthermore, the brain was washed with a phosphate buffered saline (PBS; in mM: 684 NaCL, 13 KCl, 50.7 Na2HPO2 and 5 KH2PO4, pH 7.4) for ten minutes. Next, streptavidin-Cy3 (Jackson Immunoresearch, West Grove, PA, USA), which binds to biotin, diluted in PBS (1:200, 24 hours, 4°C, or 2 hours at room temperature), was applied for enhancement of the conjugate fluorescent labelling. The brains were subsequently cleansed with PBS and dehydrated in an increasing series of ethanol (50, 70, 90, 96, 2x100 %) in steps of ten minutes each, before the brains were made transparent by methyl 2-hydroxybenzoate (methyl salicylate).

2.7 Confocal microscopy

Successfully stained preparations was scanned using a confocal laser scanning microscope (LSM 510 META Zeiss, Jena, Germany) The preparations was analysed using a 10x (EC Plan-Neofluar 10x/0.3) dry lens objective, a 20x (Plan-Neofluar 20×/0.5 NA) dry lens objective, and a 40x (C-Achroplan 40x/0.8 W) water lens objective. A 543-nm line of a Helium Neon laser excited the fluorescence biotin-streptavidine-Cy3 complex. The Cy5 in the synapsin and serotonin (see the immunohistochemistry paragraph next) immunostaining was excited by the 633-nm line of a Helium Neon laser. The distance between each section was 2 μm, the pinhole size 1 airy unit, and the resolution 1024 x 1024 pixels (if possible given the time-span). Optical sections from the confocal stacks were reconstructed by means of the LSM 510 projection tool. Brightness and contrast was adjusted using Adobe Photoshop C6 before the final figures were made in Adobe Illustrator CS6.

2.8 Immunohistochemistry

Successfully stained preparations also underwent immunohistochemistry. To obtain further information on the synaptic structures in the brain, especially the different glomeruli and the calyces, anti-synapsin labelling all presynaptic regions, and an antibody against serotonin were applied to particular preparations. Both antibodies were coupled to the fluoridising agent Cy5.

2.8.1 Synapsin immunohistochemistry

First, the preparations were rehydrated in a decreasing series of ethanol (100, 96, 90, 70, 50 % x 10 minutes) and rinsed with PBS in ten minutes. Then another series of dehydration and application of a degreasing agent Xylol (5 minutes) followed. Once again, the preparations were rehydrated and washed in PBS. Next, the preparations were pre-incubated in normal goat serum (NGS; Sigma, ST. Louis, MO, USA; 10 %) diluted in Triton X PBS (PBStx; 0.1 %) for 30 minutes. For facilitation of the antigens entrance into the neural tissue, Triton X was used. The NGS, which binds unspesific proteins, was used to minimize background staining. SYNORF 1 (Developmental Studies Hybridoma Bank, University of Iowa) diluted in the PBStx and NGS mixture (1:30), was applied next. SYNORF 1 is a monoclonal mouse antibody against the presynaptic terminal protein synapsin and thus aids the identification of synaptic neuropiles. Incubation with SYNORF 1 lasted for 48 hours at 4°C. The

preparations were washed in PBS (6 x 20 minutes) and then incubated with CY5-conjugated goat anti-mouse secondary antibody (Jackson Immunoresearch), diluted in PBStx (1:500, 48 hours, 4°C). CY5 is a hydrophilic fluorescent dye that binds to the primary antibody. The preparations were rinsed with PBS (6 x1 hour) and dehydrated in an increasing ethanol series, as previously described. Then, methyl salicylate was applied. As a last preparation before confocal laser scanning microscopy, the brains were placed on aluminium plates, frontally or dorsally positioned, in a methyl salicylate bath concealed by double-sided cover glass.

2.8.2 Serotonin immunohistochemistry

Since it was of interest to determine the neurotransmitter in one of the stained neurons a serotonin immunohistochemistry protocol was performed before the synapsin protocol in preparation pVII. The brain was taken out of the methyl salicylate and rehydrated, as described earlier. After this the preparation was washed in PBS (3 x 10 minutes, 4°C). To minimize the nonspecific binding the preparation was incubated in 5% NGS (1 hour, 4°C) followed by addition of primary antibody, rabbit antiserum against serotonin (Immunostar, Hudson, WI) diluted in PBStx containing 5% NGS (1:2000, 40 h at 4 °C). Then the preparation was washed in PBS (3 x 10 minutes, 4°C) before being incubated in CY5-conjugated goat anti-rabbit secondary antibody (Jackson Immunoresearch), diluted in PBStx (1:200, 20 hours, 4 °C). A rinsing in PBS (3 x 10 minutes, 4°C) followed before a sequence of ethanol dehydration was performed, as described above. Finally, methyl salicylate was applied.

2.9 Analytic criteria

At least one neuron had to be clearly stained and easy to identify in the confocal image stacks in each preparation for the preparation to be included in this thesis. The single spikes were quantified by measuring; the duration of the single spikes in milliseconds (ms); the amplitude of a single spike (measured in millivolt - mV), and the number of spikes fired per second (hertz - Hz) (Figure 4). The mean value and the standard deviation were to be calculated for all three parameters (ms, mV and Hz). In neurons showing bursting activity, the bursting amplitude was measured from the raised membrane potential to the top of the action potential. The spiking parameters for the spontaneous activity of each recording were measured one second before stimulus onset and one second after normal spontaneous activity was re-established after stimulus offset. These measurements were performed ten times during the total recording period.

An odour-specific excitatory response was defined as a repeatedly higher spike frequency rate compared to the representative spontaneous activity. An inhibitory response was defined as a lower frequency rate compared to the representative spontaneous activity and/or a depression in the membrane potential. Recordings including both inhibitory and excitatory responses during odour stimulation were identified as complex responses. Here a response was defined as a repeatable

excitation and inhibition pattern compared to the representative spontaneous activity. The physiological data were characterized by a subjective examination of the Spike2 data, accompanied with the quantitative measurements of the spike trains.

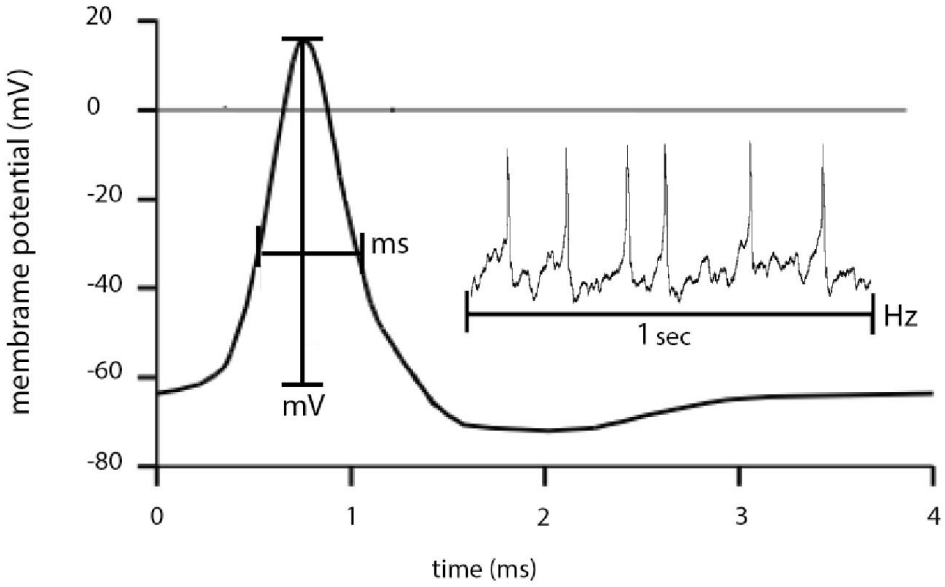

Figure 4: Illustration of the different parameters measured in this study. The membrane potential (amplitude) was measured in millivolts (mV), the duration of the single spikes was measured in milliseconds (ms), and the rate of the spike train was measured in spikes per seconds (hertz, Hz).

3 Results

Out of the 78 moths prepared for intracellular recordings, action potential activity was obtained form 54 individuals, and staining was obtained from 24 neurons. Seven preparations were selected in this thesis, comprising 13 stained neurons. An overview of the stained neurons obtained from, plus the parameters measured in each preparation, is presented in table 1 and 2. The odour response profiles of the neurons in the different preparations are summarized in table 3. The stained neurons in this thesis include the two major categories of AL neurons, PNs and LNs, as well as one CN. Among the PNs were two morphological types, one with axons following the mALT and the other following the mlALT. Also the LN category comprised two morphological types, one multiglomerular and one oligoglomerular. In general, all preparations with successfully stained neurons were prepared for immunohistochemical studies in order to determine the prominent synaptic regions in the olfactory pathway (Figure 5).

3.1 Description of the experiments performed; morphological and physiological data

Table 1: Overview of the quantitative data in this study

Number of experiments performed	78
Insect age in days	1 to 10 (median value of 3)
Species	*Heliothis virescens*
Gender	Males: 54 Females: 24
Number of intracellular contacts in shape of spike-activity	54
Number of stained brain preparations	24
*Number of successfully stained brain preparations***	8
*Number of projection neurons (PN) stained**	6 (pI, pII, pIII)
*Number of local interneurons (LN) stained**	6 (pII, pIV, pV, pVI)
*Number of centrifugal neurons (CN)**	1 (pVII)

Age, species, gender and neuron type distribution for the successfully stained preparations (pI – pVII). Also the number of experiments performed from September 2013 to February 2014 is specified. Abbreviations: pI-pVII – preparation number. *: Included in this thesis. **: Easily identified neurons with physiological data.

Table 2: Overview of parameters measured in each preparation

Preparation number: *Parameters:*	*pI*	*pII small*	*pII large*	*pIII*
Spike duration (ms)	2,2 (SD= 0,6)	1,5 (SD= 0,3)	3,1 (SD= 0,5)	1,3 (SD= 0,7)
Spike amplitude (mV)	12,30 (SD= 5,42)	2,2 (SD= 0,63)	4,29 (SD= 2,51)	12,90 (SD= 3,25)
Spiking rate (Hz)	3,18 (SD= 2,27)	24 (SD= 7,23)	13,09 (SD= 4,68)	35,73 (SD= 7,18)
	pIV	*pV*	*pVI*	*pVII*
	14,0 (SD= 1,2)	3,5 (SD= 1,0)	4,3 (SD= 1,4)	18,4 (SD= 2,1)
	33,10 (SD= 3,21)	56,60 (SD= 8,07)	24,20 (SD= 5,85)	66,40 (SD= 7,43)
	3,91 (SD= 2,07)	11,45 (SD= 9,52)	20,64 (SD= 8,67)	2,27 (SD= 0,90)

Quantitative parameters from the spontaneous activity of the intracellular recordings, performed in seven preparations (pI – pVII). pII contains two sets of spike amplitudes. The duration of a single spike was measured in milliseconds (ms), spike amplitude in millivolts (mV), and spike frequency per seconds (hertz, Hz). The parameters are given with the mean value and with standard deviation, SD (n = 10 for all parameters). All physiological data are summarized in table 7 and appendix 2.

Table 3: Odour response profiles for all preparations.

Preparation:		*PI*	*PII*	*PIII*	*PIV*	*PV*	*PVI*	*PVII*
Gender:		*Male*	*Female*	*Female*	*Male*	*Male*	*Female*	*Male*
Odour stimuli:	*Morphology:*	*PN*	*PN* *LN*	*PN*	*LN*	*LN*	*LN*	*CN*
Air		0	0	0	+	0	0	0
B10		0	0	0	0	0	x	0
Linalool		0	0	0	0	0	x	0
Germacrene D		0	0	0	0	0	x	0
2-Phenylethanol		0	0	0	0	0	x	0
3Z - Hexenyl acetate		0	-	0	0	0	x	0
Ocimene		0	0	0	0	0	x	0
Farnesene		0	0	0	+	0	x	0
Ylang-Ylang		0	0	0		0	x	0
Z11-16 Ald					0	0		
Z9-14: AL						0		
Z9-14: AL + Z11-16:AL					0	0		0
Z11-16:OH *								
Z11-16:AC *								
Z11-16 Ald + Linalool *								
Z11-16 Ald + B10 *								

Overview table for all stimuli used in all experiments (*: including four that did not elicit responses in the presented neurons), the response profile for the different preparations (pI - pVII), gender and morphological classification. Description of the different markings; + excitatory response; - inhibitory response; 0 no response; x mixed response of both inhibition and excitation. A blank space means that no stimuli were given. Air was used as the control stimuli. The plant odours are marked in green, and the pheromones and plant-pheromone blends in pale orange. Abbreviation: LN – Local interneuron; p – preparation, PN – Projection neuron. The constituents of the odour stimuli protocol and the components of the B10 mix and ylang-ylang are summarized in table 6 and appendix 1.

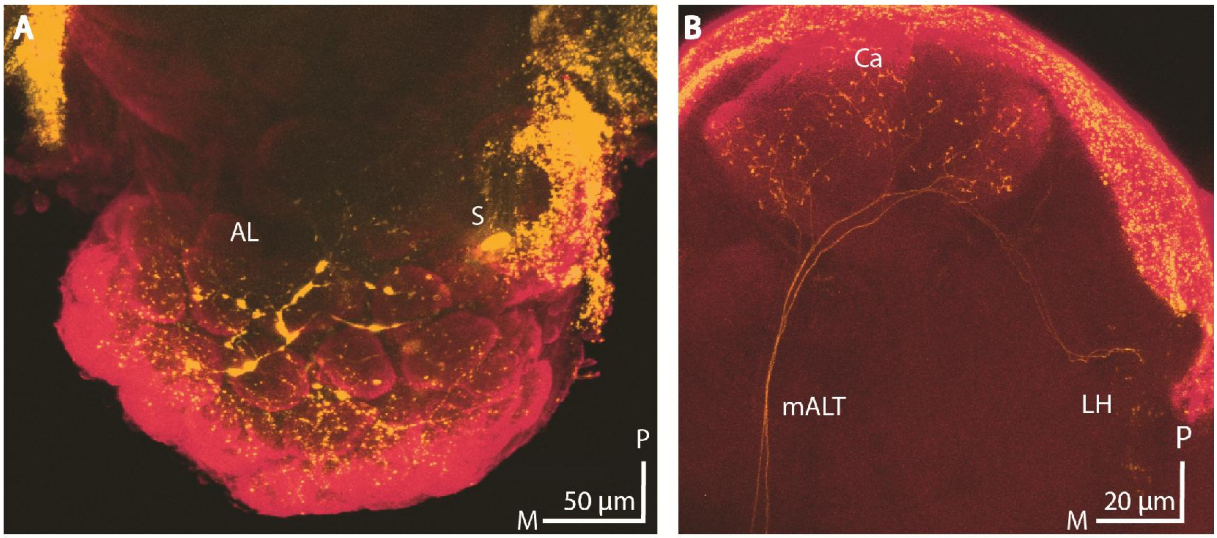

Figure 5: Immunohistochemical results. **A)** An example showing neural processes in preparation pIV innervating the characteristic glomeruli of the antennal lobe (AL). **B)** Neural processes from preparation pI innervating the calyces (Ca) of the mushroom body and the lateral horn (LH). Abbreviations: M – medial; mALT – medial antennal lobe tract; P – posterior; S – soma; µm – micrometre.

3.2 Projection Neurons

Two morphological types of PNs were successfully stained in this study, five uniglomerular PNs with axons projecting in the medial antennal lobe tract (mALT) and one multiglomerular PN passing in the mediolateral ALT. Four uniglomerular PNs were simultaneously stained in the same preparation, whereas one uniglomerular PN and one multiglomerular were stained in distinct preparations. All PNs in the current study displayed regular spiking spontaneous activity. The morphology and physiology of the stained neurons are presented in table 4.

Table 4: Overall characteristics of the PNs presented in this thesis.

PN identification:	*Soma location:*	*Soma size (µm):*	*AL innervation:*	*ALT:*	*Spiking profile:*	*Odour response:*
PN1 – 4 (pI)	MC	~10	Uniglomerular	mALT	Single spiked	None
PN5 (pII)	LC	~10/20 *	Uniglomerular?	mALT	Single spiked*	Inhibition: Hexenyl acetate*
PN6 (pIII)	LC	~10	Multiglomerular	mlALT	Single spiked	None

The soma size and location, the neuronal AL innervation pattern, the ALT pathway and the odour responses are presented for preparation I, II and III (PI - pIII). * PN5 in pII was stained together with LN2 and LN3, making it difficult to see from which of the stained neurons the somata and physiological recording in pII originates from. Abbreviations; AL – Antennal lobe; ALT – Antennal lobe tract (m – medial, ml – mediolateral); MC – medial cell cluster; LC – lateral cell cluster; p – preparation; µm – micrometre.

3.2.1 Uniglomerular projection neurons following the medial antennal lobe tract

3.2.1.1 PN 1, 2, 3 and 4

Morphology

In preparation one (pI), four axons (PN1 to PN4) were simultaneously stained. As demonstrated in figure 6, the prominent mALT possesses four labelled axons, of which two are particularly well stained (Appendix 3). Each of the four neurons innervated a single ordinary glomerulus. The glomerular innervations differed among these neurons, one showing a dense dendritic arborisation that filled the entire glomerulus in contrast to the others showing a loose innervation (Figure 6B). Three somata were visible in the confocal images (diameter ~10 μm), all residing in the medial cell cluster (Figure 6B). Three of the axons, the two well-stained and one of the weaker stained, innervated the calyces, each sending three collaterals into this structure (Figure 6C). The fourth axon bypassed the calyces dorsally. Possibly, a thin branch from the current axon innervated the lateral calyces from a lateral position (Figure 6E). The arborisation pattern of the collaterals showed numerous terminals with blebs, some of which occurred in paired blebs. The innervations of these neurons extended throughout the whole structure. After giving off the collaterals in three axons, plus the one bypassing the calyces, turned in an anterior direction innervating the most lateral part of the lateral horn (LH), positioned adjacent to the optic lobe (Figure 6D and Appendix 3). The terminals in the LH also carried bleb-like structures, but with smaller size than those in the calyces. More fibers run between the calyces and the LH than between the AL and the calyces. However, the fibers projecting to the LH is difficult to distinguish.

Physiology

Spontaneous activity as regular single spikes was recorded from one of the uniglomerular PNs being simultaneously stained. The neuron was first silent and then fired trains of spikes. The recording electrode had an initial resistance of 400 MΩ increasing to 800 MΩ during the recording period. The spike train comprised single spikes having a mean duration of 2,2 ms (SD=0,6) and an amplitude of 12,3 mV (SD=5,42). The spike firing rate was 3,18 Hz (SD=2,27) (table 2). The odour stimuli tested elicited no response (control, B10, linalool, germacrene D, phenylethanol, hexenyl-acetate, ocimene, farnesene and ylang-ylang, Table 3). All stimuli induced an artefact, possibly due to mechanical disturbance or a current fluctuation in the neural circuit (Figure 6F).

3.2.1.2 PN 5

Morphology

The second preparation (pII) contained one uniglomerular PN (PN5), together with two LNs (LN2 and 3) (Figure 11). Two somata (~ 20 μm in size) both residing in the lateral cell cluster were stained

(Figure 7B). PN5 followed the mALT and innervated the ipsilateral calyces loosely before terminating in the LH (Figure 7A and C).

Physiology

Spontaneous activity recorded form pII comprised two different spiking amplitudes, one large and one small. The large amplitude spikes has a duration of 3,1 ms (SD= 0,5), an amplitude of 4,29 mV (SD= 2,51) and a firing rate of 13,09 Hz (SD= 4,68). The small amplitude spikes has a duration of 1,5 ms (SD=0,3), an amplitude of 2,2 mV (SD= 0,63) and a firing rate of 24 Hz (SD=7,23). The neuron responded with hyperpolarization and inhibition of the spiking activity in response to hexenyl acetate-stimulations. The latency for the response according to the stimulus onset was 5,7 ms for the first stimulation and 30,5 ms for the second stimulation (Figure 7D). No response was elicited by any of the following odour stimulations: control, B10, linalool, germacrene D, phenylethanol, ocimene, farnesene and ylang-ylang (Table 3). The recording electrode had a resistance of approximately 350 MΩ during the recording period. Whether the spiking activity originated from this PN or the LNs cannot be determined.

3.2.2 Multiglomerular projection neuron following the mediolateral antennal lobe tract

3.2.2.1 PN 6

Morphology

One successfully stained PN (PN6) in preparation three (pIII), with an axon confined to the mlALT showed a multiglomerular innervation of the AL. Each glomerulus was loosely innervated by the current neuron (Figure 8A and C). This multiglomerular PN had a thick main axon passing directly to the medial LH. On its route it extended one branch in the medial LH, dorsally of the pedunculus. The soma, having a diameter of ~ 15 μm, resided in the lateral cell cluster. In addition to the main axon, two thinner branches of the current neuron projecting out of the AL towards an area in the protocerebrum medially to the LH. One of the sub-branches extended in a medial direction and the other in a lateral direction as compared to the main axon (Figure 8B and D).

Physiology

The intracellular recording comprised a high spontaneous spike activity of 35,73 Hz (SD= 7,18), a spike amplitude of 12,90 mV (SD= 3,25), a duration of single spikes of 1,3 ms (SD= 0,7) (Figure 8E). The odour stimuli tested elicited no response; control, B10, linalool, germacrene D, phenylethanol, hexenyl acetate, ocimene, farnesene and ylang-ylang (Table 3). The recording electrode had a resistance of 350 MΩ during the recording period.

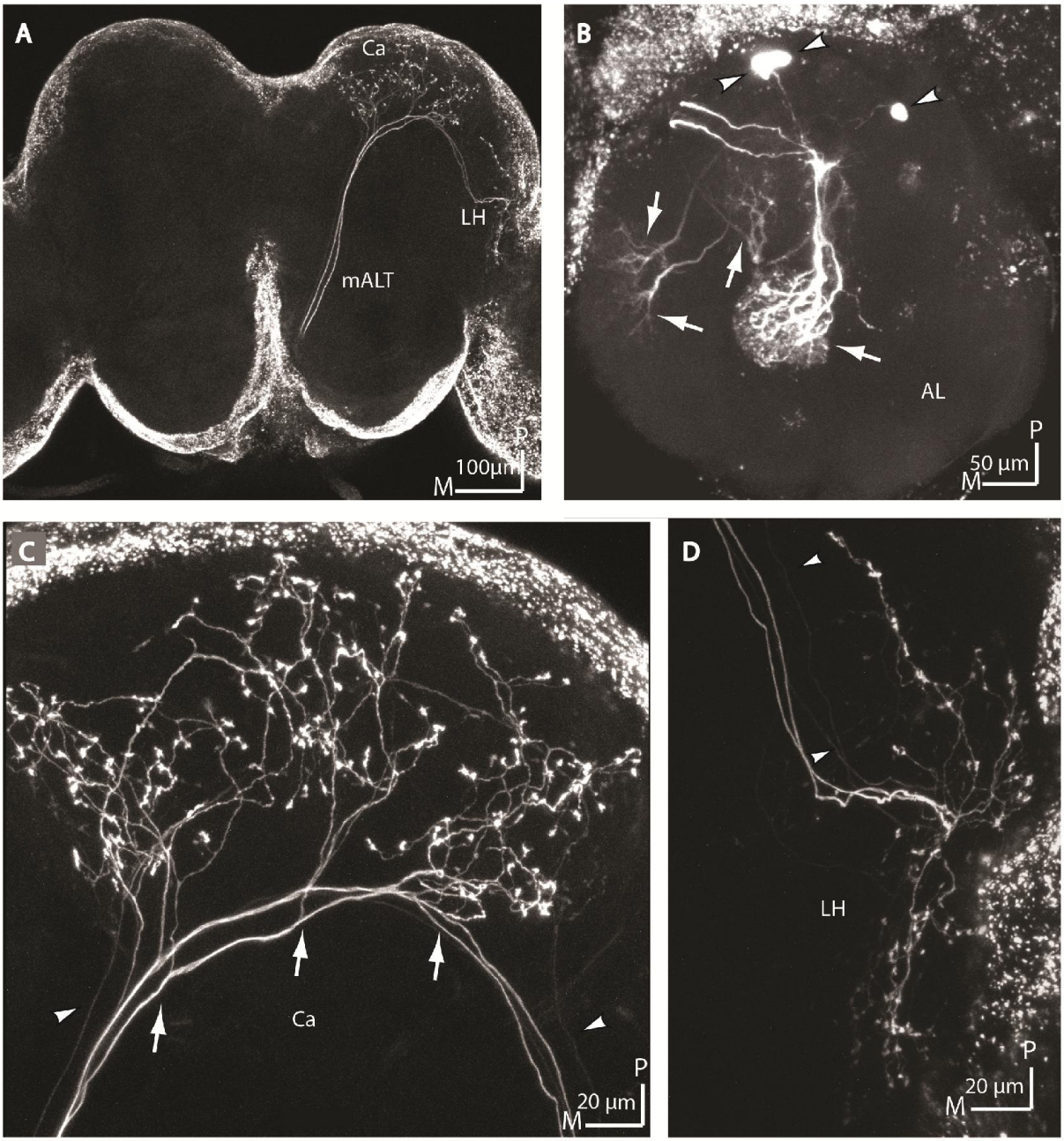

Figure: 6A, B, C and **D.**

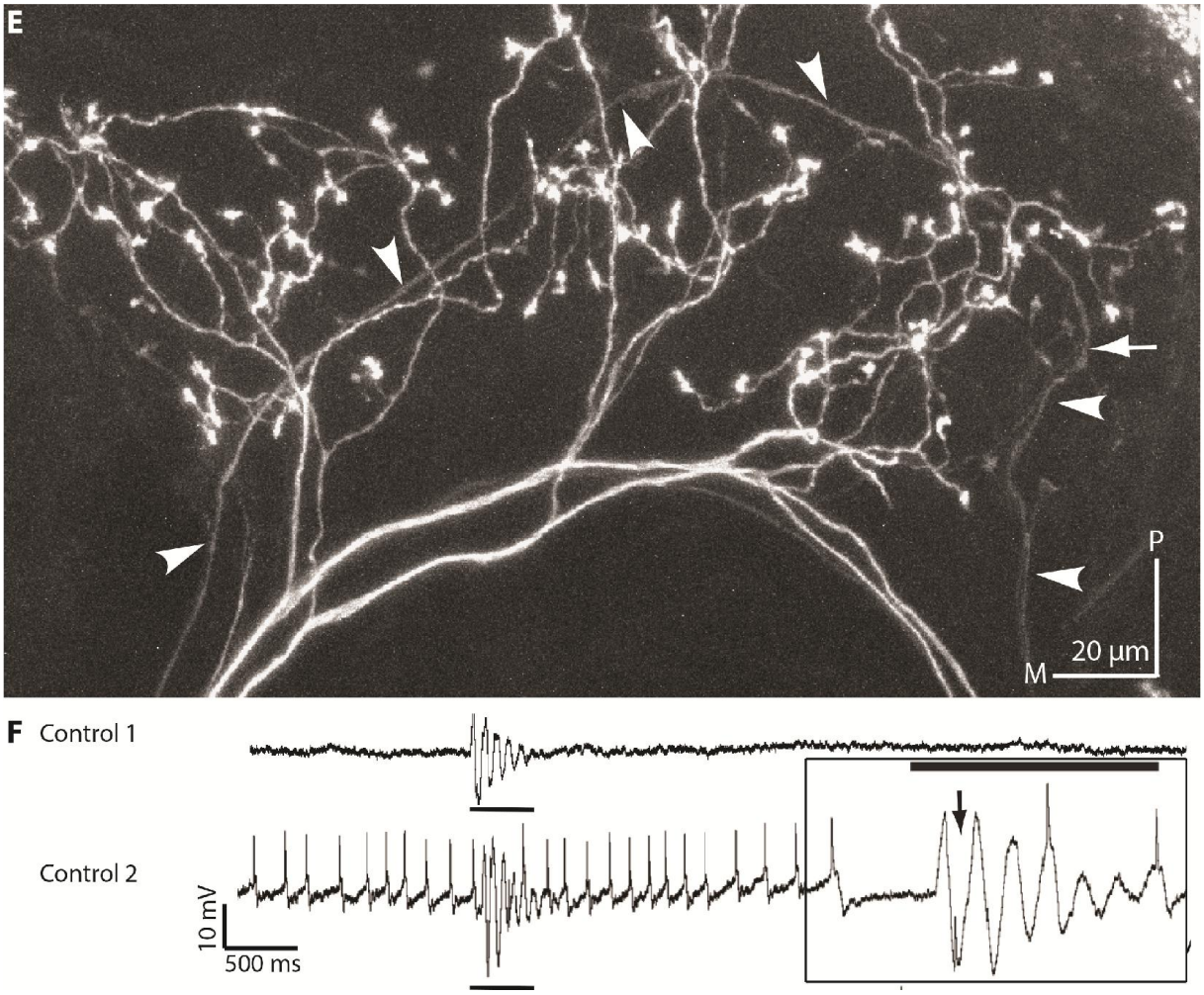

Figure 6: A) Confocal image of preparation I (pI), a male *Heliothis virescence* moth seen from a dorsal view (10x magnification). pI contain four projection neurons (PNs) (PN1 – 4) in the left hemisphere, which projected from the antennal lobe (AL), along the medial antennal lobe tract (mALT) to the calyces (Ca), where three of them innervated the Ca - a fourth bypassed the Ca dorsally, possibly innervating it from the lateral side. Then all PNs turned in an anterior direction and innervated the lateral horn (LH) laterally. Two of the PNs were more prominently stained. **B)** In the AL four ordinary glomeruli were stained, as indicated by arrows, and three somata were located in the medial cell cluster, all indicated by arrowheads (20x magnification). **C)** Ca innervation (40x magnification). Collaterals entered the Ca at three points, as indicated by arrows. Arrowheads indicate the fourth neuron bypassing the Ca dorsally. **D)** All four neurons innervated the LH in a lateral manner (40x magnification) Arrowheads indicate two less prominent axons. **E)** A dorsal selection of the confocal image stacks display the Ca innervation. A fourth neuron indicated by arrowheads. The possible lateral innervation point indicated is by an arrow **F)** Spike trains from stimulation with control one and two. Horizontal bar marks the stimulation period (400 ms). The enlarged area represents the stimulation period from second control stimulation. Notice the spike, indicated by an arrow, in the bottom of the oscillation. The fluctuations of the baseline might be due to some mechanical or electrical artefacts. Abbreviations: M – medial; ms – milliseconds; mV – millivolt; P – Posterior; µm – micrometre.

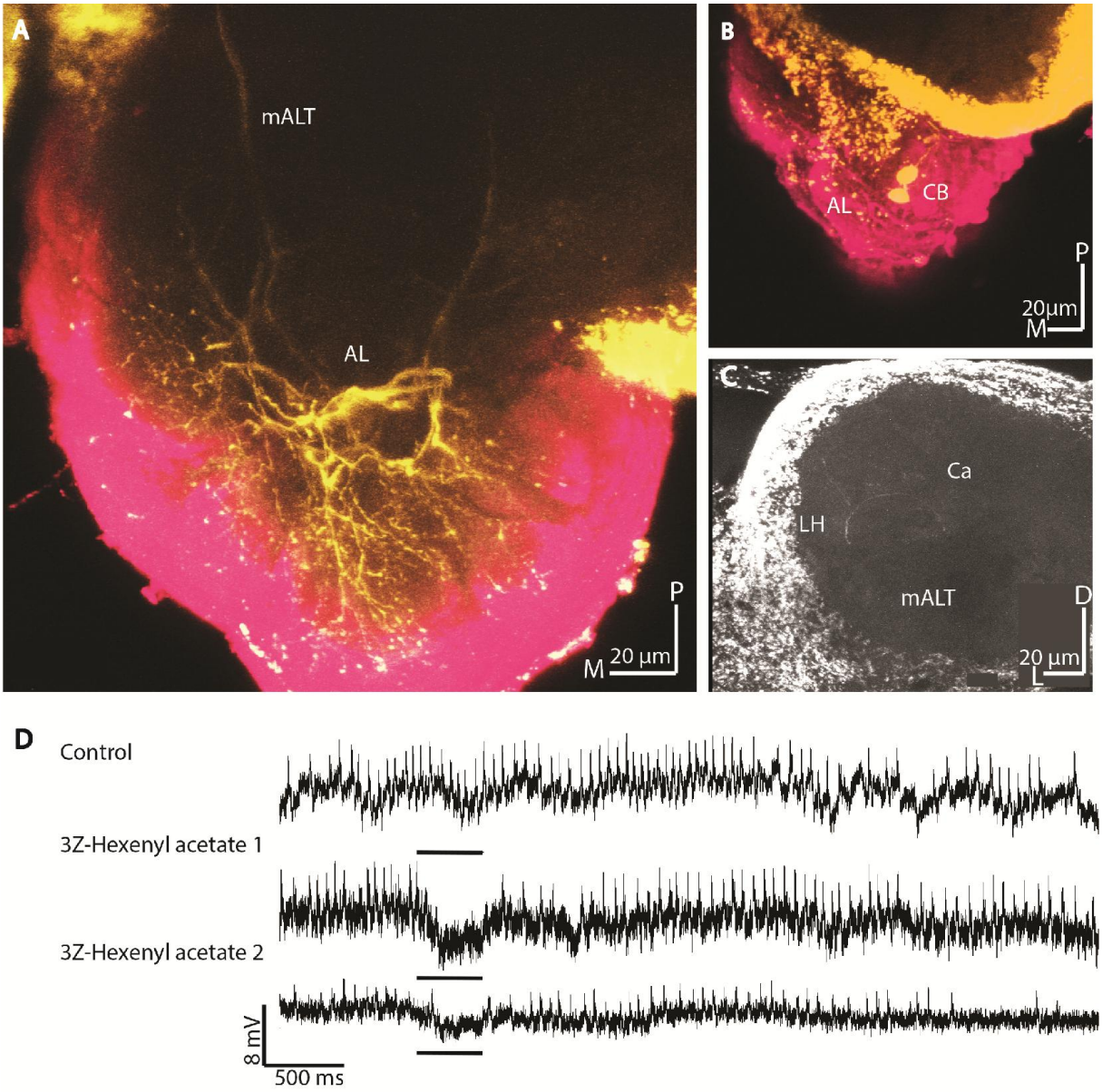

Figure 7: A) Confocal image of preparation II (pII) from the left antennal lobe (AL) in a *Heliothis virescence* female, seen from a dorsal view. One projection neuron (PN5) and at least two local interneurons (LN2 and LN3, Figure 10) were prominent (20x magnification). **B)** The two somata reside in the lateral cell cluster, seen here in a yellow colour, from a dorsal view (40x magnification). **C)** PN5 followed the medial antennal lobe tract (mALT) and innervated the calyces (Ca) and the lateral horn (LH) in a loos manner, seen from a frontal view (40x magnification). **D)** Stimulation with control and 2x 3Z-hexenyl acetate. Notice the depression of action potentials during the stimulus period in the two stimulations. Horizontal bars marks the stimulation period (400 ms). Abbreviations: D – dorsal; M – medial; ms – milliseconds; mV - millivolt L – Lateral; P– posterior; µm – micrometre.

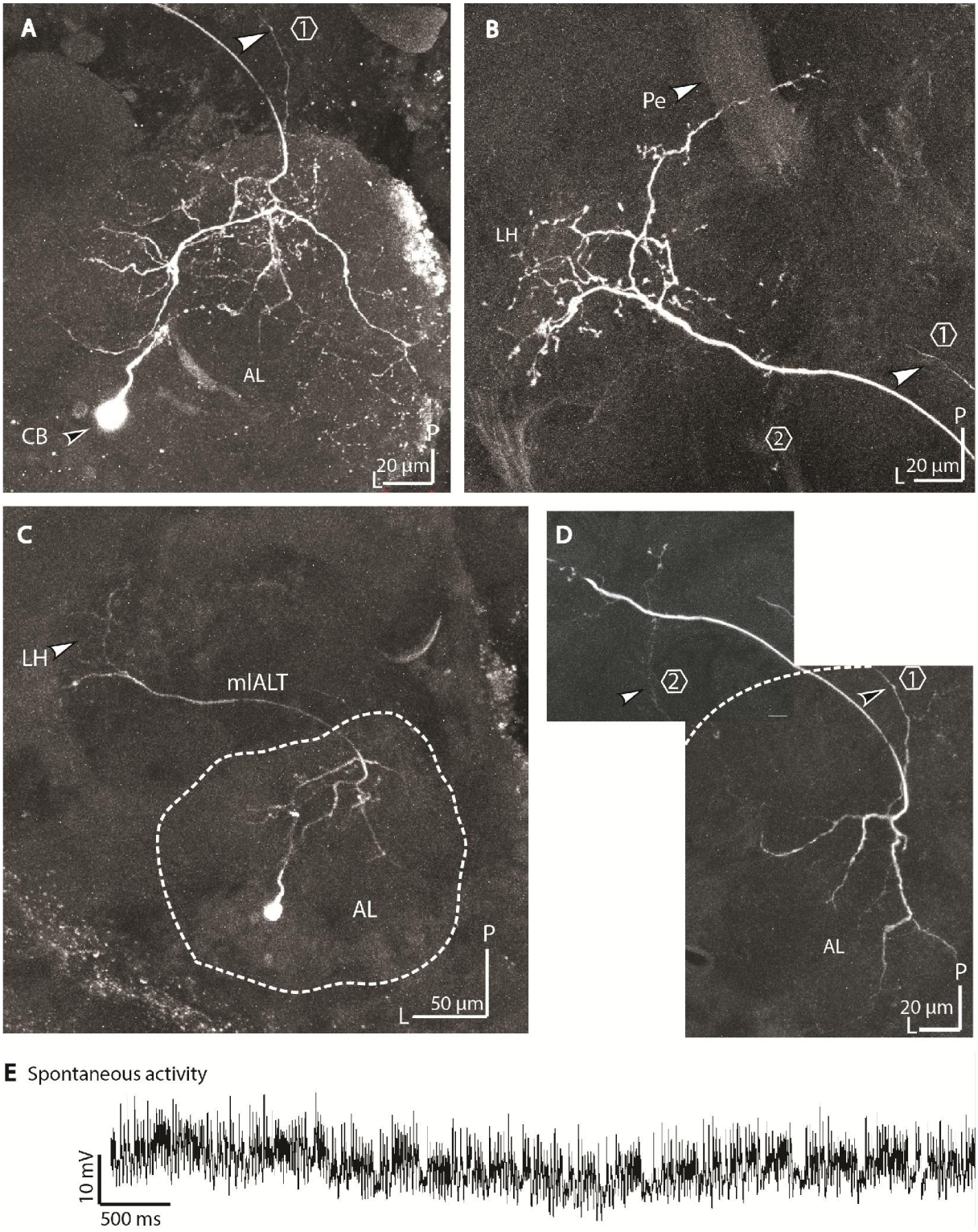

Figure 8: Projection neuron 6 **(**PN6) in from a *Heliothis virescence* female in preparation pIII, seen from a dorsal view. **A)** The soma resided in the lateral cell cluster in the right antennal lobe (AL). The main axon, along with two additional branches, projected out of the AL. Branch number one can be seen in this image (40x magnification) **B)** Arborizations in the lateral horn (LH) area, where the main axon and the two additional branches (1 and 2) (40x magnification). **C)** PN6 projects out of the AL in the mediolateral antennal lobe tract (mlALT) (20x magnification). **D)** Branch number one project towards the calyces (Ca) in a lateral direction and ended up dorsally to the pedunculus (Pe). Branch number two projects out of the AL in a more medial direction towards the same area as the first branch. **E)** Spike train from the spontaneous activity. Abbreviations: L – lateral; ms – milliseconds; mV – millivolt; P – posterior; µm – micrometre.

3.3 Local Interneurons

Six LNs originating in three preparations were successfully stained in this study. They comprised two morphological types; five multiglomerular LNs innervating most, if not all glomeruli, and one oligoglomerular LN innervating only some of the glomeruli. All neurons, which were confined to the AL, had their soma located in the lateral cell cluster. The intracellular recordings showed different spontaneous activity in these LNs, for example regular single spikes, bursting of spikes or a combination of the two. The morphological and physiological properties of the LNs are presented in table 5.

Table 5: Overall characteristics for the LNs presented in this thesis.

LN identification:	*Moth gender:*	*Soma size (μm):*	*Soma location:*	*Branching pattern:*	*Spiking profile:*	*Response:*
LN1 (pIV)	Male	20	LC	Multiglomerular	Single spiked	Excitation: Control and Farnesene
LN2 and 3 (pII)	Female	10 or 20 *	LC	Multiglomerular	Single spiked *	Excitation: Hecenyl acetate *
LN4 and 5 (pV)	Female	20	LC	Multiglomerular	Bursting	None
LN6 (pVI)	Female	20	LC	Oligoglomerular	Single spiked and bursting	Excitation: diverse Plant odorants

The moths' gender, location and size of the somata, the innervation pattern in the antennal lobe and the spike train profile for each preparation. * From which neuron-type in pIII this recording originates from is unknown. Abbreviations: LC – lateral cell cluster; LN – local interneuron; pII, pIV, pV, pVI – preparation number; μm – micrometre.

3.3.1 Multiglomerular local interneurons

3.3.1.1 LN 1

Morphology

One multiglomerular LN was stained in a male preparation (pIV) (Figure 9A). The neuron seemed to innervate all glomeruli, including the MGC units, as indicated in Figure 9B. The soma was positioned in the lateral cell cluster (Figure 9C). The neural processes had prominent swellings that measured one-third of the soma diameter, for example approximately 20 μm (Figure 9A,C). Unfortunately, the glomeruli had slipped out of the neurolemma during the experiment making further analyzation of the glomerular innervations difficult.

Physiology

This multiglomerular LN displayed a regular single spiking spontaneous activity. The neuron showed a response to the first control stimulation, but this kind of response did not appear in the succeeding B10 stimulation (Figure 9D). However, the neuron displayed an excitatory response to stimulation with farnesene (Figure 9D). The response latency was 164,5 ms and 130 ms for the first and second farnesene stimulation, respectively. Other odorants tested were: control, linalool, germacrene D, phenylethanol, hexenyl acetate, ocimene, the major pheromone component Z11-16:AL and the

pheromone blend of both minor and major pheromone component: Z9-14:AL + Z11-16:AL (Table 3). The spontaneous activity decreased significantly during the recording period (Figure 9D). The recording electrode had a high resistance of approximately 1200 MΩ during the registration period. The spike train comprised of single spikes, each showing a particular long duration of 14,0 ms (SD= 1,2) and large amplitudes of 33,10 mV (SD= 3,21). The firing rate was 3,91 Hz (SD= 2,07).

3.3.1.2 LN 2 and 3

Morphology

In preparation pII one PN (PN5) and several LNs were stained. The LNs are all housed in the left AL, where they resided together with PN5 (Figure 7A). These LNs innervated the AL in a typical multiglomerular manner. Two sets of somata were stained; two small ~ 10 μm in size (indicated by arrows in figure 10A) and two of large ~ 20 μm in diameter (indicated by the dotted line in figure 10A). All four resided ventrally in the lateral cell cluster. The two large somata do not show any obvious connection to the PN or the LNs stained in pII. Of the four somata appearing before the immunostaining procedure with synapsin (Figure 10A) only two were visible after this staining procedure (Figure 7B). The two remaining somata had a diameter of ~ 20 μm and were the same size as the somata originating in the LNs stained in this study (Table 5), and smaller than the PN somata in this study (Table 4), which is the reason why pII was decided to contain two LNs and one PN, which will be discussed later.

Physiology

The physiology of the neuron recorded in pII is presented in the previous PN subsection (Figure 7D).

3.3.1.3 LN 4 and 5

Morphology

In preparation five (pV) two LNs were stained (LN5 and LN6) (Figure 11A and B), both innervating most glomeruli in a typical multiglomerular manner. From their somata residing adjacent to each other in the lateral cell cluster, the neurites followed each other in shape of a major process passing towards the middle of the AL. Here. They extended several branches in mirror symmetry throughout the AL. The somata of the two neurons were ventrally positioned in the lateral cell cluster, both measuring to approximately 20 μm in size. In this preparation one weakly stained process projecting out of the AL in direction toward the calyces and the LH was labelled. However, the current branch had no connections to the stained LNs in this preparation.

Physiology

The intracellular recordings showed a particular pattern of bursting spikes trains superimposed on a strong depolarization. When stimulating various odorants and mixes no response appeared (Figure 11D). The spike train was comprised of spikes which was measured with a duration of 3,5 ms (SD=

1,0), with a large amplitude of 56,6 mV (SD= 8,07) and at a rate of 11,45 Hz (SD= 9,52). The recording electrode had a resistance of 300 MΩ during the recording period. Some fluctuations of the membrane potential occurred during the experiment.

3.3.2 Oligoglomerular local interneuron

3.3.2.1 LN 6

Morphology

The oligoglomerular LN (LN6) was stained in a female moth (pVI). The neuron innervated the AL glomeruli in a loose manner with thinner processes as compared to those of LN1, being multiglomerular. LN6 innervated the two female-specific glomeruli (LFGs), in addition to two neighbouring ordinary glomeruli (Figure 10A). The weakly stained soma (~ 20 μm) resided in the lateral cell cluster (Figure 12B).

Physiology

The intracellular recording showed alternating periods of bursting and single spiking activity (Figure 12C). Application of the stimulus control did not elicit visible changes in the activity. However, subsequent application induced a particular response including an inhibition followed by an excitation (Figure 12D). The irregularity of the spiking made it relatively difficult to interpret the responses, however, the LN responded to the following stimuli: B10; linalool; germacrene D; phenyl ethanol; hexenyl acetate; ocimene; farnesene and ylang-ylang (Figure 12D). The hyperpolarization, which was elicited by all odour stimuli, was followed by an excitatory response according to the bursting activity and then a subsequent inhibition according to the single spike frequencies. A repeated burst of action potentials occurred at the end of the stimulation period in response to almost all odour stimuli, except for ocimene. Furthermore, a repeated decrease in the single spike activity can be seen after the stimulation period, especially for the phenyl ethanol and hexenyl acetate. The recording electrode had a resistance of approximately 300 MΩ during the recording period, and the single spikes had a duration of 4,3 ms (SD= 1,4) and an amplitude of 24,20 mV (SD= 5,85). The spiking rate was 20,64 Hz (SD= 8,67).

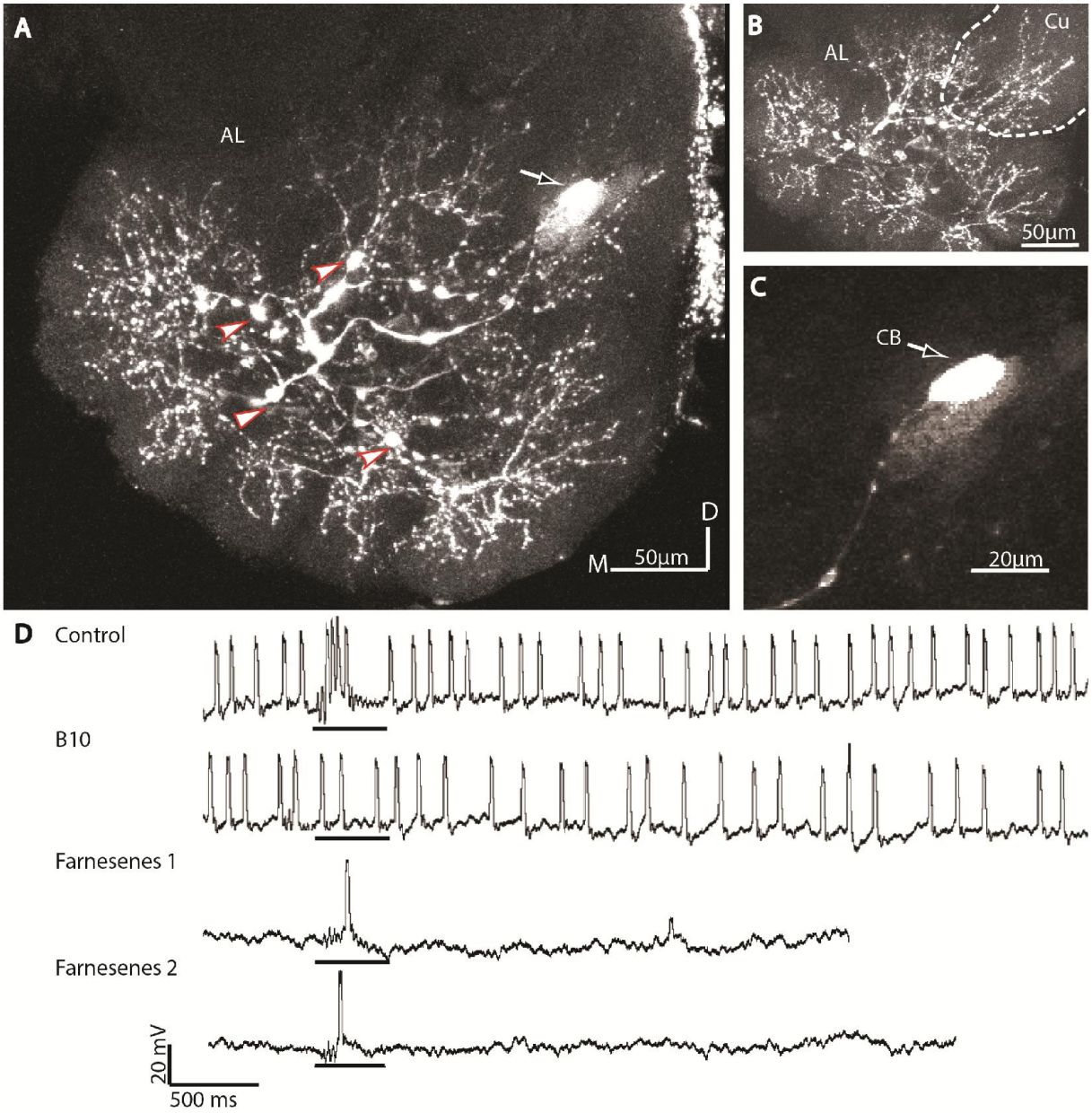

Figure 9: Confocal images of local interneuron 1 (LN1) in the left antennal lobe (AL) in a *Heliothis virescence* male (preparation pIV), seen from a frontal view (40x magnification). **A)** The four arrowheads marks swellings in the dendrites and the arrow indicates the soma. **B)** A different sample of stacks than in figure A, oriented in a more anterior direction. The dotted line indicates the cumulus (Cu) of the macroglomerular complex (MGC). **C)** The soma resided in the lateral cell cluster. **D)** Spike trains from stimulations with control (air), B10 and farnesene. Notice the response to the control but not to the sequential B10 stimulation. Also notice the response to the farnesene stimulation during the silent spontaneous activity period. Horizontal bars marks the stimulation period (400 ms). Abbreviations: D – dorsal; M – medial; ms – milliseconds; mV – millivolt; µm – micrometre.

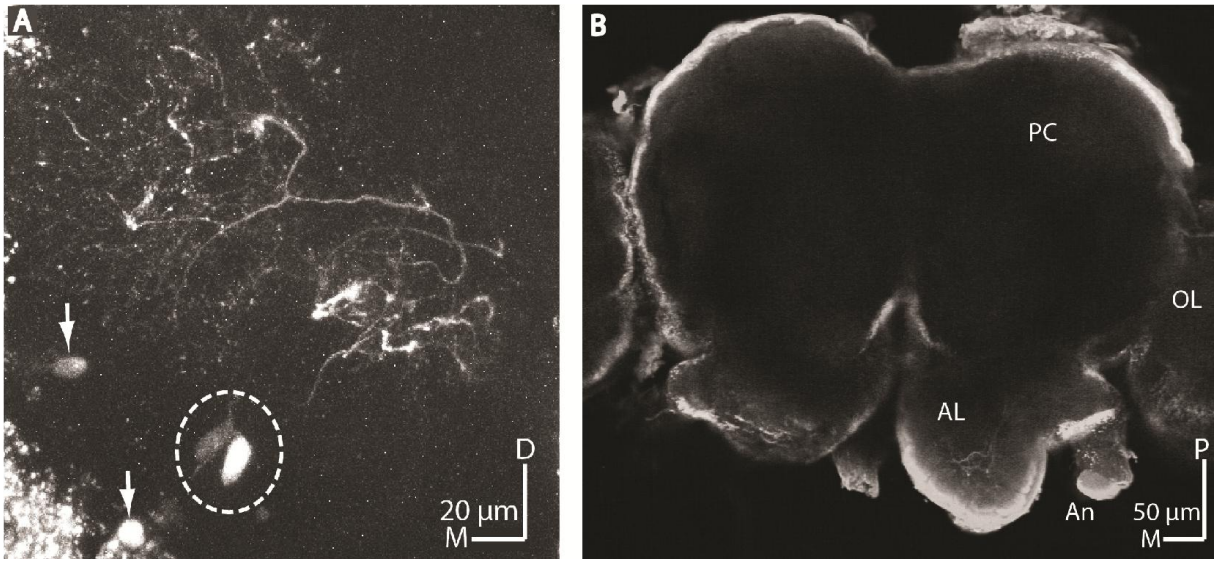

Figure 10: A) Confocal image (40x magnification) of preparation pII in the left antennal lobe (AL) from a *Heliothis virescence* female, seen from a frontal view. pII contained one projection neuron (PN) and at least two local interneurons (LNs) (LN2 and 3). The LNs innervated the AL in a mutiglomerular manner. The two somata seen in connection with the neuronal arborizations are marked with a dotted line, the latter somata are marked by arrows. **B)** The moth brain preparation seen from a dorsal view (10x magnification). Abbreviations: An – antennal nerve; D – dorsal; M – medial; ms – milliseconds; mV – millivolt; P – posterior; OL – olfactory lobe; µm – micrometre.

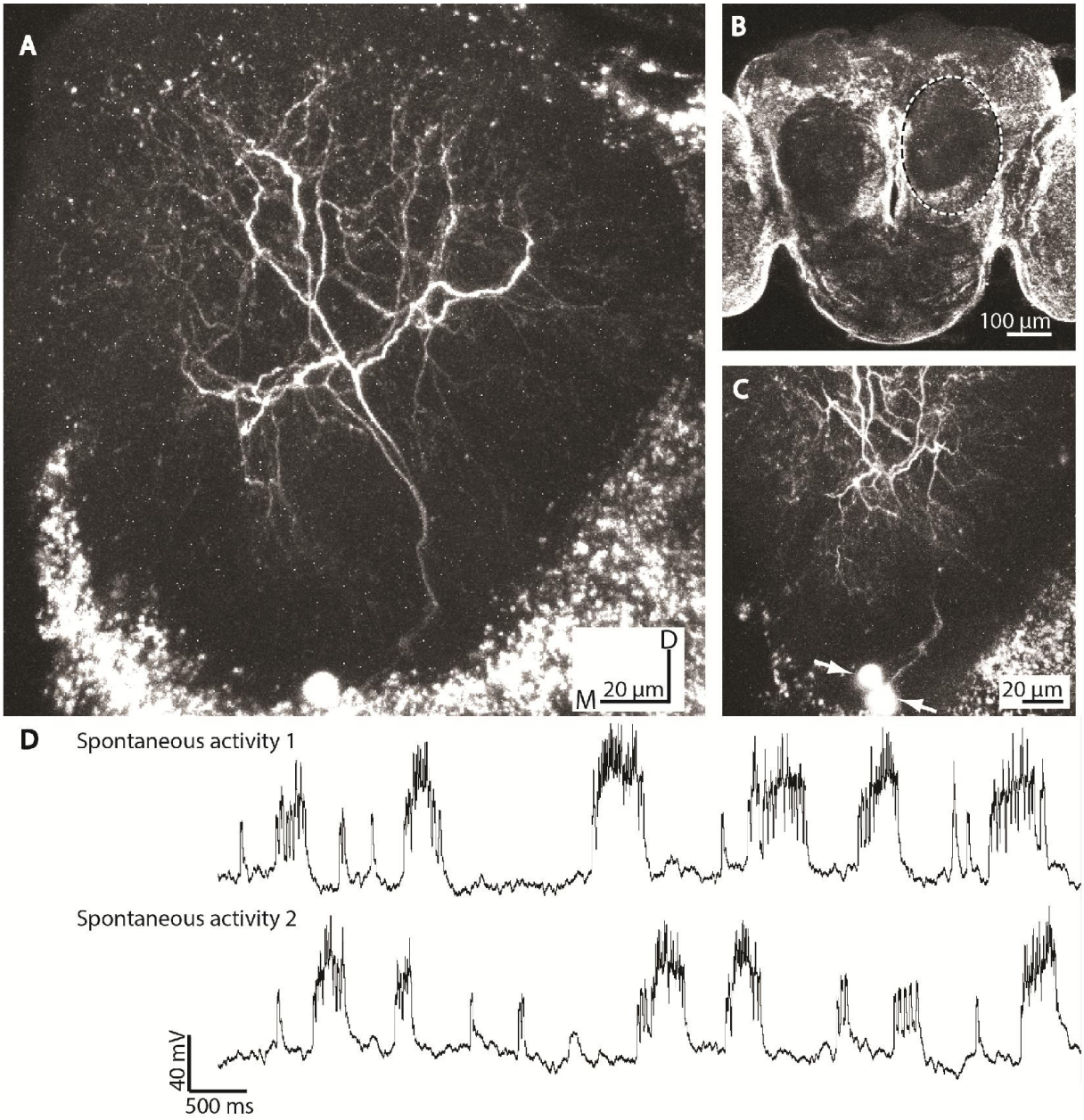

Figure 11: Confocal image of the local interneurons (LNs) stained in the left antennal lobe (AL) of preparation pV from a *Heliothis virescence* female, seen from a frontal view. **A)** This staining comprised two LNs (LN4 and LN5), arborizing through the entire AL and innervated all glomeruli (40x magnification). **B)** The stained neurons indicated by the dotted white line (10x magnification). **C)** Both somata were located side by side ventrally in the lateral cell cluster (LC), as indicated by the two arrows (40x magnification). **D)** Spike trains from the spontaneous activity recorded in pV. No responses were seen for this recording to any of the odour stimulations. Abbreviations: D – dorsal; M – medial; ms – milliseconds; mV – millivolt; µm – micrometre.

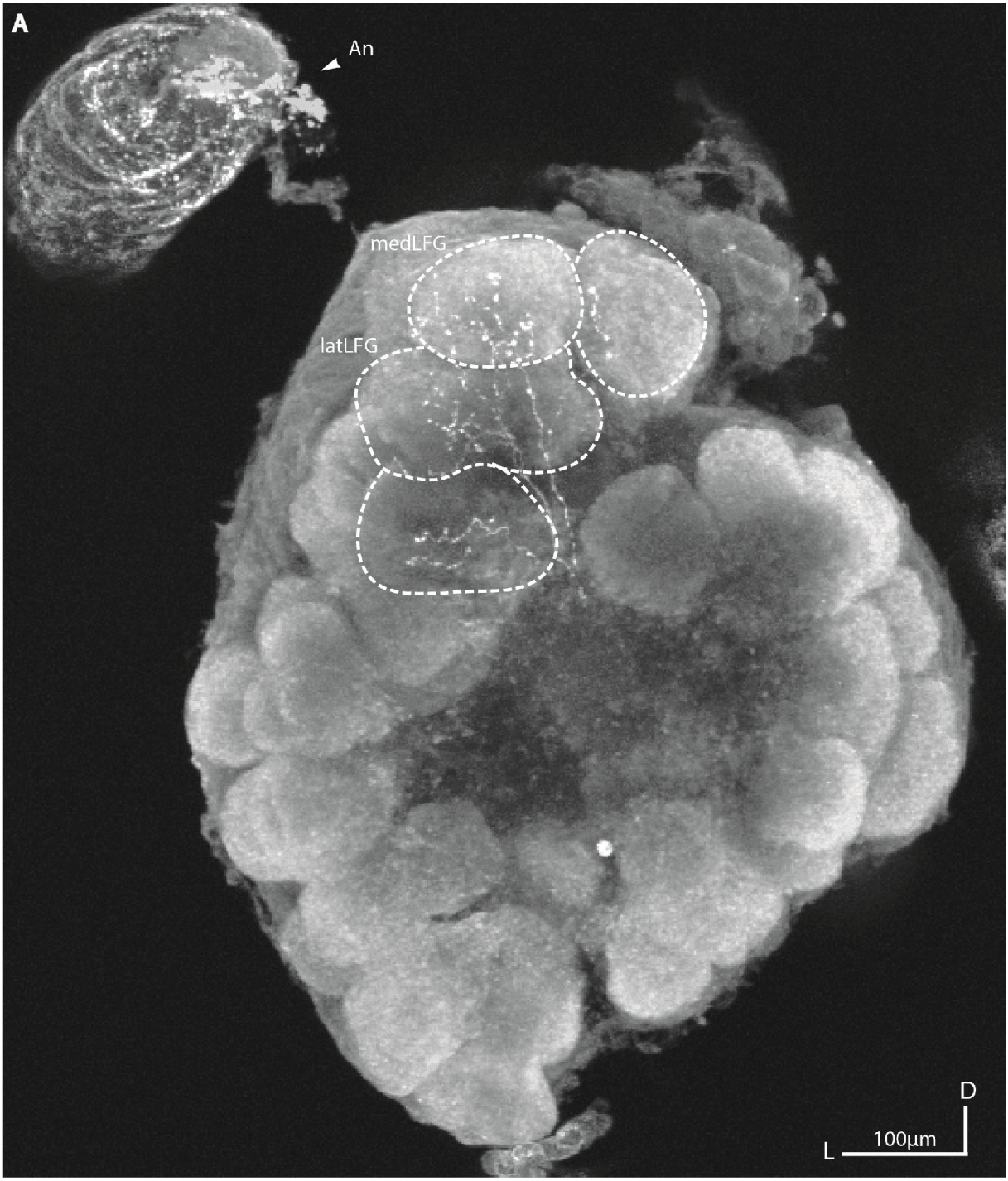

Figure: 12 A

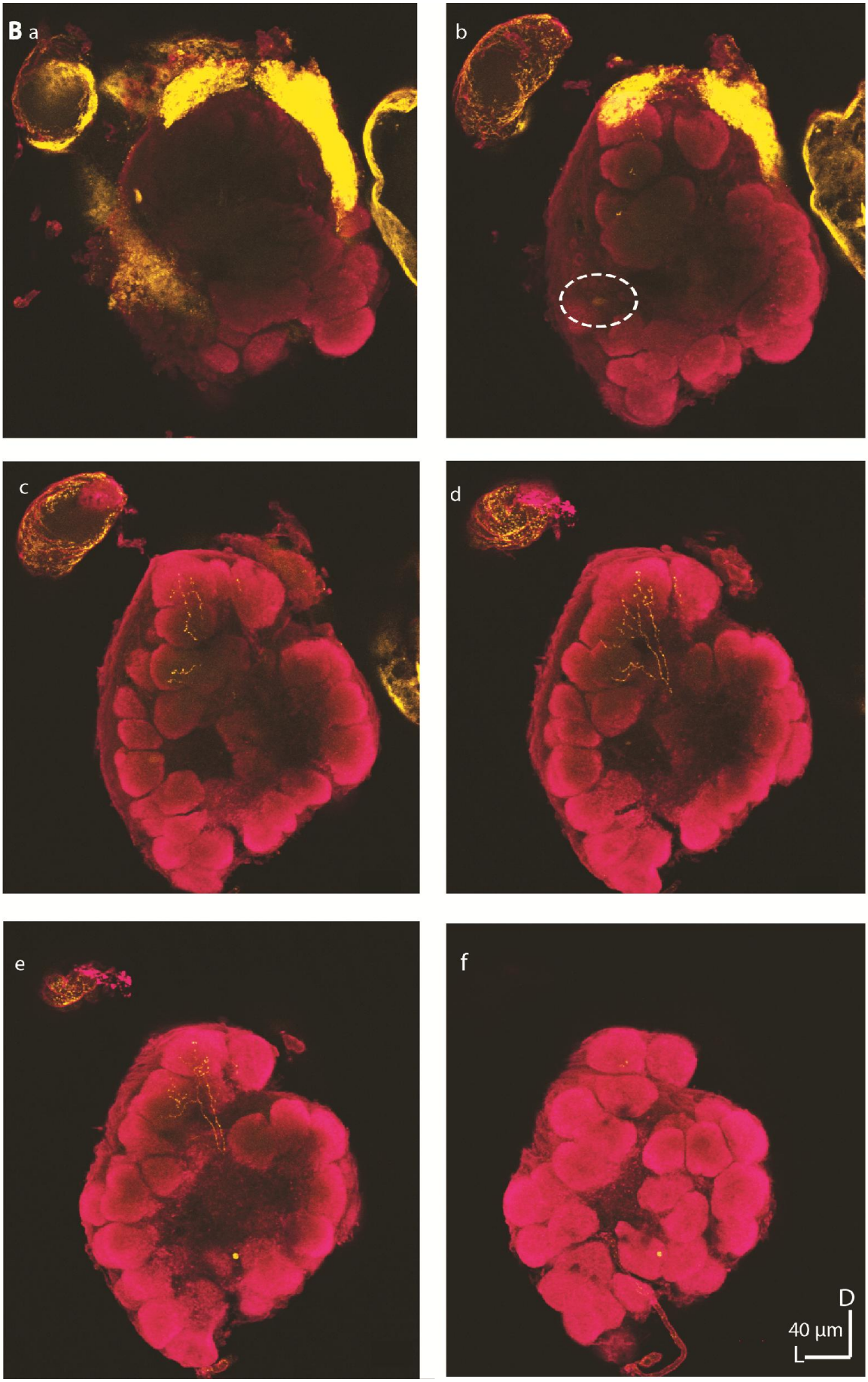

Figure: 12B

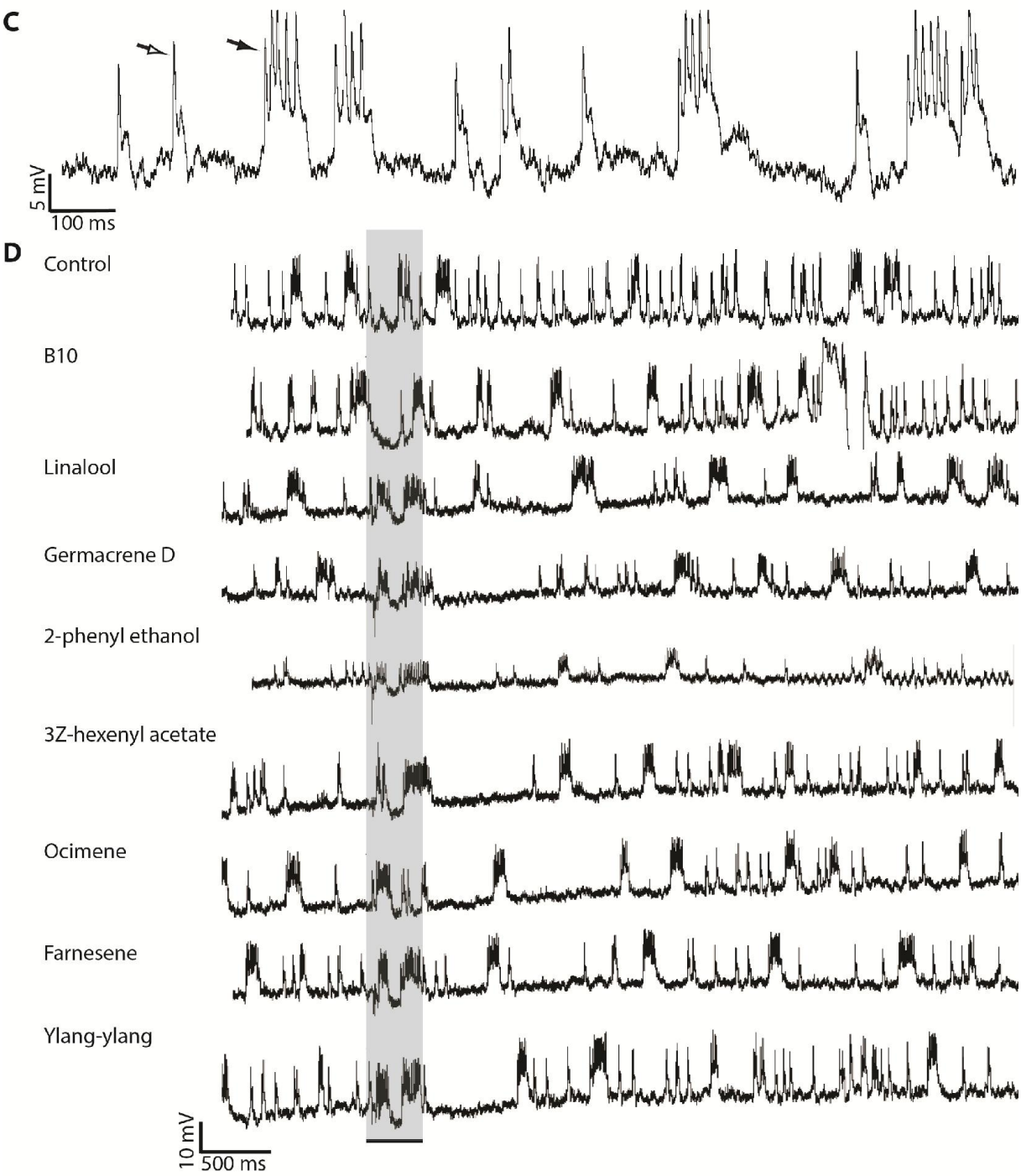

Figure 12: LN6 in preparation pVI in the right antennal lobe (AL) from a *Heliothis virescence* female, seen from a frontal view (20x magnification). **A)** The local interneuron (LN6) soma was located in the lateral cell cluster, anteriorly positioned. The arrowhead indicates the position of the antennal nerve (An). The dotted white line demonstrates the contour of the innervated glomeruli, two ordinary glomeruli and the two large female glomeruli (LFGs) **B)** Serial slices (posterior-anterior progression in a frontal view): a) From 94 to 74 µm into pVI. b) From 74 to 54 µm. Notice the soma indicated by the dotted circle. c) From 54 to 44 µm. d) From 44 to 34 µm. e) From 34 to 24 µm. f) From 24 to 4 µm. **C)** The enlarged spike train in this figure display the two different spiking profiles represented in this recording, a single spiked spontaneous activity, indicated by a white filled arrow, and a bursting firing pattern, indicated with a black filled arrow. **D)** Spike trains show different response patterns to the stimulations presented in the order they were applied. Notice the excitatory response from the bursting spikes and the inhibitory effect in the single spikes. Horizontal bars marks the stimulation period (400 ms). Abbreviations: D – dorsal; L – lateral; latLFG – lateral large female glomeruli; medLAT – medial large female glomeruli; ms – milliseconds; mV – millivolt; µm – micrometre.

3.4 Centrifugal Neuron

3.4.1 CN 1

Morphology

In preparation seven (pVII) one centrifugal neuron (CN) was stained (CN1) (Figure 13A). The large soma resided in the lateral cell cluster and was measured to ~ 20 μm in size. The neuron projected from the AL housing the soma with a thick unbranched axon, following the mALT and branching towards the ipsilateral calyces. The axon also crossed over to the contralateral side giving off a branch innervating the calyces on this side as well. In both hemispheres, a second thin process extended in an anterior direction towards the central body (Figure 13B). The main axon passed on in an anterior direction to the contralateral AL (Figure 13C). The processes in the contralateral AL were relatively weakly stained, however, the neuron had multiglomerular innervations.

Physiology

The intracellular recording was performed in the AL housing the soma. The spontaneous activity appeared as trains of spikes of relatively low firing rate that even decreased during the recording period. The spike amplitude was particularly large 66,4 mV (SD= 7,43), and the spike duration long, 18,4 ms (SD= 2,1). The spiking rate was 2,27 Hz (SD= 0,90). The recording electrode had a high resistance of ~1200 MΩ when the dye was iontophoretically injected. At that time the neurons showed no spiking activity.

Immunohistochemistry

After the recording and staining procedure in this preparation, a serotonin-specific labelling immune-labelling procedure was carried out. Based on the physiology and the morphology this neuron was assumed to be a serotonin immumoreactive-neuron-type. Therefore, a serotonin procedure was performed, expecting the 633-nm line of a Helium Neon laser to excite the CY5-label. However, as seen in Figure 14A, the soma is not visible in this figure. Figure 14B is from the same preparation, and here the soma excited by a 543-nm line of a Helium Neon laser is prominent in a yellow colour. It means that the neuron stained in pVII was not a SI-neuron-type, or that the procedure used had technical problems.

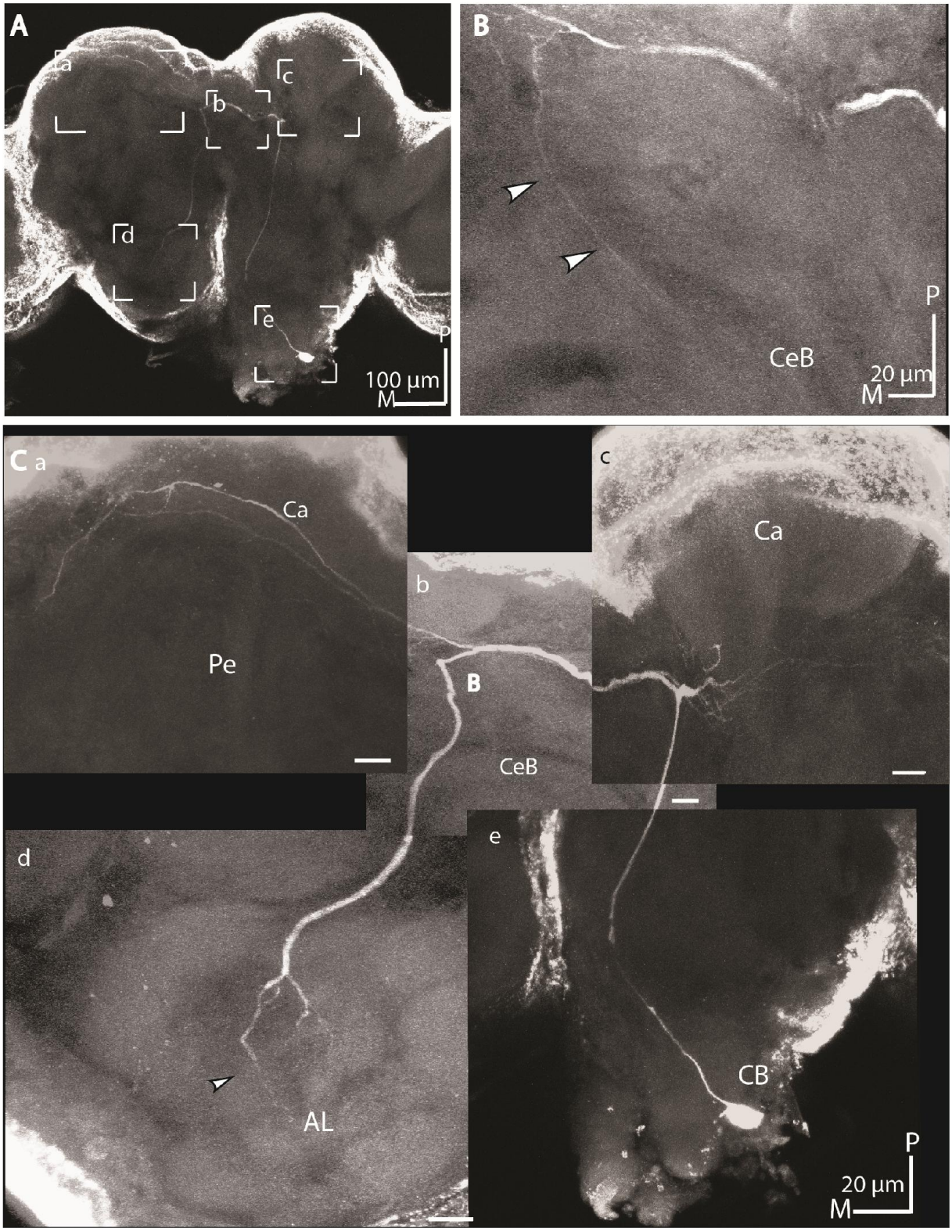

Figure 13: A and **B.**

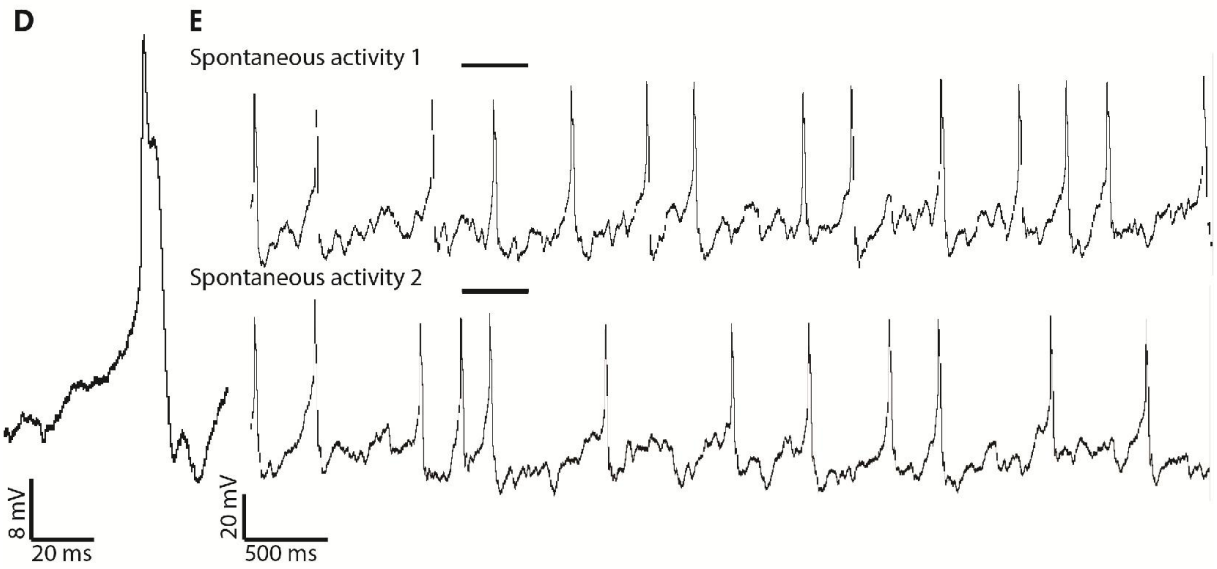

Figure 13: A) Confocal image of a whole-brain-scan of preparation pVII from a *Heliothis virescence* male seen from a dorsal view (10x magnification). pVII contains one centrifugal neuron (CN1) with its soma located lateral cell cluster in the left antennal lobe (AL). The axon followed the medial antennal lobe tract (mALT) and innervated the area dorsal of the ipsilateral calyces (Ca). After crossing the midline, the neurite projected towards an area dorsal to the contralateral Ca. Then, it turned anteriorly through the mALT an arborized in the contralateral AL. **B)** A thin branch projected in a medial and ventral direction towards the central body (CeB) from the axon which crossed the midline (40x magnification). **C)** Projection images of the CN, composed of single image stacks (40x magnification). The contra- and ipsilateral area dorsal to the Ca was both innervated (Figure C a and C c). The pedunculus (Pe) is indicated with an arrowhead (Figure C a). After bypassing the CeB (Figure C c) the CN crossed the midline and gave off a branch towards the CeB (Figure C b). The contralateral AL seemed to be innervated by CN1 in a multiglomerular manner (Figure C d). The large soma was located in the ipsilateral AL (Figure C e). **D)** The spontaneous spiking activity of CN1 comprised large spikes. **E)** The spike train gave no response to any of the odour stimuli. Notice the high amplitude spikes with a relatively low frequency rate. Horizontal bar marks the stimulation period (400 ms). Abbreviations: M – medial; ms – milliseconds; mV – millivolt; P – posterior; µm – micrometre.

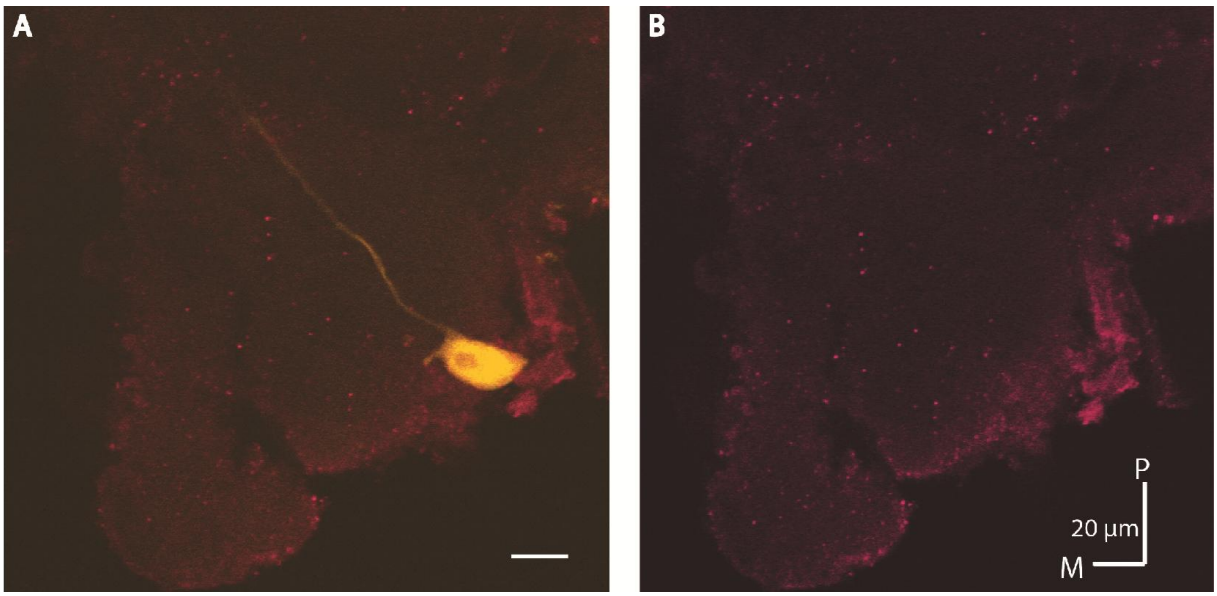

Figure 14: Centrifugla neuron one (CN1) in preparation pVII from a male *Heliothis virescens*, seen form a dorsal view. An immunohistochemistry procedure was used to label the serotonin protein with Cy5, as described in subsection 2.8.2 in this study. The 543-nm line of a Helium Neon laser excited the Micro Ruby labelled with Cy3 **(A)**, and the Cy5 was supposed to be excited by 633-nm line of a Helium Neon laser **(B)**, respectively. Abbreviations: M – medial; P – Posterior; µm – micrometre.

4 Discussion

AL neurons have been characterized in several insect species of different families. In all species, the neurons` morphology fall inn to three categories, PNs, LNs and CNs, which corresponds to the neurons identified in the present study. The motivation for continuous studies of AL neurons in *H. virescens* was to further explore the morphology and physiology of the neuronal subtypes. While the main innervation patterns of the neuron types are well known, the appurtenant physiology and morphological details still need further exploration. Studies on *H. virescens* are of particular value as the primary odorants for single receptor neurons are described and facilitates further investigations on the odour specificity and coding mechanism in the AL. However, in this study, the physiological data are too limited to make any conclusions regarding specific response patterns to distinct odours.

4.1 Projection Neurons

4.1.1 Morphology of the uniglomerular projection neurons following the medial antennal lobe tract

The stained PNs confined to the mALT in this study (pI and pII), innervating the calyces and the LH (Figure 6A and 7C), showed in principle the same projection pattern as uniglomerular mALT PNs previously described in moths, e.g. in *M. sexta* (Homberg *et al.*, 1988; Kanzaki *et al.*, 2003) and *H. virescens* (Berg *et al.*, 1998; Rø *et al.*, 2007; Løfaldli *et al.*, 2010; Løfaldli *et al.*, 2011). Accordingly, preparation pI contained four simultaneously stained PNs following the mALT with collaterals innervating the calyces and axons terminals ending up laterally in the LH (Figure 6 and Appendix 3). The PNs` somata of these simultanously stained neurons were located in the medial cell cluster, and the neurons innervated glomeruli in the dorso-medial part of the AL (Figure 6B), as earlier described in *M. sexta* (Homberg *et al.*, 1988) and *H. virescens* (Rø *et al.*, 2007). In general, PNs in mALT have their somata located in the cell cluster positioned closest to the innervated glomeruli (Homberg *et al.,* 1988). The four PNs varied in their innervation pattern of the glomeruli, from arborizing densely throughout the entire glomerulus to a loose spacy partial innervation (Figure 6B). This is also in accordance with the previously described innervation patterns in *H. virescens* (Rø *et al.* 2007) and *M. sexta* (Sun *et al.* 1997).

After leaving the AL, the medial-tract PNs presented here projected to the calyces, innervating the entire structure via the three collaterals (Figure 6C). This is according to the literature reporting three, four and five collaterals sent off from the main axon on its rout along the anterior edge of the calyces (Homberg *et al.*, 1988; Rø *et al.* 2007). The numerous terminal swellings of knob-like structures or blebs innervating the entire calyces, as demonstrated in figure 6C, also corresponds to pervious

previous studies of moths where hundred to two hundred terminal knobs were counted (Homberg *et al.*, 1988; Rø *et al.*, 2007).

In the LH, the axonal ramifications expanded into small blebs, being considerably smaller and thinner than those of the calyces (Figure 6D), as previously described in other moth species (Homberg *et al.*, 1988; Kanzaki *et al.*, 2003). The medial-tract PNs in pI (PN1-4) innervated ordinary glomeruli and typically projected in the most lateral part of the LH (Figure 6F), and this innervation pattern is reported in various articles (Christensen and Hildebrand, 1987; Homberg *et al.*, 1988; Kanzaki *et al.*, 1989; Anton and Hansson, 1995; Hansson, 1995; Martin *et al.*, 2011; Zhao *et al.*, 2014 submitted article).

One of the previously stained PN in pI (PN4) of the current study showed a unique innervation pattern that has not previously been described. This neuron did not send collaterals into the calyces but passed along its dorsal edge running directly to the LH (Figure 6E). The meaning of the unusual projection pattern is unclear. After all, why follow the mALT and not innervate the calyces? As this neuron was weakly stained in the current area, the interpretation of the neuron`s detailed innervation pattern is difficult. Analysing the confocal images, however, more fibers seem to project between the calyces and the LH than between the AL and the calyces, and one might speculate that PN4 may send branches along the main axon back to the calyces from the LH, having a modulatory effect in this multimodal integration centre, as described for some medial ALT PNs in Rø *et al.* (2007). In the study by Rø *et al.* (2007) a medial tract PN named P1c was described as possibly multiglomerular innervating ordinary glomeruli. This neuron bypassed the calyx anteriorly but did not send any branches to innervate it. Furthermore, this neuron innervated extensively the medial part of the LH. However, some doubts have been raised whether this neuron was co-stained with a LN in the same preparation and that the PN might in fact be uniglomerular (Personal communication, Hanna Mustaparta). Thus, the PN described by Rø *et al.* (2007) resemble the unique neuron stained here, in pI, by bypassing the calyces. However, the two neurons projected along different parts of the calyces, dorsal versus anterior. Also, the ramifications in the LH differed, the neuron in pI terminated more laterally than the neuron found by Rø *et al.* (2007). Another exception from the classical innervation pattern of uniglomerular PNs, as described by Homberg *et al.* (1988), is that some PN fibers passing in the mALT innervated the superior protocerebrum anterior to the calyces before projecting to the LH (Homberg *et al.,* 1988). However, this innervation pattern was not observed in the present study. Obviously, the neuronal innervation in the superior protocerebrum have distinct functions, but they are unknown.

In preparation pII, PN5, which passed in the mALT, was co-stained with two LNs (LN2 and LN3). (Figure 7A). Also this PN innervated the calyces and the LH (Figure 7C), but the innervation in the AL did not appear in the confocal images. The location of the small somata of this PN (~ 10 μm) was

located in the lateral cell cluster showing the typical size for PNs stained in this study (Table 4). Another interesting aspect is the staining of four somata in the same preparation (Figure 10A), with two of them disappearing after the immunostaining procedure (Figure 7B). The two remaining somata had a diameter of ~ 20 μm, the same size as the other LNs stained in the present study (Table 5). The size difference between somata of LNs and PNs has been pointed out in an early study by Matsumoto and Hildebrand (1981).

4.1.2 Morphology of the multiglomerular projection neuron following the mediolateral antennal lobe tract

One successfully stained projection neuron (PN6) innervating the AL in a loose multiglomerular manner (Figure 8A), had its soma located medially in the lateral cell cluster (figure 8A and C). The axon followed the mlALT projecting directly to the LH and extending a side-branch to the superior protocerebrum (Figure 8B). This is the typical morphology of a neuron confined to the mlALT and PNs of this category are previously identified in several species, e.g. in *H. virescens* (Løfaldli *et al.*, 2012), *M. sexta* (Hoskins *et al.*, 1986; Homberg *et al.*, 1988), and *Tricholusia ni* (Anton and Hansson, 1999). Since the PN6 innervated ordinary glomeruli exclusively it most likely conveys plant odour information of some sort, even though there was a lack of responses to the plant odours tested here. In the study by Løfaldli *et al.* (2012), several mlALT PNs appearing from a mass staining had sparse dendritic arborizations in each innervated glomerulus. This kind of sparse innervation is shown in individually stained PNs as well, both in *H. virescens* (Rø et al., 2007) and *T. ni* (Anton and Hansson, 1999). Generally, most of the innervated glomeruli were located laterally and medially in the AL. All the neurons following the mlALT in the study of Løfaldli *et al.* (2012) had slightly different arborizations in the LH-area, some extending branches posteriorly towards the ipsilateral calyces and dorso-medially towards the pedunculus and the lobes. The innervation in the two latter structures corresponds to the innervation shown in PN6 in this study (Figure 8B). The multiglomerular neurons recorded by Løfaldi *et al.* (2012) had a long latency (300 ms) and a long excitation to the B10 mixture but not to the single odorants tested. The sparse innervation of the AL and the response to the odorant mix exclusively was interpreted as a particular feature of the current neuron implying a high response threshold. This corresponds to findings in the honey bee demonstrating that the mlALT neurons most often show a sparse innervation patterns in the AL and have a high threshold for being activated (Abel *et al.*, 2001; Kirschner *et al.*, 2006) Also, Høydal (2012) reported about one multiglomerular mlALT PN responding exclusively to the ten component blend whereas another PN, passing in the mALT and innervating two glomeruli, one densely and one sparsely, responded to the two single components linalool and hexenyl acetate only. The mlALT PNs are not as well described in the literature as the mALT PNs. However, it is well known that several of the PN fibers of the mlALT are GABAergic (Hoskins *et al.*, 1986; Berg *et al.*, 2009).

Noticeably, the two sub-branches of PN6 projecting out of the AL in addition to the main axon (Figure 8A, B and D) have not previously been described in the current PN type.

4.2 Local interneurons

The LNs presented in this study displayed a typical morphology including neuronal processes confined in the AL, somata located in the lateral cell cluster (Table 5), and primary neuritis projecting towards the centre of the AL, as previously described in *H. virescens* (Berg *et al.*, 2009) and other moth species, like *M. sexta* (Matsumoto and Hildebrand, 1981; Christensen *et al.*, 1993), *Spodoptera littoralis* (Anton and Hansson, 1994), and *B. mori* (Seki and Kanzaki, 2008).

4.2.1 Morphology of the multiglomerular local interneurons

LN1 in preparation pIV, which contained the most prominently stained LN in this study, had large symmetrical ramifications throughout the AL innervating most glomeruli, including the MGC units. To the author's knowledge, this type of LN, including appurtenant physiological data has not been described before in *H. virescens*. Evident in this neuron was large swellings along the processes that resemble stubby dendritic spines increasing the innervation area of the processes. These coarse processes ramifying widely in the AL are typical and have earlier been described in *M. sexta* by Matsumoto and Hildebrand (1981), and in *B. mori* by Seki and Kanzaki (2008). Interestingly, the innervation pattern and morphological details in PN1, achieved from *H. virescens*, is particularly similar to the type IIb in *M. sexta* (Matsumoto and Hildebrand, 1981). Regarding the innervation of all glomeruli, including the MGC units, LN1 may possibly be participating in general functions, such as gaining global control of the whole AL where it would naturally benefit from a large innervation surface. The fact that LN1 innervates all areas of the AL might also indicate that information about pheromones and plant odours are indeed integrated in the AL as discussed by Seki and Kanzaki (2008), Berg *et al.* (2009), and Reisenman *et al.* (2011). It is commonly found that numerous LNs in the moth AL are GABAergic (Seki and Kanzaki, 2008), and that they contribute with inhibitory interactions within and between olfactory subsystems (pheromonal and non-pheromonal subsystems), potentially to enhance contrast and strengthen odorant discrimination. These inhibitory networks might optimize olfactory information processing by generating oscillatory synchronization, lateral inhibition, and thus gaining control of the AL output (Sachse and Galizia, 2002). The existence of lateral inhibitory effects in response to behaviourally significant odorant stimuli makes the computation in the AL complex (Heinbockel *et al.*, 2013). However, these inhibitory computational functions have not yet been directly demonstrated in AL LNs (Seki and Kanzaki, 2008). LNs with other transmitters, most of which acting as co-transmitters, are also present in the AL of *H. virescens* (Berg *et al.*, 2007).

The two LNs in pV (LN4 and LN5) identified in *H. virescens* female in the present study had thinner processes than LN1 and did not show prominent swellings. Like LN1, these neurons displayed striking

similarity to particular LNs previously described in *M. sexta* (Christensen *et al.*, 1993), all of which showed a type of symmetric mirror-image innervation including a primary neurite that sends thick fibres into the AL giving rise to thinner arborizations that innervate most glomeruli. The present LN4 and LN5 have adjacently located somata and parallel neuritis projecting in the same manner throughout the AL. The function of this kind of parallel innervations is difficult to understand. However, it might indicate a function depending on the ratio of input and output synapses in the different glomeruli. It would be interesting to know whether one, of both, of the neurons are GABAergic, in the latter case possibly exerting reciprocal inhibition. Alternately, one LN might be excitatory and the other inhibitory perhaps regulating the response strength of the PN output.

4.2.2 Morphology of the oligoglomerular local interneuron

In clear contrast to the multiglomerular LNs, the oligoglomerular LN6 in pVI (Figure 12A) innervated only particular glomeruli, in this case the LGFs and two adjacent ordinary glomeruli. The LFG identification was based on its location at the base of the antennal nerve entrance to the AL in the female moth preparation, as demonstrated in the AL atlas of the *H. virescens* female (Løfaldli *et al.*, 2010). The response of this neuron to all tested plant odours show its role in processing plant odour information. This is in agreement with previous findings in *M. sexta* showing that PNs innervating the LFGs respond to stimulation with host plant odours (Rospars and Hildebrand, 1992). The term female-specific LFGs was first suggested by Rössler *et al* (1998) in *M. sexta*, and is later used in other moth species, including *H. virescens* (Berg *et al.*, 2002; Løfaldli *et al.*, 2010). By functional tracing, Hillier et al. (2006) have shown that OSNs responding to the second principal pheromone component, Z9-14:AL, project in the LFGs in *H. virescens*. One might speculate whether plant odour responding OSNs also project in the LFGs of *H. virescens*. However, this has not been shown yet. Plant odours and mixtures eliciting activity in ordinary glomeruli are well demonstrated in *H. virescens* by the use of calcium imaging, eliciting activity in one, two, or three glomeruli (Galizia *et al.*, 2000; Skiri *et al.*, 2004). Due to the limited scope of the current project, identification of the glomeruli innervated by the oliglomerular LN, LN6, according to the atlas has not been made. Anyway, the restricted glomerular innervation by LN6 may serve as a substrate for interaction between specific glomeruli, i.e. those that compute information about particular components in a behaviourally significant odour blend as discussed by Sadek *et al.* (2002) and Reisenman *et al.* (2011). Considering the fact that LN6 innervates the LFGs as well as ordinary glomeruli, it may also play a role in integrating information about plant odours and pheromones.

4.3 Physiology of the projection and local interneurons

Whereas the morphology of LNs and PNs in the present study showed two well-defined classes of AL neurons, the physiology obtained by intracellular recordings showed less distinct differences between the neuron classes.

4.3.1 Spontaneous activity

In an early study, Christensen *et al.* (1993) reported that the spike shapes of LNs and PNs in *M. sexta* differed by LN spikes having longer duration than the PN spikes (Christensen *et al.*, 1993). This is indeed the case for the multiglomerular LN1 in this study wich had the unusual long duration of 14,0 ms (SD= 1,2) (Table 2). However, particular long spike duration did not apply to all LNs. In the other LNs, the duration varied from 1,5 ms (SD= 0,3) to 4,3 ms (SD= 1,4). The spikes of the two relevant PNs, on the other hand, showed spike durations of 2,2 ms (SD= 0,6) and 1,3 ms (SD= 0,7), respectively. Therefore, no conclusion could be made concerning the single spike duration of the two antennal-lobe neuron categories in this study. Varying spike durations within LNs and PNs is also shown in other moth species like *M. sexta* (Reisenman *et al.,* 2011), as well as the fruit fly *D. melanogaster* (Chou *et al.*, 2010).

Another general characteristic of the LN spikes is the occasional appearance of two different sets of amplitudes. As first reported by Matsumoto and Hildebrand (1981), this feature was originally observed in LNs of the moth *M. sexta* (Matsumoto and Hildebrand, 1981; Christensen *et al.*, 1993), and later found in *S. littoralis* as well (Anton and Hansson, 1994). In the present study, the recording from a neuron in pII (PN or LN) showed spikes of two different amplitudes (Figure 7A). This might either be due to two sets of spikes occurring from one neuron or recording of spikes originating from each of the two neurons being stained. It has been suggested that the appearance of several spike amplitudes displayed by one neuron could be due to different spike-initiating zones, as discussed by Zhao and Berg (2009) as regards the SI-neuron in *H. assulta.* Interestingly, some LNs displayed a spontaneous activity characterized by smaller spikes superimposed on a strong depolarization. Also, the irregular bursting pattern was sometimes combined with single spike activity.

Altogether, the spontaneous activity of some LNs differed from that of the PNs by showing bursting spikes superimposed on a strong depolarization as well as a complex pattern of activity by bursting combined with single spikes (Table 5). This is in clear contrast to the regular single spiking activity recorded in the PNs in this study (Table 4). Generally, a large variation in spiking rate was found in both LNs and PNs, without an obvious difference between the two classes (Table 2).

4.3.2 Odorant responses to relevant odours

Intracellular recording from neurons in small insects, particularly from the AL neurons, is a challenging task, and data on responses from these neurons are in general limited. This applies especially to plant odorant AL neurons, whereas responses to pheromones have been easier to obtain. The technical challenges are the main reason why the data on responses in the present study is also limited. Concerning LNs, few studies on intracellular recordings providing data on the neurons' response properties have been carried out, as pointed out by Galizia and Rössler (2010).

Complex response patterns to many odorants, like in LN6, may reflect the innervation of several ordinary glomeruli. Interestingly, in *M. sexta,* linalool is found to activate uniglomerular PNs innervating the LFGs. Here, the excitatory phase of the response to linalool was followed by a membrane hyperpolarization, resulting in cessation of spiking activity. Also, the duration of both the excitatory and inhibitory phases increased with increasing concentration (King et al., 2000). These responses are comparable to those elicited by LN6 responding to all plant odours tested, including linalool (Figure 12), indicating that responses to one particular odour substance may be elicited in the different types of neurons. Linalool is a relevant primary odorant in *H. virescens* (Røstelien *et al.*, 2005) and a typical flower odour involved in nectar feeding and learning as shown by conditioning experiments in *H. virescens* (Skiri *et al.*, 2005). In addition to unspecific responses to all plant odours tested, like in LN6, a more specific response to one of the tested odorants only, i.e. farnesene, as in LN1, was recorded as well. Thus, the present study indicates that the LNs consist of distinct types showing varying response properties, including both broadly tuned neurons requiring an odour blend for being activated and specifically tuned neurons needing just one component to respond.

Among the PNs, no responses were recorded except for an inhibition elicited by one single odorant, hexenyl acetate, in pII where two LNs and one PN were stained (Figure 7D). Thus it is uncertain wether the response is elicited in the PN or one of the LNs. In addition, the recording is of low quality and should not be over interpreted. The lack of responses in the recorded PNs might be expected considering the fact that five of them were uniglomerular and one innervated a few glomeruli. Based on the fact that the OSNs are tuned to one primary plant odorant, and the assumption that each projects to one specific glomerulus, each uniglomerular PN might respond primarily to one of these primary odorants. In the present study, ten primary odorants were tested. These constitute about 1/6 of the biologically significant odorants presumed to be relevant based on the number of glomeruli in the AL. In addition, the short recording period did rarely allow all ten odorants to be tested. This may explain the low chance of obtaining responses from plant-odour PNs in the AL of *H. virescens*.

4.3.3 Activity in projection neurons according to different antennal lobe tracts

An interesting question in the present study was whether the PN axons of the three ALTs show different physiological properties. Findings in the honey bee have shown that PNs of the two major AL output pathways of this species, the m- and lALT, both innervate the calyces and the LH (Menzel *et al.*, 1994; Krofczik *et al.*, 2008). Based on these findings, Galizia and Rössler (2010) suggested that: "one system might filter odor quality information, and the other the time-structure of a stimulus and its concentration, or one might process blend information, while the other might extract odor mixture components. This arrangement would be similar of the parallel streams in the visual systems of many species. The two systems may also accomplish different tasks for olfactory learning and memory, one

coding odor quality in an experience-dependent way and the other in an experience-independent way". They further expressed that the relevance of temporal parameters, such as synchronizations and oscillations of spikes in PN populations, and their connection via the two PN pathways to the MBs, are still unclear. Since there are parallel tracts in *H. virescens* as well (Figure 1), it has been speculated whether the PNs in these tracts also mediate different properties of the odour information. However, in the present study the identified PNs belonging to the mALT and the mlALT did not show responses and cannot be ascribed to different physiological properties. In a study of the moth, *T. ni,* no obvious differences in the response patterns of PNs projecting in the mALT, lALT, and the mlALT were found, indicating that the same information is processed in the parallel pathways (Anton and Hansson, 1999).

Intracellular recordings and morphological studies of uni-glomerular PNs in the mALT have been performed in several moth species; *H. virescens* (Christensen *et al.*, 1995; Berg *et al.*, 1998; Vickers et al. 1998; Zhao and Berg, 2010; Løfaldli *et al.*, 2011; Løfaldli *et al.*, 2012), *M. sexta* (Matsumoto and Hildebrand, 1981; Christensen and Hildebrand, 1987; Homberg *et al.*, 1989; Kanzaki *et al.*, 1989) and *H. zea* (Christensen *et al.*, 1991; Vickers et al. 1998). However the PNs of the mlALT and the lALT in moth are rarely physiologically described (Rø *et al.*, 2007; Galizia and Rössler, 2010). Whether the PNs of different ALTs might be ascribed to mediate different features of the odour stimulus needs to be further investigated in moths as well as in other insects.

4.4 Centrifugal neurons

4.4.1 Centrifugal neuron morphology

The staining of one bilateral neuron having its soma located in one AL and ramifications in the contralateral antennal lobe, plus neural processes in both protocerebral hemispheres, was indeed an unexpected finding. As mentioned in the results, the unique morphology of the neuron indicates that it is identical with the so-called serotonin-immunoreactive (SI) neuron, previously identified in several moth species, *H. assulta* included (Zhao and Berg, 2009). The current neuron has not been identified in *H. virescens*, however. Even though the serotonin labelling in this study did not work out, the morphology and physiology was sufficient for concluding that this is in fact a SI-neuron. For example, the diameter of the soma stained in the current study, which measured ~ 20 μm, is similar to the soma size of SI neurons in several other species (Sun *et al.,* 1993; Hill *et al.,* 2002; Zhao and Berg, 2009), Also the characteristic location of the soma found here, being situated in the postero-ventral part of the lateral cell cluster, corresponds with that previously reported for this neuron type.

Morphological studies of the SI neuron, based on immunocytochemistry, have been performed in different insect species, moths included. This unique neuron, being one in a pair, was first discovered in *M. sexta* (Kent *et al.,* 1987). Ultrastructural studies have shown the presence of mainly output synapses in the antennal lobe not housing the soma, indicating the modulatory role of the neuron – possibly through signals received at the protocerebral level (Sun *et al.* 1993; Dacks *et al.* 2006). In accordance with these findings, functional investigations have reported that serotonin enhances responses in antennal-lobe neurons (Kloppenburg and Hildebrand, 1995). However, so far, physiological properties of the SI neuron itself have been reported two times only, in both cases from a moth species, i.e. *B. mori* (Hill *et al.,* 2002) and *H. assulta* (Zhao and Berg, 2009).

There is no doubt that serotonin has a prominent role as a modulatory neurotransmitter, both in vertebrates and invertebrates. For example, the well-known learning mechanisms studied in the sea slug *Aplysia california* involve modulatory neurons releasing serotonin (Brunelli *et al.,* 1976). In the moth antennal lobe, serotonin is reported to influence neuronal responses. Thus, a low concentration of serotonin was shown to reduce the number of action potentials produced by LNs and PNs, whereas a high concentration had the opposite effect (Kloppenburg and Mercer, 2008). Taking into account the varying level of serotonin in the moth during day and night, these neuromodulatory effects may explain why the male moth responds to the female sex pheromones at night but not during the day. Generally, serotonin is reported to enhance central olfactory neuron responses during stimulation with both pheromones and plant odours (Hill *et al.,* 2002; Dacks *et al.,* 2005).

In spite of the generally similar branching pattern of SI neurons identified in moths, there seems to be certain morphological distinctions in the various species. For example, the thin fibres innervating the central body in *H. virescens*, as found in the present study (Figure 13B), were not found in the closely related species *H. assulta* (Zhao and Berg, 2009). In *M. sexta*, on the other hand, similar innervations have been reported (Figure 2; Kent *et al.* 1987). The central complex, comprising the central body, the protocerebral bridge, and the noduli, is a series of interconnected neuropils that span the midline of the PC (Homberg *et al.,* 1988). Neurons of the central body are known to respond to mechanosensory, visual, and chemical stimuli. Interference with its function seems to disrupt normal motor patterns of behaviour (Homberg *et al.,* 1988; Chapman et al., 2013). The path of information in and out of the central complex, however, is still far from completely understood. The role of the central complex in the processing visual information from the dorsal rim region of the compound eye, which is sensitive to polarized light, is relatively thoroughly described. The plane of polarization varies systematically across the sky depending on the position of the sun, but can be detected even on a cloudy day (Homberg *et al.,* 1988, Chapman *et al.,* 2013). This could therefore be the information needed for the 24-hour modulation cycle of the AL neurons, and may also explain why the serotonin levels alters (Chapman *et al.,* 2013, Homberg *et al.,* 1988). Yet another feature characterizing the SI neuron in *B. mori*, is its innervation of the lateral accessory lobe (LAL) in the hemisphere housing the soma (Hill *et al*., 2002). The LAL is also an olfactory neuropil, and it constitutes a part of the central complex. The functional significance of this innervation is unclear, but the LAL has known connections to major descending interneurons (Chapman *et al*., 2013). In summation, the different arborisation patterns of the SI-neurons in various species can possibly cause slightly different modulatory mechanisms in the species. As regards *H. virescens* and *H. assulta,* being allopatric species (Yeon *et al.* 1998; Waldvogel and Gold 1990), the geographical isolation may have played a role for the slightly different morphologies of their SI neuron. The relatively weak staining of the contralateral antennal lobe in *H. virescens* as compared to that of *H. assulta*, however, might be caused by the high resistance in the registration electrode (~ 1200 MΩ) during staining of the SI-neuron in this study (Figure 12Cd).

4.4.2 Centrifugal neuron physiology

This is the third time intracellular recordings have been performed from the SI-neuron, and the first time in *H. virescens*. Also, this is the first time anyone has recorded from the AL housing the soma. All physiological parameters stand out compared to those of the other neuronal classes in this study (Table 2); the duration of the single spikes is exceptionally long, i.e. 18 ms (SD=2,1), with LN1 being the only comparable neuron from this study (Figure 9D), and the amplitude is also very high. The neuron had a low spiking rate of 2,27 Hz (SD=0,90) and no apparent response to any of the odour stimuli. The lack of odour responses is in accordance with results from the study by Hill *et al.,* (2002),

who recorded from the LAL in *B. mori*. Zhao and Berg (2009), on the other hand, recorded two distinct types of action potentials; one large spike amplitude spiking train with low spontaneous activity (1,3 Hz) and with no observable response to the pheromones and the plant odours, and one small with high spontaneous activity (13,3 Hz), which showed observable odour responses. The recording which was performed from the AL not housing the soma was suggested to include various spike-initiating zones acting in such a way that the large amplitude spikes were present globally whereas the small spikes were present in local parts of the antennal lobe only (Zhao and Berg, 2009). Different spikes in the same recording have previously been observed in local AL neurons of the *M. sexta* (Matsumoto and Hildebrand 1981; Christensen *et al.* 1993). Altogether, the previous and present results indicate that the large amplitude spikes originate in the axon hillock located in the AL housing the soma, whereas the small amplitude spikes originate from another spike initiating zones in the contralateral AL.

4.5 Methodical assessments

The analytic criteria set in this study (subsection 2.9) were set in order to compare the different AL neurons within the different neuronal groups, hopefully the neuronal classes (PN, LN or CN) would give the same response to the same odour stimuli. The neurons in this study can, however, not be compared to each other as they belonged in different neuron classes (PN, LN or CN) and had different responses to the odorants. Nevertheless, the different spike parameters can reflect an overall trend for the different groups. Therefore, the mean value and the standard deviation of the different parameters, durations (ms), amplitude (mV), and spiking rate (Hz), were calculated for the spontaneous activity to be able to comment on this variety. The different neuronal classes and the response patterns they elicited, in addition to the small sample size, makes a statistical approach not applicable as this would produce data without significance and add no further insight to the results in this study. As a statistical approach was invalid, the responses were interpreted in a subjective manner.

The result of the physiological recordings from the neurons could be dependent on where in the neuron they took place, for example in the thick central axon, the decentralized thinner ramifications, or in the soma. For example, LNs will typically receive signals from multiple PNs and LNs and the electrophysiological responses might therefore differ between the different local sites. Some responses will be stronger in distinct parts of the neuron (can be caused by summation), and smaller in others (might be inhibited). Also, the resistance in the recording electrodes, in this study varying between 300 and 1200 MΩ, might influence the quality of the recording. The high resistance in the recording electrode could affect the spiking amplitudes Likewise, if the amplitudes are low, the contact between the recording electrode and the neurons could be of a weak character and therefore would effect the resulting measurements. This should be considered when interpreting the responses in this study.

Several neurons were simultaneously stained in many of the preparations (pI, pII and pVI). If the intracellular recording comprised several spike amplitudes is it possible that the registration electrode could be in contact with several neurons at the same time. On the other hand, dye diffusing through gap junctions in electrically coupled neurons might be the cause when the registration comprises one set of spiking amplitudes. Gap junctions are present in the vertebrate olfactory bulb (Kosaka and Kosaka, 2005). Even so, there is no evidence for the presence of gap junctions in glomerular neuropils of the moth AL (Tolbert and Hildebrand, 1981).

5 Further studies

Research in moth olfaction has come a long way, and the general odour detection system of moths holds a valuable tool for understanding odorant information coding in the CNS. By combining the obtained results with previous knowledge is it possible to comment on the connection between the morphology and physiology of the different AL neurons stained. However, many more neurons need to be stained, and many olfactory responses need to be recorded, to gain more insight in the dynamics between single cells and the computation of the olfactory information detected in multiple cells. Pheromone and host-plant related behaviour remain highly active fields of study when it comes to moth olfaction, not least because of the economic importance the moth play as a number of moth species are agricultural pests in the still widely used monocultures. Naturally, it would be of interest to determine the specific primary odourants represented in the glomeruli in the *H. virescens* AL to gain more knowledge about the specific relationship between the moth and its host plant. Further, it would be interesting to demonstrate the base of olfactory memory and how this information is stored, using insect models to help explain the Proustian phenomenon.

6 Conclusions

- The categories of neurons characterized in this thesis correspond with the previously identified AL neuron classes, LNs, PNs, and CNs.
- Most uniglomerular PNs following the mALT in this study display the classic innervation pattern of P1a-neurons, including the bleb-like innervations in the calyces and the LH.
- One unique mALT PN, not previously described, following the mALT seemed to bypass the calyces, or alternatively innervate the current structure from its lateral part.
- Uniglomerular mALT PNs innervating ordinary glomeruli project in the lateral part of the LH, close to the optic lobe.
- Multiglomerular PNs following the mlALT seem to terminate more medially in the LH than the uniglomerular mALT PNs innervating ordinary glomeruli.
- One multiglomerular PN following the mlALT had additional branches projecting out of the AL.
- Two morphological types of LNs were stained in this study, multiglomerular LNs and oligoglomerular LNs, and individual LNs display distinct morphology in respect to thickness of neural processes as well as glomerular innervation pattern.
- Individual LNs may innervate both ordinary and sex-specific glomeruli, including MGC units in males and LFG units in females.
- Individual LNs displays varying spontaneous activities with regular single-spiking activity, bursting spiking activity, or a complex spiking pattern including both single and bursting spikes.
- The species studied here, *H. virescens*, possesses an SI-neuron types having similar morphological and physiological properties to those previously described in other moth species.
- The SI-neuron in *H. virescens* display species-specific morphological properties by innervating the central body.

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Appendix 1

Table 6: Distribution of the different odorants in the experiment protocol in the order they were used, constituent parts of B10 (Løfaldli et al., 2012), and the single odours also represented in ylang-ylang.

Experimental protocol:	*Single odorants in B10:*	*Single odorants also represented in ylang-ylang*:*
Air		
B10		
Linalool	Linalool	Linalool
Germacrene D	Germacrene D	Germacrene D
2-Phenylethanol	2-Phenylethanol	2-Phenylethanol
3Z - Hexenyl acetate	3Z-Hexenyl acetate	3Z-Hexenyl acetate
Ocimene		Ocimene
Farnesene	Farnesene	Farnesene
Ylang-ylang		
Z11-16 Ald	3Z-Hexanol	3Z-Hexanol
Z9-14: AL	3Z-Hexen-1-ol	3Z-Hexen-1-ol
Z9-14: AL + Z11-16:AL	Oscimene	
Z11-16:OH	Geraniol	Geraniol
Z11-16:AC	(+)3-carene	(+)3-carene
Z11-16 Ald + Linalool	E-verbenol	E-verbenol
Z11-16 Ald + B10	Methyl benzoate	Methyl benzoate

*Ylang-ylang is an essential oil comprising 49 different odorant components (Stashenko, 1996).

Appendix 2

Table 7: Quantitative data for the spike trains; duration in ms – milliseconds, Hz - spiking rate in hertz; mV - amplitude in millivolt; SD – Standard deviation.

Preparation number:	*pI*	*pII small*	*pII large*	*pIII*	*pIV*	*pV*	*pVI*	*pVII*
Duration:	*ms*	*ms*	*ms*	*ms*	*ms*	*ms*	*ms*	*ms*
	2,6	1,3	2,5	0,9	16,9	3,9	5,2	19,9
	1,3	1,7	2,1	0,9	13,0	4,6	5,3	19,7
	3,2	1,7	3,5	0,9	14,4	2,3	4,3	19,7
	3,1	1,7	3,4	2,0	13,0	4,0	4,0	15,8
	1,8	2,0	3,4	1,2	13,6	3,4	5,2	14,8
	1,8	1,6	3,5	2,4	13,6	4,2	5,3	19,7
	2,0	1,2	3,2	0,8	13,6	2,1	5,0	14,8
	2,5	1,2	3,2	1,0	15,7	2,3	2,0	19,7
	2,2	1,2	3,2	1,1	14,3	3,2	1,8	19,7
	1,8	1,1	2,3	0,8	13,2	3,0	5,9	19,7
	1,5	1,3	3,4	2,5	13,2	5,0	2,8	18,8
Average	2,2	1,5	3,1	1,3	14,0	3,5	4,3	18,4
SD	0,6	0,3	0,5	0,7	1,2	1,0	1,4	2,1
Amplitude:	*mV*	*mV*	*mV*	*mV*	*mV*	*mV*	*mV*	*mV*
	12	2	2	15	34	60	26	63
	9	2	2	16	31	67	28	72
	19	3	5	12	29	56	14	61
	2	1,5	2	11	31	65	29	54
	18	1,5	3	12	30	64	26	72
	11	1,5	3	12	34	54	19	61
	20	3	3	12	35	58	17	60
	11	2,5	8	20	38	46	24	71
	10	2	8	10	31	54	33	75
	11	3	7	9	38	42	26	75
Average	12,30	2,2	4,29	12,90	33,10	56,60	24,20	66,40
SD	5,42	0,63	2,51	3,25	3,21	8,07	5,85	7,43
Spiking rate per second:	*Hz*		*Hz*	*Hz*	*Hz*	*Hz*	*Hz*	*Hz*
	4	30	9	37	2	15	17	2
	6	33	7	41	0	7	24	2
	5	12	20	38	3	21	13	2
	4	29	13	37	4	8	13	3
	6	24	11	27	3	4	28	3
	0	32	15	42	4	25	4	0
	4	14	6	42	5	11	32	2
	1	24	18	42	4	3	26	2
	4	25	17	39	4	4	31	3
	1	29	11	24	6	0	17	3
	0	17	17	24	8	28	22	3
Average	3,18	24	13,09	35,73	3,91	11,45	20,64	2,27
SD	2,27	7,23	4,68	7,18	2,07	9,52	8,67	0,90

Appendix 3

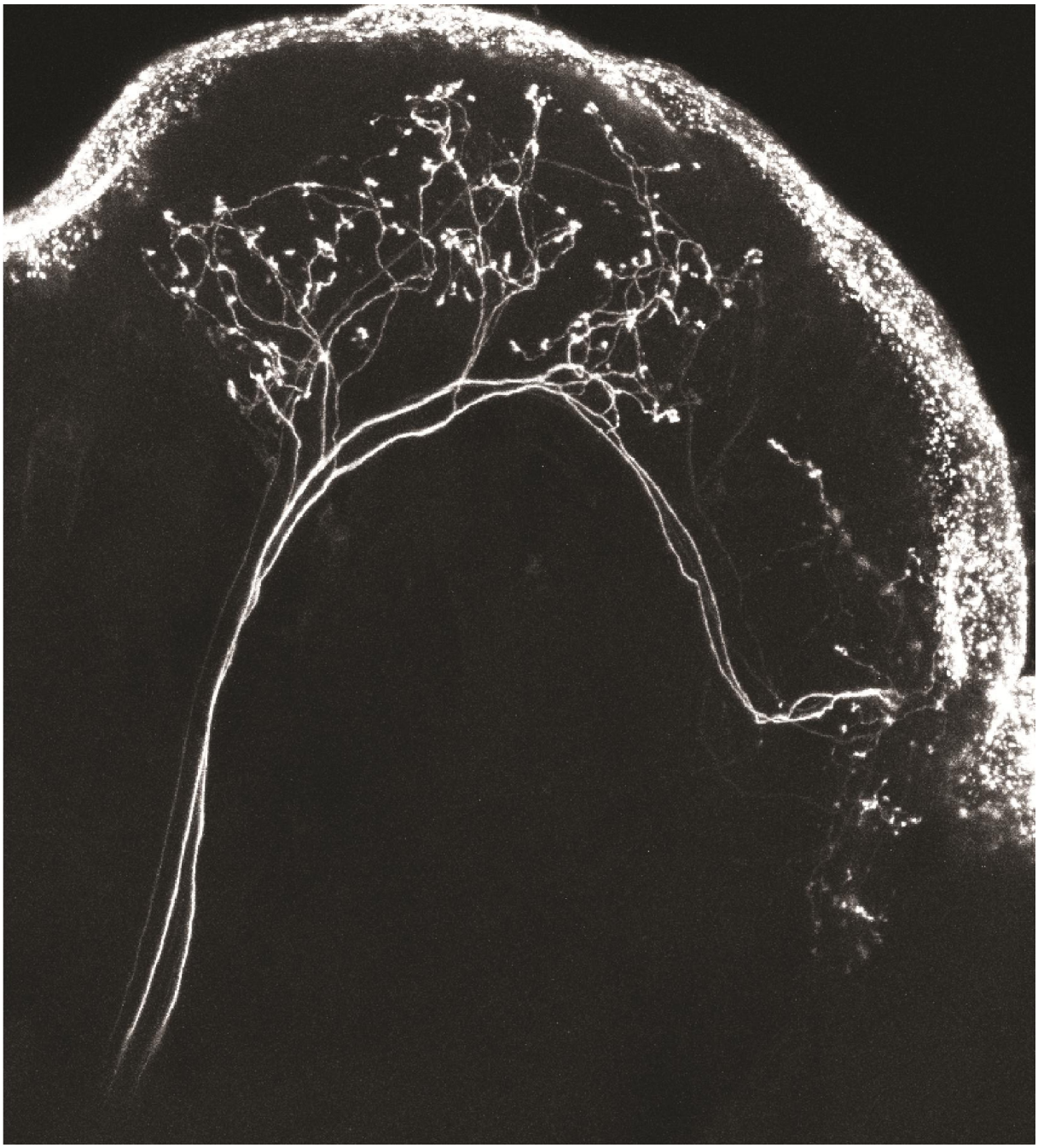

Figure 15: Confocal image of four simultaneously stained projection neurons (PN1-IV) from preparation I, a male *Heliothis virescens* moth seen from a dorsal view. Evident are the protocerebral arborizations in preparation pI projecting from the antennal lobe to the calyces for then to turn in an anterior direction ending up in the lateral horn (20x magnification).