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Characterization of functionally different projection neurons in the olfactory pathway of the male moth, *Heliothis virescens*

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Preface

This Master's thesis has been written at the Department of Biology at the Norwegian university of Science and Technology (NTNU), Trondheim. The laboratory work was conducted in the Chemosensory Laboratory, Department of Psychology.

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Sammendrag

Den kjemiske sansen regnes som den evolusjonært sett eldste sanse-modaliteten, og de fleste organismer er avhengige av luktesystemet for å overleve. Hos møll detekteres duftmolekyler av sanseceller som er lokalisert på antennen. Disse sensoriske nevronene projiserer direkte inn til antenneloben, det primære luktesenteret i insekthjernen, der de kontakter to hoverdtyper av andre ordens nevroner, projeksjonsnevroner og lokale internevroner. Projeksjonsnevronene sender aksoner til høyere hjernesenter som mushroom body calyces og det laterale horn. Mens de lokale internevronene er involvert i prosesseringen av duftinformasjon lokalt i antenneloben. Til sammen gjør dette systemet insektene i stand til å oppfatte små, flyktige molekyler fra omgivelsene, prosessere denne informasjonen og reagere i samsvar med duften. Hann-møll kan detektere stoffer som sendes ut av hunner av samme art, kalt feromoner, samt stoffer frigitt av hunner av andre arter, kalt interspesifikke substanser. Antennelobens karakteristiske synapseområder, kalt glomeruli, er anatomisk delt inn i det såkalte makroglomerulære kompleks som er ansvarlig for prosessering av informasjon om feromoner og interspesifikke substanser, og de så kalte ordinære glomeruli som behandler planteduftsignaler.

For å forstå hvordan sanseinntrykk om duftsubstanser kodes og prosesseres i hjernen er det viktig å kartlegge nevrontypene involvert i prosessering av lukt på ulike nivå. I dette studiet er ulike typer av projeksjonsnevroner i hjernens primære luktsenter karakterisert, både fysiologisk og morfologisk, ved hjelp av intracellulær registrering og farging.

To funksjonelt ulike typer av projeksjonsnevroner ble funnet, én som innerverte det største glomerulus i makroglomerular-komplekset og én som innerverte ordinære glomeruli. Dataene fra dette studiet viser at de to funksjonelt forskjellige nevrontypene terminerer i adskilte områder i det høyere hjernesenteret, det laterale horn. Blant projeksjonsnevronene knyttet til ordinære glomeruli, ble det funnet morfologisk distinkte typer i form av projeksjonsnevroner knyttet til to ulike antennelobe-trakter.

Abstract

Most organisms, ranging from mammals to bacteria, are dependent of their sense of smell in order to survive and reproduce. In moths, the detection of odorants is performed by olfactory sensory neurons located on the antenna. The sensory neurons bring olfactory information directly to the primary olfactory center in the brain, the antennal lobe. Onwards, projection neurons following one of three main tracts bring the information to higher olfactory centers including the mushroom body calyces and the lateral horn. In addition, neurons confined to the antennal lobe, local interneurons, influence the processing within the current region. This arrangement enables the animal to detect and identify the olfactory input, and to behave appropriately according to the odor. In addition to the detection of ordinary odorants, male moths have the ability to detect pheromones and interspecific substances released by conspecific and heterospesifics females, respectively. In the antennal lobe, the characteristic synaptic structures, termed glomeruli, are anatomically separated in to two systems, one male-specific compartment called the macroglomerular complex, and one consisting of ordinary glomeruli.

In order to increase the knowledge about odor processing in the moth brain, intracellular recordings and staining from second order neurons was conducted in the current study. Two functionally different types of projection neurons were characterized, one male-specific type innervating the macroglomerular complex, and one plant odor type innervating ordinary glomeruli. Among the projection neurons innervating ordinary glomeruli, distinct morphological categories associated with different antennal lobe tracts, were found. Among the scientifically interesting results of the present study, is the finding that antenna lobe output neurons underlying different behaviors target seemingly non-overlapping regions of the lateral horn.

Table of Contents

Introduction

The chemical sense is considered to be the evolutionary oldest sensory system. Organisms use chemical stimuli for locating nutritious food, finding potential partners, determining their place in a social hierarchy, and discovering potential dangers like predators, toxins, or competitors. The ability to detect chemicals from the ambient environment is thus crucial for survival of most animals (Kaupp, 2010; Mustaparta, 2002; Sachse and Krieger, 2011).

All organisms, from bacteria to mammals, have a chemical sense, and the olfactory system is surprisingly conserved through evolution (Ache and Young, 2005; Blair, 1995). This can be illustrated by comparing the olfactory system of phylogenetically distant groups of animals such as insects and mammals. The similarities are especially evident in the structure and function of the peripheral sensory neurons and the primary olfactory center in the brain; the antennal lobe in insects and the olfactory bulb in mammals. As compared to mammals, insects have a somewhat simpler system which is also easier accessible for experimental research. Thus, insects are good models for studying basic principles connected to this modality (Kaupp, 2010). In addition, many species of insects influence human society by acting as pollinators, pests, and vectors of disease. The insect olfactory system is essentially involved in all of their activities. Thus, more knowledge about the olfactory system is required to better understand the behavior of this diverse group of animals (Martin et al., 2011, Sachse and Krieger, 2011).

The insect olfactory system

The insect olfactory system is, in spite of its relative simplicity compared with that of mammals, a complex arrangement involved in both innate and learned behaviors (Hansson and Stensmyr, 2011). Among the most well-known innate behaviors evoked by odor molecules is the characteristic zigzag flight of an aroused male moth flying in the pheromone plume released by a conspecific female (Kennedy and Marsh, 1974). Ever since the first pheromone was identified from the females of the silk moth *Bombyx mori* (Beutenandt et al., 1959), the neuronal pathway underlying this stereotypic response has been thoroughly studied in several moth species.

Some of the most studied species of moths belong to the subfamily heliothine (Lepidoptera: Noctuidae), which includes over 350 species of moths found on all continents. Some members of this large sub-family, including the genum *Heliothis*, are considered major pests on food

and fiber crops (Cho et al., 2008). This is one of the reasons why the heliothine moths have become such popular organisms for researchers. Also, the heliothine are particularly interesting objects to study because they use their male-specific olfactory system to communicate, not only intraspecifically, as in many other species, but also interspecifically. Many different species of heliothine moths utilize the same molecule as their major sex pheromone. However, because they use different secondary components and unique ratios of the binary blends they are still able to segregate conspecifics from heterospecifics (Berg et al., submitted article).

The peripheral olfactory system

The neurons responsible for detecting odorants in insects, olfactory sensory neurons (OSN), are bipolar cells, as their equivalent in mammals (Kaupp, 2010). The dendrites of OSNs are surrounded by aqueous solution called receptor lymph inside hair-like cuticle structures called sensilla (Sachse and Krieger, 2011). The OSNs responsible for detection of substances released by females are found within long, male-specific sensilla called *sensilla trichodea* (Steinbrecht et al., 1992). The male-specific OSNs have been found to be highly specific, usually responding to one single type of molecule only (Almaas et al., 1991; Berg and Mustaparta, 1995). In the moth *Heliothis virescens*, two types of sensory neurons involved in detection of pheromones have been found. These two neuron types respond specifically to the major and the secondary sex pheromones identified for this species, *cis*-11-hexadecenal (Z11-16:ALD) and *cis*-9-tetradecanal (Z9-14:ALD), respectively. In addition, this moth has two neuron types tuned to two substances released by sympatric heterospecific females, *cis*-11-hexadecenyl (Z11-16:AC) and *cis*-11-hexadecenol (Z11-16:OH) (Almaas and Mustaparta, 1990; Berg et al., 1995; 1998). Thus, the male-specific olfactory system in *H. virescens* can be divided into two subsystems, one involved in detection of pheromones, i.e. chemical substances released by conspecific females eliciting the sexual response (Karlson and Lüscher, 1959), and one responsible for recognizing substances released by heterospecific females, so called interspecific substances, inhibiting the sexual behavior. Because of their antagonistic effect, pheromones and interspecific substances ensure attraction to conspecifics and reproductive isolation from heterospecifics (Mustaparta, 1996; Vickers and Baker, 1997).

Moths also use their olfactory system for detection of plant odors and other volatile substances such as CO_2 (Gurenstein et al., 2012). The OSNs responsible for the detection of plant odors are found in particular sensilla, different from the male-specific type. Examples of

other morphological types include the cone shaped basiconic sensilla and pitted coeloconic sensilla (Sachse and Krieger, 2011).

Which kind of information the OSNs mediate will generally depend on what olfactory receptor (OR) is expressed in that particular neuron. It has been found that the "one neuron – one receptor" principle applies to OSNs of insects (Krieger et al., 2004; Kaupp, 2010). Also, physiological studies have shown that most OSNs are relatively narrowly tuned, responding in a highly selective manner (Røstelien et al., 2000; Røstelien et al., 2005). The ORs in insects were not discovered until 1999, eight years after the identification of the mammalian OR (Buck and Axel, 1991; Clyne et al., 1999). Insect ORs are, like mammalian ORs, seven transmembrane proteins. However, the proteins in the two groups show inverted membrane topology. Insects have the N-terminal on the intracellular side, while mammals have the N-terminal on the extracellular side of the membrane (Benton et al., 2006). In addition, the insect ORs are always co-expressed with a second highly conserved receptor protein called Or83b, forming a heteromeric receptor (Sato et al., 2008; Wicher et al., 2008). In this dimerized receptor, consisting of two seven-transmembrane proteins, it is the ligand binding protein that is responsible for the selectivity of the OSN. However, the function of the OSNs and the trafficking of ORs to the dendritic membrane during development seem to be dependent on Or83b (Larsson et al., 2004). The ORs responsible for detection of pheromones and interspecific substances have been identified for several species of insects (Krieger et al., 2004; Van der Goes van Naters and Carlson, 2007). Among the first pheromone receptor gens identified, was a particular group in *H. virescens* (Krieger et al., 2004). Later, physiological studies have identified four male-specific odor receptors, each tuned to one of the pheromones or interspecific compounds the specific OSNs in male *H. virescens* respond to (Große-Wilde et al., 2007; Wang et al. 2011).

Insects are depending on olfactory binding proteins (OBP) to transport the hydrophobic olfactory molecules through the aqueous antennal lymph to the receptor. The OBP are, as the ORs, highly specific and only bind a single or a few molecules. In this way they are assumed to contribute to the overall specificity of the neurons situated within a sensillum (Sachse and Krieger, 2011; Hansson and Stensmyr, 2011).

Upon binding an odorant, a transduction mechanism takes place, resulting in a change in the activity in the OSN. The axons of the OSN make out the antennal nerve, in which olfactory

information is brought from the antenna directly to the insect brain (Sachse and Krieger, 2011).

The central olfactory system

Glomerular organization of the antennal lobe

The axons of OSNs project directly to the antennal lobe (AL), which is the primary olfactory center in the insect brain. This is the first site in the brain where integration and processing of olfactory information takes place (Hansson and Anton, 2000; Homberg et al., 1988). In the AL, the olfactory sensory fibers terminate in spherical structures called glomeruli. The projections enter the AL in a chemotopic manner, as all OSNs expressing the same receptor type terminate in the same glomerulus (Martin et al., 2011; Vosshall et al., 2000). This predicts a one-to-one relationship between the number of OR types and the number of glomeruli (Mustaparta, 2002). In many insects, a sexual dimorphism concerning the number of glomeruli in the AL has been found (Hansson and Stensmyr, 2011). Male moths have a few male-specific glomeruli located dorsally in the AL, the so called macroglomerular complex (MGC). The MGC is innervated by OSNs responsible for detecting pheromones and interspecific substances. The input to the MGC is highly segregated from the rest of the AL that receives input from plant-odor sensitive OSNs (Galizia et al., 2000). In *H. virescens* for instance, the male has four MGC glomeruli located at the base of the antennal nerve entrance (Berg et al., 1998; 2002; Galizia et al., 2000). These glomerular units include the cumulus, which is innervated by OSNs detecting the major pheromone component, Z11-16:ALD, the dorso-medial compartment, receiving information about the second pheromone component, Z9-14:ALD, and two ventrally located glomeruli receiving information about the two interspecific substances, Z11-16:AC and Z11-16:OH (Berg et al., 1998). A physiological tuning of the MGC units has been found in other species of moth as well (Christensen et al., 1991; Hansson, 1995). Some female-specific glomeruli have also been found, further underlining the sexual dimorphism in the olfactory systems of particular insect species (Løfaldli et al., 2010). The number of glomeruli both in the MGC and the rest of the AL vary between different species (Hansson et al., 1991; Kanzaki et al., 2003). The AL of male *H. virescens* consists of 62 ordinary glomeruli in addition to the four MGC glomeruli (Berg et al. 2002; Løfaldli et al., 2010).

AL neurons

In addition to the terminals of OSNs, three other neuron types are found in the AL. These are local interneurons (LN) confined to the AL, projection neurons (PNs) responsible for bringing the olfactory information to higher brain areas, and centrifugal neurons (Homberg et al., 1989). The latter have their cell bodies and dendrites outside the AL, and send input to the glomeruli (Homberg et al., 1989). This type of neuron will not be further discussed in this thesis. Particular connections between the different types of AL neurons have been described, and the different arrangements include both excitatory and inhibitory circuits (Mustaparta, 2002). First, the ORNs give excitatory input both to the PNs and the LNs. Onwards, the PNs give excitatory input to the LNs, and the LNs give inhibitory input to PNs and other LNs. The LNs can thus, give rise to both inhibition and excitation of the PNs, by the process of inhibition and disinhibition respectively (Christensen et al., 1993; Mustaparta, 2002).

LNs, being confined to the AL, are responsible for modulating the output from the AL. Generally, most antennal lobe LNs are inhibitory, using gamma amino butyric acid (GABA) as their neurotransmitter, but excitatory cholinergic LNs have been found as well, in *Drosophila melanogaster* (Huang et al., 2010; Shang et al., 2007). Furthermore, the presence of particular neuropeptides acting as co-transmitters has been documented in LNs of *H.virescens* (Berg et al., 2009). The majority of LNs are multiglomerular, and by interglomerular inhibition they are involved in sharpening the contrast between activated and non-activated glomeruli (Lei et al., 2004). Both the MGC and the ordinary glomeruli may be innervated by the same LN and therefore, the activation of ordinary glomeruli may affect the response of PNs innervating the MGC region (Namiki et al., 2008). Also, the LNs are probably involved in synchronizing the activity in populations of PNs (Mustaparta, 2002; Sachse and Galizia, 2002). In addition to the multiglomerular LNs, a smaller number of oligoglomerular LNs, innervating only a few glomeruli are present in the AL as well (Martin et al., 2011; Namiki et al., 2008).

The other major category of second order neurons, the PNs, bring olfactory information from one of the two divisions of the AL, the MGC or the ordinary glomeruli, to higher brain centers such as the mushroom body calyces and the lateral horn (LH) (Kanzaki et al., 1989; Sachse and Krieger, 2011,). So far, no PNs innervating both the MGC and ordinary glomeruli have been found (Berg et al., submitted article). In moths, the PNs project in one of three main tracts; the medial antennal lobe tract (mALT), the mediolateral antennal lobe tract (mlALT), and the lateral antennal lobe tract (lALT). The mALT, being the most prominent tract,

contains mainly uni-glomerular PNs (Homberg et al., 1988; Ito et al., 2014; Rø et al., 2007; Zhao et al., 2010). However, PNs innervating two or three glomeruli have also been described (Kanzaki et al., 2003; Løfaldli et al., 2010; Namiki and Kanzaki, 2011). The target regions of mALT PNs in the protocerebrum include the calyces and the LH, in the current order (Homberg et al., 1988). PNs in the mlALT usually innervate more than one glomerulus while both uniglomerular and multiglomerular PNs have been found in the lALT (Rø et al., 2007). PNs in the mlALT and the lALT project directly to the LH, and some neurons from the lateral tract send projections further to the calyces (Homberg et al., 1988; Rø et al., 2007).

Higher olfactory centers

As previously described, the PNs bring olfactory information mainly to two higher brain centers, the mushroom body calyces and the LH (Homberg et al., 1988; Rø et al., 2007). The calyces is an easily recognizable structure located posteriorly in the insect brain and is, in moths, shaped like two fused cuplike structures (Fahrbach, 2006; Martin et al., 2011; Rø et al., 2007). The calyces is part of a larger structure called the mushroom body, consisting of several neuropils built up of Kenyon cells (Heisenberg, 1998). The dendrites of the Kenyon cells make up the calyces. Another essential structure of the mushroom body is the pedunculus, which runs anteriorly, and branches into three lobes, the α-, β- and γ- lobe. In addition, there is a second lobe system called the Y- lobe (Rø et al., 2007). Even though the calyces of some insects receives input from multiple sensory modalities, the main input in moth originates from the AL (Fahrbach, 2006).The second higher olfactory center, the LH, is a more diffuse neuropil as compared to the calyces, but never the less, it receives input from antennal lobe PNs carrying olfactory information (Ito et al., 2014; Martin et al., 2011). It has been found that the two different neuropilic areas, the calyces and the LH, are involved in different tasks regarding odor information processing. The mushroom bodies are found to be involved in learning and memory in many species of insects (Fahrbach, 2006; Heisenberg, 1998; Heisenberg, 2003). For instance, *D. melanogaster* missing 90% of the Kenyon cells, lost their ability to condition presented odor stimuli (Heisenberg, 1998). However, the fly still showed attractive and repulsive behavioral responses to some odorants, indicating that processing of information being relevant for innate behavior responses takes place at a different location, most likely the LH (Heimbeck et al., 2001; Heisenberg, 1998).

Each Kenyon cell contacts a number of PNs, and thus the calyces is a likely candidate for integration of signals originating from various odor stimuli (Heisenberg, 2003; Namiki et al.,

2013). As concerns the LH, Ruta et al. (2010) have reported, in a publication describing the pheromone- sensitive pathway in *D. melanogaster* from the OSN to the descending output, that the neurons in the LH respond very specifically, indicating a labeled line arrangement. However, in locusts, the LH neurons responded to a wide range of tested stimuli contradicting the findings in *D. melanogaster* (Gupta and Stopfer, 2012). This may indicate that there are differences between species. All three ALTs have target regions in the LH, and the projecting areas of different tracts have shown a high degree of overlap. This indicates integration of olfactory information from the tracts at the level of the LH (Løfaldli et al., 2012). Due to the relatively well-described olfactory pathway of the *H. virescens* male, including a male-specific system being driven by four signal molecules and an arrangement for general odorants, it is indeed interesting to investigate more in detail how odor signals within each category is represented in the LH of this species.

Aim of thesis

Olfactory information is mediated to the higher olfactory centers via PNs. In order to better understand how olfactory information is coded in the insect brain, it is relevant to learn more about the morphology and physiology of these neurons. The main aim of this thesis was therefore to identify different categories of antennal lobe PNs, including male-specific and plant odor responding neurons. More specific aims were to map the target regions of male-specific and plant odor projection neurons in the lateral horn, as well as characterizing physiological and morphological properties of projection neurons passing in different antennal lobe tracts.

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Materials and methods

Experimental species

The noctuid moth, *H. virescens* (Heliothine; Lepidoptera), were reared in the chemosensory laboratory at Dragvoll, NTNU. The culture originated from Bio Crop science, Germany.

Eggs and larvae were kept in a climate chamber (66% air humidity, 28 ^{o}C). When the larvae transformed into pupae they were sorted according to sex and kept in separate incubators. Upon hatching, they were moved to cylindrical plexiglas containers with perforated lids for air supply. Food (5% sucrose solution) was given *ad libitum*. Maximum eight moths were kept in each container. Both pupae and adult moths were kept in climate chambers (Refritherm 200, Struers-Kebolab, Albertslund, Denmark) at 27 ^{o}C and 70 % humidity with a reversed photoperiod 14:10h light:dark.

Preparation of the odorants

Both the pheromones and ordinary odorants were prepared in the same way. The odorants were diluted in hexane to the preferred concentration (10^{-5} M). The odor solution (usually 100 µL) was then transferred onto a small circular filter paper so that it contained 10 ng of the odorant. The pheromone mixture (Z11-16:ALD +Z9-14:ALD) was applied to the filter paper in a 16:1 ratio at a total concentration of 10 ng. The two mixtures containing the major pheromone component (Z11-16:ALD) and the plant mixture B10 and linalool respectively, where both applied in a 1:100 relationship with 10 ng of the pheromone and 10 x 100 ng of the plant odors. The loaded filter paper was positioned in a flow of nitrogen, ensuring evaporation of the hexane, before it was wrapped up and placed within a clean glass cartridge. A control was prepared using only hexane. The plant mixture B10 contains 3Z-hexenol, 3Z-hexenyl acetate, ocimene, linalool, geraniol, 3-carene, E-verbenol, methyl benzoate, hexanol and phenylethanole. The odorants were stored at -18 °C and renewed approximately ones a month. All of the odor substances used in the experiments, their concentration and the order of stimulation are presented in table 1 below.

Table 1: Amounts (ng) of odor substances used as stimuli. The odor substances are presented in the order they were given during experiments.

	Odor substances used as stimuli	Amount (ng) of the stimuli
	Control (Hexane)	
Pheromone stimuli	Z11-16:ALD +Z9-14:ALD (16:1)	9.41 + 0.59
	Z11-16:ALD	10
	Z9-14:ALD	10
	Z11-16:OH	10
	Z11-16:AC	10
	Z11-16:ALD + B10 (1:100)	10 + 100
	Z11-16:ALD + Linalool (1:100)	10 + 100
Plant odorants	B10	10
	Linalool	10
	Geraniol germacrene d	10
	2-phenylethanol	10
	3Z-hexenyl acetate	10
	Ocimene	10
	Farnesene	10
	Ylang-ylang	10

Preparation for electrophysiological recordings

Insects (one to six days old) were anaesthetized by cooling at 4 ^{o}C for approximately 10-15 min prior to experiments. They were then mounted in a pipette tube (1 mL) where the tip had been cut off to expose the head of the moth. Moths were immobilized by packing dental wax (Kerr Corporation, Romulus, MI, USA) around the neck. Scales and hairs on the head of the insect were removed, before a opening was made in the cuticle between the antennae by means of a razor blade knife and tweezers. Muscles and some trachea were removed to expose the brain. The neurolemma covering the brain was carefully removed from the AL using tweezers. Ringer's solution (150 mM NaCl, 3 mM $CaCl_2$, 3 mM KCl, 25 mM $C_{12}H_{22}O_{11}$, 10 mM TES, pH 6.9) was applied throughout the experimental procedure to keep the brain moist.

Intracellular recording and staining

The animal was placed under the microscope in a way that gave easy access to the MGC-area of the AL. In order to minimize noise from electrical devises close-by, registrations took place inside a Faraday chamber. A chlorinated silver wire that served as the reference electrode was

carefully inserted into the compound eye of the insect contralateral to the visualized AL. A glass micro electrode pulled with a Flaming-Brown horizontal puller (P97; shutter instruments, Navato, CA, USA) and serving as the recording electrode was inserted into the MGC area of the antennal lobe using a micro manipulator (Leica). Together these two electrodes made up an electrical circuit, making it possible to record changes in voltage across cell membranes, i.e. nerve impulses. The tip of the electrode was filled with the fluorescent dye tetramethylrhodamine-biotin dextran (Micro-Ruby, Invitrogen, Germany) and then backfilled with potassium-acetate (0.2 M). The electrical signals recorded from the neural tissue were visualized on an oscilloscope and made audible through a loudspeaker. The neuronal signal was digitalized by means of a data acquisition unit CED (micro 1401 mk II, Cambridge Electronic Design Limited, Cambridge, UK). This was possible because the electrode was connected to a preamplifier (Axonprobe-1A, multipurpose microelectrode amplifier, Molecular devises, CA, USA) through a chlorinated silver wire inserted into the fluid filled electrode. The electrical signals were registered in the software Spike 2 (version 7, CED).

The test stimuli were delivered to the preparation via the odor-bearing cartridge placed in parallel with a cartridge providing a continuous airflow (400 mL/min). Both cartridges, connected to a magnetic valve system via Teflon-tubes, were placed approximately 2 cm from the antenna. Upon neuronal contact, stimulation with the odorants was given as an air puff (100 mL/min, 400 ms) through the odor-containing glass cartridge. The commands to change the airflow were given via a script running in Spike 2. The delay from the valve opened until the odorant was delivered to the antenna has been estimated to be 200 ms. This delay was compensated for in the Spike 2 script (Høydal, 2012).

The most advantageous order of stimuli delivery was found after some experimental testing. The control was always tested first (table 1), and if possible it was tested again several times throughout the registration period. After the control, the mixture of the two sex pheromone components (Z11-16:AL + Z9-14:AL, 1:16) was tested before the two substances were tested separately. Next, the two interspecific substances Z11-16:OH andZ11-16:AC, were given. Two mixtures, containing the major sex pheromone Z11:16 AL and linalool or the plant mixture B10, respectively, was the next to be tested. Finally, the ordinary plant odorants B10, linalool, geraniol germacrene d, farnesene, 2-phenylethanol, 3Z-hexenyl acetate, ocimene and ylang-ylang were tested. The stimuli were given at an interval of at least 10 seconds, and each

stimulant was tested two times or more. The plant odorants were only given if there was a stable connection between the electrode and the neuron.

After testing the odor stimuli, the neuron was ionotrophoretically stained using a positive pulse current (0.7-1.2 nA, 2 Hz) for 9-11 min. Then, the insect was covered with wet paper and left at 4 ^{o}C in a moist box overnight allowing neural transportation of the dye.

Dissection and chemical protocols

The head was cut off and placed in melted dental wax in a dissection bowl. Ringer solution was added before the antenna and maxillary palp were cut off and the rest of the cuticle, muscles, and trachea were removed.

The brain was fixed in 4 % formaldehyde for 24 hours at 4 ^{o}C or for 2 hours at room temperature. After the fix had been removed, the brain was rinsed in phosphate buffered saline (PBS) for 10 min. To enhance the fluorescent labeling, Streptavidin-CY3 (Jackson immunoresearch, west Grove, PA, USA) was added and left for at least 24 hours in 4 ^{o}C. The brain was then washed in PBS for 2x10min before it was dehydrated with an ascending ethanol series (50, 70, 90, 96, and 2 times 100%; 10 min each). Finally, the brain was made transparent by mounting it in methyl 2-hydroxybenzoate (methyl salicylate).

For visualizing surrounding neuropils and identifying glomeruli innervated by the stained neurons, synapsin immunostaining was performed. The dehydrated brains mounted in methyl salicylate were rehydrated in a decreasing ethanol series (2 times 100, 96, 90, 70 and 50 % ; 10 min each) and washed in PBS for 10 min before they were dehydrated and placed in Xylol for 5 min. Xylol was added to increase the permeability of the cell membranes. This was followed by a second rehydration and rinsing in PBS for 10 min. In order to minimize non-specific staining, the preparations were pre-incubated in normal goat serum (NGS; Sigma, ST. Louis, MO, USA; 10%) diluted in PBS x Triton X (PBStx; 0,1%) for 30 min.

SYNORF 1 (Developmental Studies Hybridoma Bank, University of Iowa) is a monoclonal mouse antibody that marks synaptic regions by binding to the presynaptic terminal protein synapsin. The preparations were incubated in SYNORF 1 diluted in NGS PBStx (1:30) for 46 h at 4 ^{o}C. After the incubation, the brains were rinsed in PBS for 6 x 20 min before the secondary antibody CY5-conjugated goat anti-mouse (Jackson immunoresearch, west Grove, PA, USA) and PBStx was added (1:500) for a period of 46 hours in 4 ^{o}C. Finally, the brains were rinsed in PBS 6 x 60 min, dehydrated, and mounted in methyl salicylate.

Scanning and imaging

To visualize the neurons, the brains were placed on aluminum plates and scanned by a confocal laser-scanning microscope (Zeiss LSM 510 META, Jena, Germany). The brains were scanned with helium/neon (HeNe) 1 laser (543 nm) in order to excite the biotin-streptavidine-CY3 complex (Ex/Em: 555/580) used for the ionotophoretic staining of the neurons. In order to excite CY 5 (Ex/Em: 650/670) and thereby visualize the neuropil structures in the brain, the preparations were scanned with the HeNe 2 laser (633 nm). The objectives EC Plan-Neofluar 10x/0.3, Plan-Neofluar 20x/0.5 I and C-acroplan 40x/0.8 W were used in connection with both lasers. The pinhole size was set to 1 airy unit, the brightness and contrast adjusted, and the thickness of each images in the stack were optimized according to the objective to improve the quality of the image. The scan resulted in a z-stack that was further analyzed and modified in the LSM image browser. Adobe Photoshop CS6 was used to adjust the contrast and brightness of the images attained in LSM, before the final figures were made in Adobe Illustrator CS6.

Analyzing the physiological data

The physiological data were examined and characterized by a subjective examination of the data set obtained in Spike 2. A response was defined as a change in activity seen during stimuli that was visually and quantitative different from the control. The latency of inhibitory changes in activity was measured from the onset of the stimuli to the last spike before the inactive period. To be considered as an inhibitory response, the inhibitory period had to start during the stimulation period (0-400 ms) and had to last for $\geq$ 100 ms. Complex responses, where both excitation and inhibition was found in response to the same stimuli, were identified based on the following criteria: (1) Both the inhibitor and excitatory phases of the response had to be repeated, (2) the inhibitory response had to start within the stimulation period, (3) the excitatory phase seen after the stimuli offset had to start within two seconds after the stimuli offset and (4) the inhibition period and time before stimuli onset had to remain similar for repeated stimulation periods. If the visual examination did not correspond with the gathered quantitative data, the visual examination was use for determination of the respons.

The frequency (Hz, spikes/sec) for the spontaneous activity was found by measuring the activity during a period of one sec before the stimuli onset of all stimuli. The frequencies during the stimulation were found by measuring number of spikes during the stimulation

period and divide this on 0.4 sec to get spikes/sec. In order to determine if there was a difference between the spontaneous activity and the activity during stimulation, the ratio between them was found. This ratio was then compared between the different stimuli and the control to see if there were notable differences. Neuronal height and width were measured to see if the different neuron types could be determined based on their physiological characteristics.

Results

All together experiments were performed on 65 individuals of the moth *H. virescens*, and intracellular registrations from 56 neurons were obtained. Of these, 31 neurons were attempted stained resulting in six preparations containing 13 successfully labeled antennal lobe neurons and one neuron confined to the protocerebrum. The antennal lobe neurons include ten PNs, nine passing in the mALT and one in the lALT, and two LNs. An overview of the successfully stained neurons achieved from the different preparations can be found in table 2. An additional table (table 4) presenting the responses registered during the electrophysiological recordings is included in appendix I. For practical reasons, the neurons are presented according to the brain preparation they were found in. Also, all figures are presented in the end of the results.

Table 2: Brain preparations containing stained neurons found in the present study. The neurons are presented according to the brain preparation (B) they belonged to. MGC PN: projection neuron innervating the macroglomerular complex, OG PN: projection neuron innervating ordinary glomeruli, u: uniglomerular. m: multiglomerular, mALT: medial antennal lobe tract, lALT: lateral antennal lobe tract, LC: lateral cell cluster, MC: medial cell cluster

Preparation ID	**BI**	**BII**	**BIII**	**BIV**	**BV**	**BVI**
Neuron classification	MGC uPN	OG uPN	OG uPN, 3.order neuron	OG uPN	OG mPN	mLN
Number of stained neurons in the preparation	2	2	2	4	2	2
Antennal lobe tract	mALT, lALT	mALT	mALT	mALT	mALT, lALT	-
Location of terminal projections in the LH	Antero-medial	Lateral, anterior	Lateral	Lateral	Lateral	-
Cell body location	MC	-	LC	MC	-	LC
Cell body size (µm in diameter)	~11	-	~10	~8	-	~13

PNs

PN innervating the MGC

BI: One uniglomerular PN innervating the MGC was stained. This neuron, found in preparation BI, arborized in the large MGC-unit located at the entrance of the antennal nerve, the cumulus (figure 1A). The neuron had two main dendritic branches in the AL, both sending off fine processes that filled the entire cumulus (figure 1A). The cell body, which had a diameter of 11 µm, was located in the medial cell cluster (figure 1A, B and E). The neuron followed the medial ALT passing in an anterior-posterior direction along the medial part of the protocerebrum. At the anterior border of the calyces it turned laterally sending off branches into this structure on its route. At the lateral part of the calyces it turned anteriorly terminating in an anterior region of the medial LH (figure 1E). Unfortunately, the calyces were not completely intact at the time of scanning; however, based on what was still there, the neuron seemed to innervate the current neuropil area relatively sparsely. Only a few thin branches could be observed in the calyces. This differed from the ramifications in the LH, showing a more elaborate projection pattern (figure 1C, D and E).

The neuron, which had a relatively high spontaneous activity, showed no response to the major pheromone component, Z11-16:AL. Also, the pheromone blend induced no response. The two interspecific substances, Z11-16:OH and Z11-16:AC, on the other hand, both elicited a decreased spike firing rate. The inhibitory response to each of these two substances was repeated all three times the two stimuli were given. The response was more evident for Z11-16:AC with a longer inhibitory period than seen for Z11-16:OH. For both odorants, the inhibitory period was longest during the second stimulation period, i.e. 330.9 ms and 253.2 ms for Z11-16:OH and Z11-16:AC, respectively. The duration of the inhibitory periods is stated in table 3. None of the other stimuli tested gave any responses (table 4, appendix I). The frequency of the spontaneous activity changed somewhat during the registration period from approximately 30 spikes per second in the beginning to ca 10 spikes per second in the end.

Table 3: Duration of repeated inhibitory responses elicited by the two interspecific substances (Z11-16:AC and Z11-16:OH).

Stimuli	First stimulation period	Second stimulation period	Third stimulation period
Z11-16:AC	243.08 ms	330.85 ms	270.08 ms
Z11-16:OH	124.91 ms	253.20 ms	199.18 ms

PNs innervating ordinary glomeruli

Eight PNs passing in the mALT and one PN in the lALT, all innervating ordinary glomeruli, were stained. These neurons originated from four preparations BII-BV. Three of these (BII, BIII, and BIV) possessed stained neurons projecting in the mALT, while in preparation, BV, two PNs projecting in separate tracts, one in the mALT and the other in the lALT, were stained. The PNs are classified as uniglomerular, innervating a single glomerulus, or as multiglomerular, innervating more than one glomerulus. Most of the PNs in the mALT were found to be uniglomerular. However, one PN in the mALT innervated two or three glomeruli. In addition, the neuron in the lALT innervated an undefined number of glomeruli.

BII: In the current preparation, two antennal-lobe PNs were stained both passing in the mALT (figure 2). One of them showed dense arborizations within one ordinary glomerulus in the AL (figure 2A, B). Due to a slight damage of the ventro-lateral part of the AL, the glomerular arborizations of the other neuron were not identified. Also, the lack of stained cell bodies in the AL is probably due to the current rupture. Both projections passed in the mALT to the calyces and the LH. In the calyces, several processes were given off innervating the whole structure (figure 2C). The terminal projections in the lateral horn innervated the most lateral part, located adjacent to the optic lobe (figure 2D). As can be observed in figure 2D, the neurons innervated the current area relatively sparsely.

The physiological data from the present recording included a small decrease in firing rate to most tested stimuli including the control (Table 4, appendix I). However, due to the quality of the recording, the physiological data are not included in the results. They are presented figure 8 in appendix II, however.

BIII: The single PN stained in this preparation, also densely innervated one ordinary glomerulus in the AL (figure 3A). One cell body located in the lateral cell cluster having a diameter of approximately 10 µm, was labeled (figure 3A). The innervated glomerulus and the cell body of the PN were situated relatively close together. The PN projected in the mALT to the calyces innervating the whole neuropil area (figure 3B and C). The final projections in the lateral horn targeted the most lateral area (figure 3D). In addition to the uniglomerular PN, one stained process belonging to another neuron, confined to the protocerebrum, was seen in the current preparation (figure 3B). A figures of this neuron are presented in appendix III (figure 9).

The intracellular recording showed no response to the stimuli tested, except for a small inhibition to the initial stimulation with the control (figure 3E). The spontaneous activity of this neuron changed quite dramatically during the registration period, from tall spikes and a spike frequency of 13 spikes per second, to no activity what so ever.

BIV: Four PNs following the mALT were simultaneously stained in this preparation (figure 4C). Three cell bodies located in the medial cell cluster were found, all having a diameter of approximately 10 µm. Processes from the three cell bodies innervated glomeruli in the lateral part of the AL (figure 4A). Unfortunately this preparation was not successfully immunostained and identification of the glomeruli was thus impossible. Despite of this, and based on the onwards projections, the neurons are most likely uniglomerular PNs innervating ordinary glomeruli and will be considered as thus in the current thesis. From the AL, the neurons projected in the mALT to the calyces where they sent of branches. The calyces was not completely intact at the time of scanning and it is therefore difficult to make out to what extent the structure was innervated (figure 4B). After sending off branches in the calyces, the neurons project to the lateral horn, innervating the lateral part, as found in BIII (figure 3C and 4C).

The only physiological result of interest in this preparation was a change in the spontaneous activity of the neuron after stimulating with the mix of the two sex pheromone substances (Z11-16:ALD + Z9-14:ALD). This change took place 139.87 ms after stimulation offset, but was not repeated in the second stimulation period (figure 4D). The amplitude of the spikes decreased during the registration period; from approximately 53 mV before any of the stimuli were given, to about 5 mV further into the registration. Also, the spontaneous activity of the neuron varied during the recording period, from being quit high before stimulation (9 spikes per second) to zero after stimulating with the control the first time and then back to 9 spikes per second after stimulation with the pheromone blend.

BV: In this preparation, two simultaneously stained PNs, both innervating more than one glomerulus were identified. The neurons followed different ALTs, i.e. the mALT and the lALT. The PN following the mALT was found to have three branches in the AL with dense arborizations in two or three glomeruli; one glomerulus in the lateral part of the AL and one or two located more ventrally (figure 5A1). The innervations in the individual glomeruli seemed to be restricted to particular parts, not filling the entire structure. Onwards, the current

neuron sent a single axon to the calyces where it emitted branches before it ended up in the LH (figure 5 A2 and A3). The arborizations in the calyces were characterized by thick branches projecting within a relatively small area. Also in the lateral horn, the ramifications were thicker and more defined than those of uniglomerular PNs. However, the arborization area in the LH originating from this neuron was similar to the area innervated by uniglomerular PNs in the mALT.

The second neuron in preparation BV innervated several glomeruli in the AL loosely (figure 5 B1 and B2). The axon of the current PN projected in the lALT, connecting directly to the lateral horn. Here, it had ramifications in an area similar to that of the simulatnously stained PN, which was confined to the mALT (figure 5 B3 and B4). Along the path from the AL to the lateral horn, the PN in the lALT sent off several short branches innervating undefined areas in the protocerebrum. The axon of the neuron was notably thinner than that of the mALT PNs (figure 5 A2).

In figure 6A, a typical spike from the recording underlying the current staining is presented. The curve path was characterized by an unusual course during the repolarizing phase. A complex response including an inhibitory phase followed by an excitatory phase was recorded during initial stimulations with several odor stimuli. However, the response was only repeated when stimulating with the pheromone mixture (figure 6B).

LNs

In preparation BVI, two multiglomerular LNs were simultaneously stained. Both neurons had their cell bodies located in the lateral cell cluster and innervated most, if not all glomeruli. Immunostaining was attempted on this preparation, but without satisfying result. However, as shown in figure 7A, a few stained processes innervated a region that may correspond to the MGC-area.

The neurons did not respond to any of the tested stimuli (figure 7B). The spike width recorded was relatively large (figure 7C).

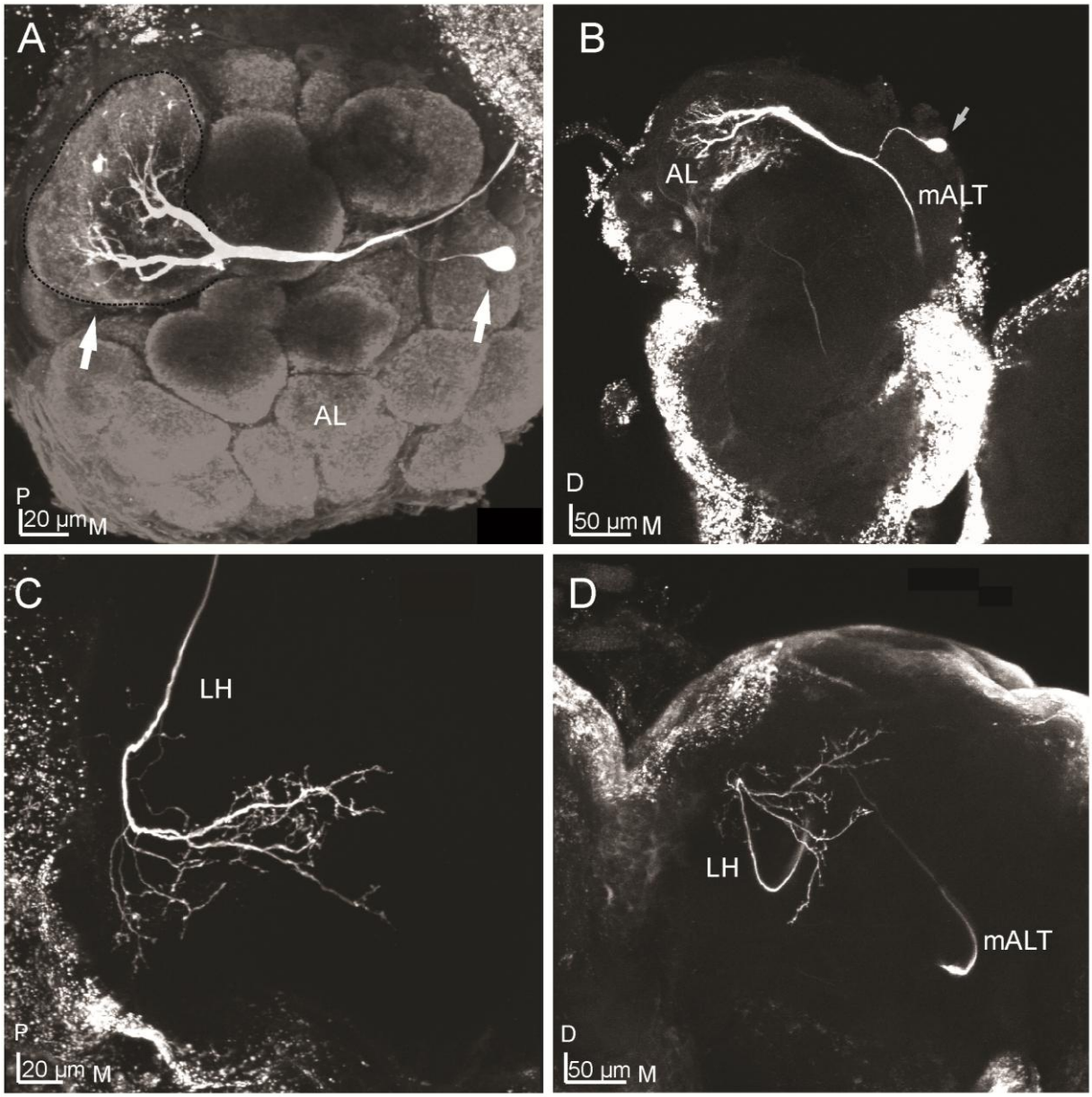

Figure 1

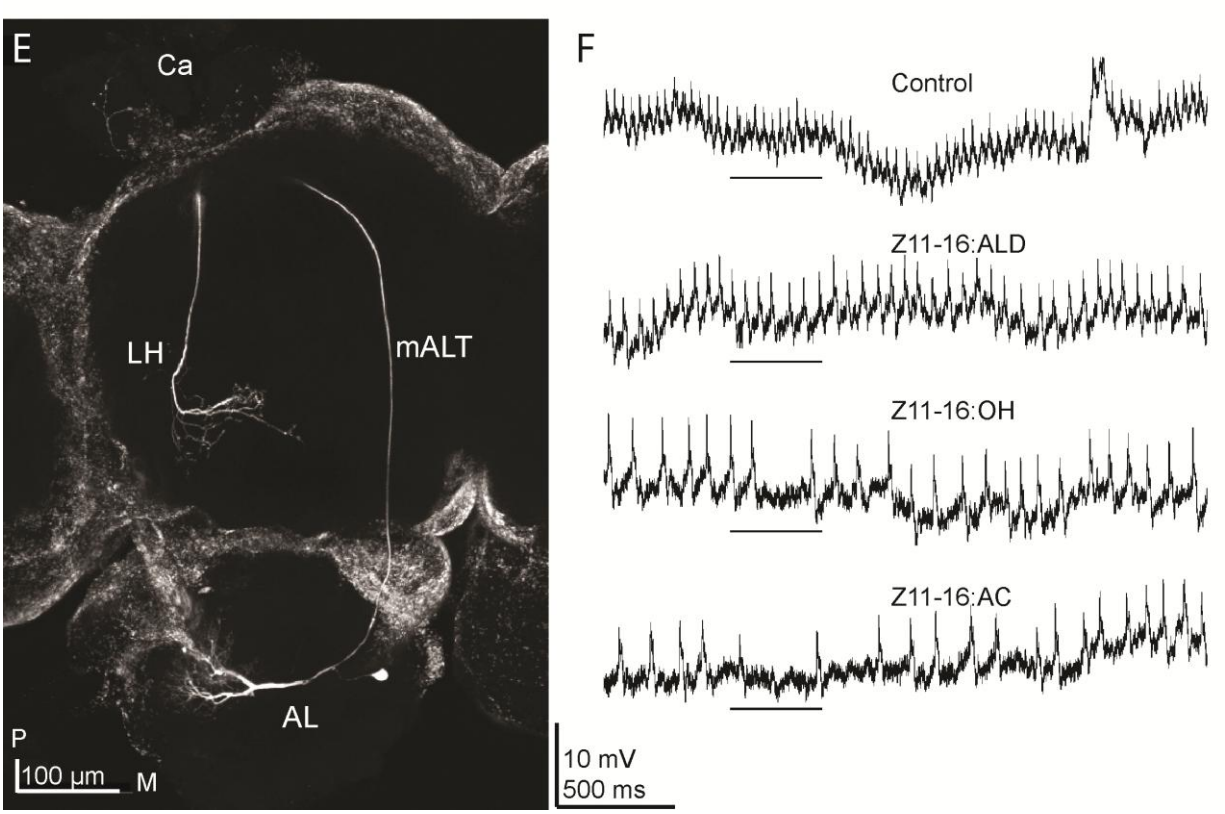

Figure 1: Morphology and physiology of one uniglomerular projection neuron (PN) innervating the cumulus in the macroglomerular complex (MGC), located dorsally in the antennal lobe (AL). The PN projects in the medial antennal lobe tract (mALT) to the calyces (Ca) where it sends off branches before it turns anteriorly and end up in the anterio-medial part of the lateral horn (LH). **A:** Dorsally scanned confocal image (40x0.8, projection view) of the immunostained AL. The dotted black line and the white arrow on the left, indicate the cumulus. The other white arrow marks the cell body situated in the medial cell cluster. **B:** Frontal confocal scan of the AL (20x0.5, projection view) indicating the mALT and the position of the cell body (arrow). **C:** Confocal image of terminal projections in (40x0.8). The brain is seen in a dorsal view. **D:** Projection image of the same projections in a frontal view. **E:** Confocal image (10x0.3, projection view) of the whole neuron. The brain is seen from a dorsal view. **F:** The lack of neural response to the control and the main sex pheromone Z11-16:ALD is shown. An inhibitory response to the two interspecific signals Z11-16:OH and Z11-16:AC is demonstrated. The scale bars situated beneath the response profiles indicate the stimulation period (400 ms). Abbreviations not mentioned above: D: dorsal, M: medial, P: posterior, mV: millivolt, ms: millisecond and µm; micro meter.

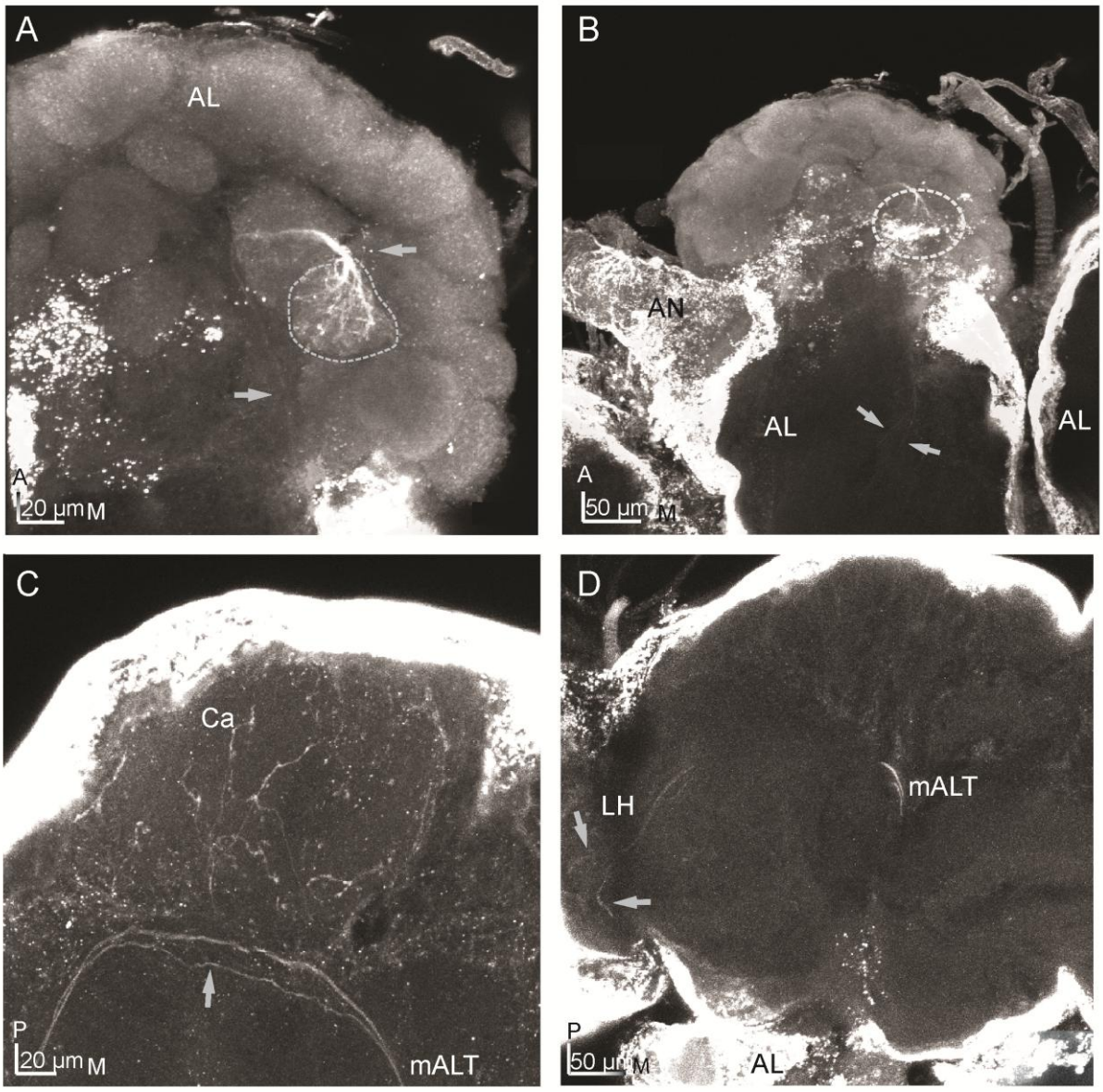

Figure 2: Two projection neurons (PNs) passing from the antennal lobe (AL) to the calyces (Ca) and the lateral horn (LH) via the medial antennal lobe tract (mALT). **A:** Confocal image of the immunostained AL (40x 0.8, projection view). The glomerular arborizations onging to one of the stained PNs is marked with a white dashed circle. The white arrow point to the projection leaving the the glomerular cluster located outside the AL. Due to damage of the AL, the glomeruli are not located in their natural position. **B:** Projection view (20x0.5) showing the immunostained preparation including the innervated glomerulus (dashed white line). The two white arrows indicate the two neurons that were observed leaving the AL. The antennal nerve (AN) is marked. **C:** Close up vie of the calyces (confocal image, 20x0.3 zoom: 1.8, projection view). The neurons had ramification in the entire structure. **D:** This confocal image (20x0.5 projection view) shows the ramifications in the lateral horn (LH). Abbreviations not mentioned above A:anterior, M: medial, P: posterior, and µm: micro meter

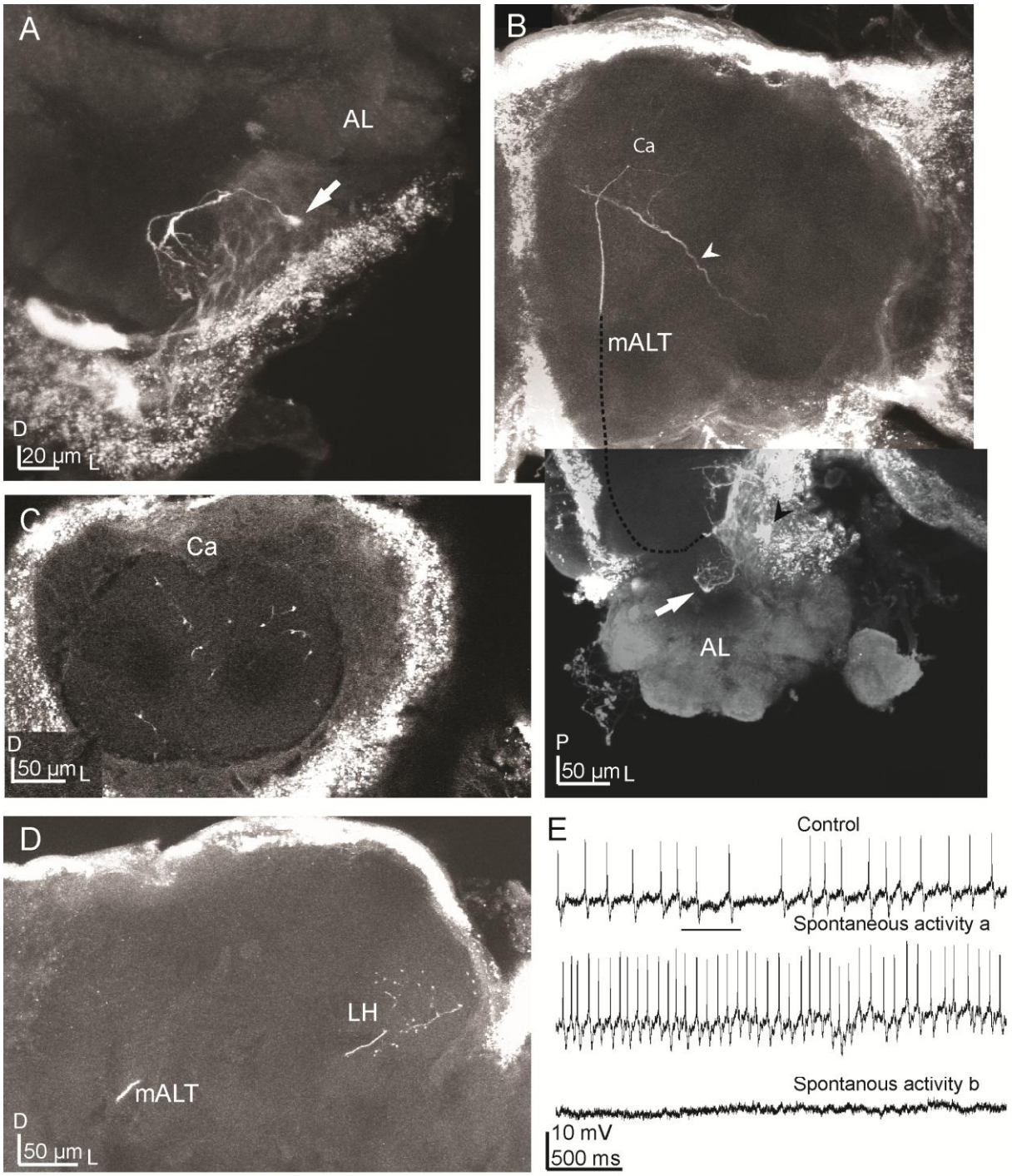

Figure 3: Confocal image of one uniglomerular projection neuron (PN) following the medial antennal lobe tract (mALT) to the calyces (Ca) and lateral horn (LH). One additional neuron, confined to the protocerebrum was also stained (marked with white arrow had in B). The latter neuron will not be further commented here. **A:** A 40x0.8 confocal image of the immunostained antennal lobe (AL). The white arrow points to the cell body located in the lateral cell cluster. **B:** An image (20x0.5, projection view) put together from to images from the same z-stack of the immunostained preparation. The neural arborizations in the AL (white arrow) and the axon up to the calyces, is visualized. The black arrow points to the cell body. The mALT is indicated by a drawn line (black dotted line) **C:** Confocal image showing the Ca and some of the ramifications found there. **D:** Confocal image, projection view showing the arborizations in the LH. **E:** The spiking pattern during stimulation with the control. The different spontaneous activity at two different times points during the registration period is also shown. The scale bars situated beneath the response profiles indicate the stimulation period (400 ms). Abbreviations not mentioned above: D: dorsal, P: posterior, L: lateral, mV: millivolt, ms: millisecond and µm: micro meter.

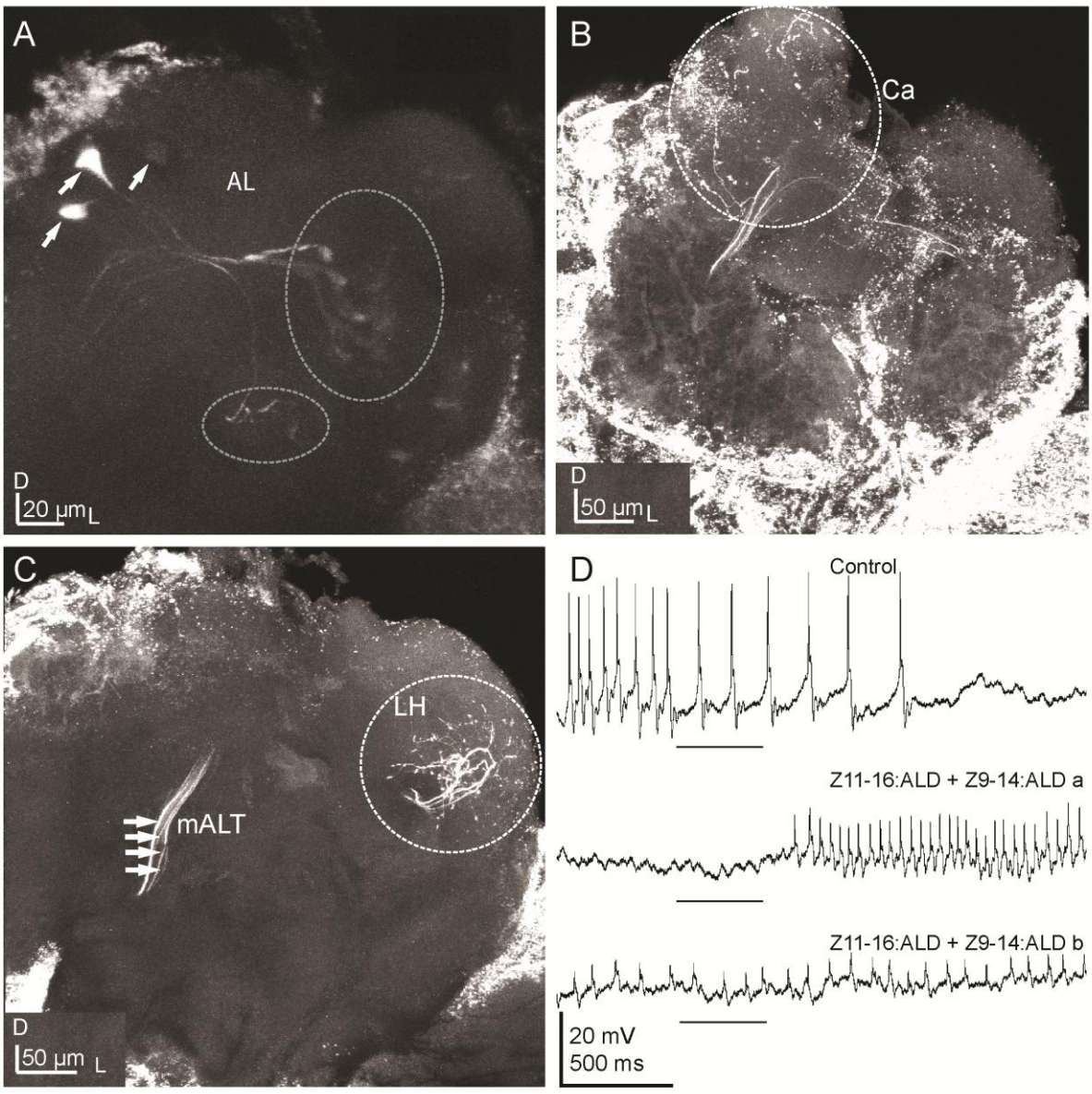

Figure 4: Confocal images of four simultaneously stained projection neurons (PNs) from preparation BIV. **A**: Confocal image of the antennal lobe (AL) (20x0.5) showinge three stained cell bodies located in the medial cell cluster (arrows) The neurons innervate glomeruli in the lateral part of the AL, and the visible arborizations are marked with gray dotted circles. **B:** The calyces (Ca) of the current preparation were slightly damaged making it difficult to identify the arborizations in this structure. **C:** The four arrows indicate the axons of the four stained PNs following the medial antennal lobe tract (mALT). The arborizations in the lateral horn (LH) are encircled. **D**: Physiological data obtained from the current preparation. The scale bar situated beneath the response profiles indicates the stimulation period (400 ms). Abbreviations not above: D: dorsal, L: lateral, P: posterior, mV: millivolt, ms: millisecond and µm: micrometer.

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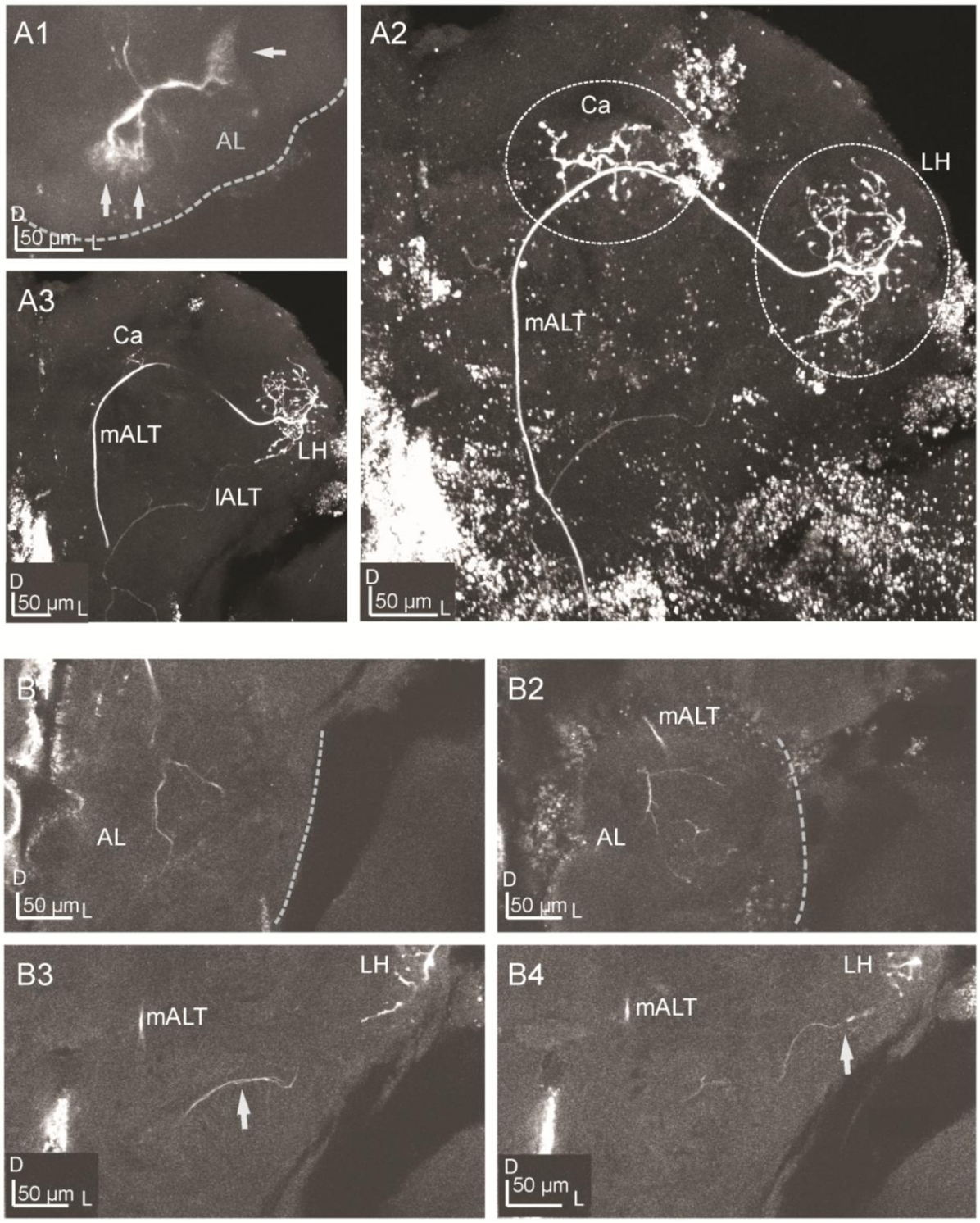

Figure 5: Confocal images of two simultaneously stained projection neurons (PNs) following different tracts, i.e. one in the medial antennal lobe tract (mALT) and one in the lateral ALT (lALT). **A1-A3:** Images of the mALT PN having dendritic branches in two or three glomeruli in the antennal lobe (AL), as indicated by arrows in A1. The neuron innervated a restricted region of the calyces (Ca) with thick processes before terminating within the lateral horn (LH). **B1-B4:** The lALT PN arborized loosely within several AL glomeruli and targeted a region of the LH that overlapped with that of the mALT PN. Abbreviations not mentioned above: D: dorsal, L: lateral and µm: micro meter.

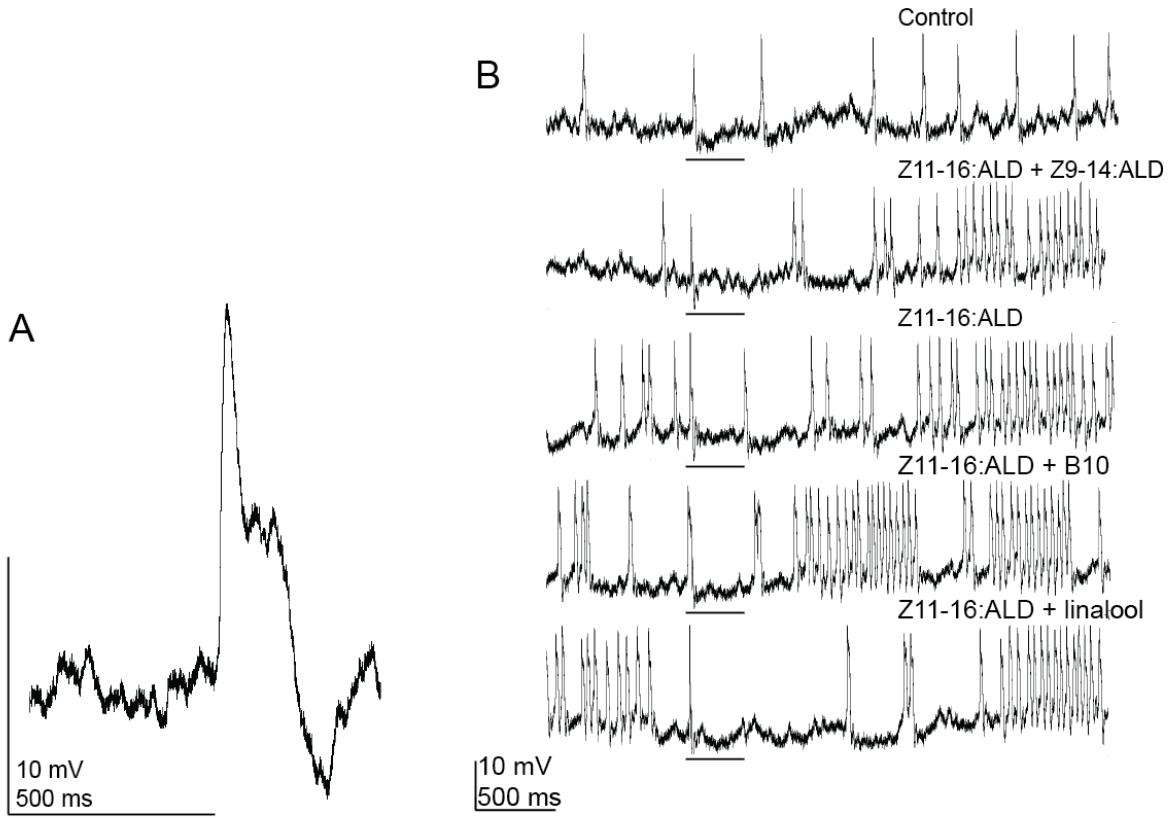

Figure 6: Physiological data from preparation BV, containing the two PNs presented in figure 5. **A:** The typical spike seen during registration. **B:** A complex response, including an inhibitory phase followed by an excitatory phase, to various odor stimuli, all including the primary pheromone component, Z11-16:AL, can be seen. The stimulation period is indicated by the black line placed under the recordings (400 ms). The response were only repeated in response to the mixture of the two pheromone components (Z11-16:ALD + Z9-14:ALD). Abbreviations: ms: millisecond and µm: micrometer.

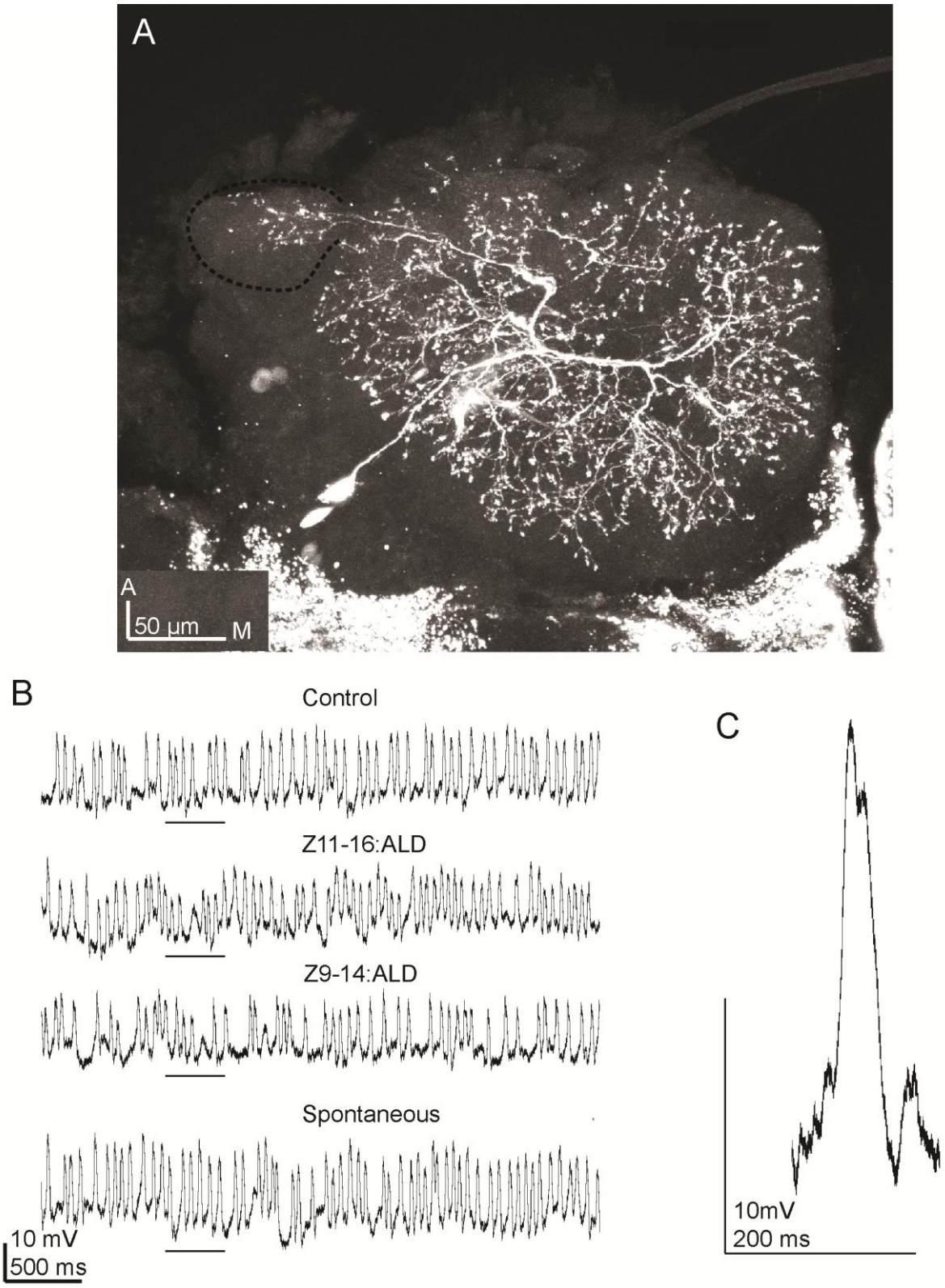

Figure 7: Morphology and physiology of two multiglomerular local interneurons (LNs) in the antennal lobe (AL). **A:** Confocal image (40x0.8) of the two LNs having cell bodies located in the lateral cell cluster. One of the LNs innervated a glomeruli in the area of the macroglomerular complex (MGC) (black dotted line) **B:** Spiking activity recorded from the current preparation when stimulating with the control, Z11-16:ALD and Z9-14:ALD respectively. The spontaneous activity is also shown. The scale bare located beneath the spike data mark the stimulation period (400 ms). **C:** example of a single spike. Abbreviations: ms: millisecond, mV: millivolt, A: anterior, M: medial

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Discussion

The main findings in this study include physiological and morphological characterization of two functional antennal-lobe PN types; one pheromone type and one plant odor type. Also, distinct PNs confined to two different tracts were identified, one type passing in the mALT and one in the lALT. Of particular interest is the morphology of one multi-glomerular PN confined to the mALT which has not previously been reported. Finally, in addition to the PNs, two multiglomerular LNs were stained in the current study.

Morphology

Target regions of PNs innervating the MGC and the ordinary glomeruli

There is little doubt about the functional and anatomical division of the AL into the MGC, which is involved in processing male-specific information, and the rest of the AL being responsible for processing plant odorant signals (Galizia et al., 2000). One of the specific goals of this thesis was to determine whether these two systems remain segregated also in higher olfactory centers. As demonstrated in figure 1E, 2D, 3D, 4C and 5A3, this seems to be the case. The termination region of the male-specific PN innervating the cumulus is obviously located more medially and anteriorly than that of PNs innervating the ordinary glomeruli. The different target regions in the LH are quite evident only by visual examination. Also, a similar projection pattern of axon terminals originating from PNs innervating the two different compartments of the AL was recently found by Zhao et al. (submitted article). They reconstructed PNs of the two functional types in *H. virescens* and *Helicoverpa assulta* and found, for both species, that the two categories of PNs targeted non-overlapping regions in the LH. Moreover, they also found a separated, but partly overlapping innervation pattern originating from the two male-specific PN types mediating information about pheromones and interspecific signals, respectively (Zhao et al., submitted article). No PNs innervating other MGC glomeruli than the cumulus was stained during the present study, and therefore the projection pattern of functionally different MGC PNs is not described here. However, several previous studies have reported similar findings concerning the separation of pheromone and plant odor signals in the LH (Fruit fly: Jefferis et al., 2007; Lin et al., 2007, Moth: Homberg et al., 1988; Kanzaki et al. 2003). Generally, terminal projections of PNs seem to be separated in the LH based on the behavior they elicit.

Also in the calyces, a separation of the two systems associated with pheromones and plant odors, respectively, has been reported (Homberg et al., 1988; Kanzaki et al., 2003; Namiki et al., 2013). In the current study, the calyces in the preparation possessing the male-specific PN was partly damaged and a comparison of the two functional types was therefore not feasible.

Morphological properties of PNs projecting in different tracts

In addition to the PNs passing in the mALT discussed above, this study also found one PN confined to the lALT. There were distinct morphological differences between the PNs projecting in these two tracts. Firstly, the PN in the lALT had a thinner axon than those in the mALT. Similar findings have also been found in previous studies (Homberg et al., 1988). Secondly, the axon of the lALT neuron sent off thin processes on its way to the LH, whereas the mALT PNs did not branch until they reached the calyces. Finally, the lALT neuron innervated several glomeruli loosely (figure 5 B1 and B2), quite different from all of the mALT PNs stained, which had dense ramifications within one or a few glomeruli (figure 2A, 3A and 5A1).

In spite of the morphological differences between neurons projecting in the two tracts, their termination area in the LH seemed to overlap. This was apparent as the lALT neuron was stained together with one PN passing in the mALT (figure 5A2 and 5B4). Overlapping terminals from PNs of the three main ALTs have previously been described in *H. virecens* (Løfaldli et al. 2012). The functional significance of the current projection pattern is not understood. However, the overlapping terminals might indicate that information from the two tracts is integrated in third order neurons in the LH.

Regarding the processes sent off by the lALT PN, they might be responsible for bringing input to the current neuron from other sensory modalities as suggested by Homberg et al. (1988). Generally, the final behavioral response of odor stimuli will depend both on the detection of the odor molecule, and on the external and the internal environment of the insect (Martin et al., 2011). This suggests that integration of different sensory modalities takes place at some level in the system. Most likely, the main part of this integration takes place in third order neurons, but as mentioned, there could be some extent of integration on the level of PNs. In this study, no other sensory modalities besides olfaction were tested.

Generally, the role of carrying odor information in parallel tracts is poorly understood. From studies in the honey bee, *Apis melifera*, it has been suggested that the mALT and the lALT

carry different qualities of the tested odorants. Müller et al. (2002) suggested that the neurons in the lALT were particularly involved in the temporal properties of the signal information whereas the neurons in the mALT were more important in providing information regarding the specificity of the stimuli. Comparing studies in *A. melifera* to what is found in the present study, however, may not be relevant because the arrangement of ALTs in the honey bee is different from that of moths. In the honeybee, the mALT and the lALT are equally prominent and all PNs in both tracts are uniglomerular. In addition, neurons in both tracts project to both the calyces and the LH, but in a reversed order (Galizia and Rössler, 2010).

Two morphological types of mALT PNs originating from ordinary glomeruli

All stained PNs projecting in the mALT found in the present study were uniglomerular, except for one. Thus, a unique neuron innervating two or three glomeruli in the AL was identified. This PN had quite distinct morphology as compared to that of the common uni-glomerular type. Its dendritic branches innervated the glomeruli densely, but without filling their entire core (figure 5A1). This was in marked contrast to the stained uniglomerular PNs, which clearly filled the whole glomerulus (figure 2A and 3A). Also in the higher protocerebral centers, the morphology of the multiglomerular neuron differed from that of the uniglomerular. While the latter had fine ramifications throughout the calyces (figure 2C and 3C), the multiglomerular PN had few, thick branches in the current structure (figure 5A3). Furthermore, in the LH, the uniglomerular PNs were found to have thin arborizations without a very elaborate branching pattern (figure 3D and 4C), whereas the multiglomerular PN had thicker branches and a more defined and elaborate arborization pattern (figure 5A3)

Multiglomerular PNs following the mALT have previously been described in both *B. mori* (Namiki and Kanzaki, 2011) and *H. virescens* (Løfaldli et al., 2010; Rø et al., 2007). In all of these studies, the multiglomerular neurons innervate the glomeruli densely, as found in the present study. However, the branching in the calyces seems to differ between multiglomerular neurons. Løfaldli et al. (2010) stained and reconstructed a multiglomerular PN in the mALT which sparsely innervated the calyces with fine ramifications. Similary, Rø et al. (2007) described a neuron with ramifications in the calyces. This neuron innervated the medial calyces on its projection towards the LH, but in addition it sent a branch to the lateral calyces after entering the LH. Rø et al. (2007) also reported findings of a multiglomerular PN in the mALT that did not enter the calyces at all. Thus, the branching in the calyces found in the current study does not resemble any of the previous findings in *H. virescens*. Also, the thick

branches and elaborate projection pattern in the LH did not resemble the previously described ramification in the LH.

Based on the fact that some multiglomerular PNs in all three main tracts in the moth brain seem to bypass the calyces, it has been suggested that these neurons do not contribute to associative learning, but instead is more involved in innate behavior (Rø et al., 2007). However, as described above, the multiglomerular neuron in the mALT both in this and other studies, have ramification in the calyces, indicating that at least some multiclomerular PNs provide information important for learning and memory.

Morphology of the LNs

Two multiglomerular LNs, simultaneously stained in the same preparation, were identified in the current study. Interestingly, one of them seemed to innervate both ordinary glomeruli and parts of the MGC while the other only innervated ordinary glomeruli (figure 7A). Whereas similar LNs have been found in the silk moth, *B. mori* (Seki and Kanzaki, 2008), this kind of data has previously not been reported in any heliothine species. This arrangement indicates that the separation of the AL into the ordinary and male-specific glomeruli, is not absolute.

Physiology

Physiology of the PN innervating the cumulus of the MGC

The input to the cumulus originates from OSNs being sensitive to the main pheromone component in *H. virescens*, Z11-16:ALD (Hansson et al., 1995; Berg et al., 1998). Thus, it would be reasonable to assume that PNs with dendrites in this glomerulus would respond excitatory to stimulation with the current substance. However, this was not the case. During the registration period, no excitatory responses were measured, neither when stimulating with Z11-16:ALD alone, nor when stimulating with the mix of the two pheromone components. However, the current neuron displayed a repeated inhibitory response to the two interspecific signals; Z11-16:AC and Z11-16:OH. This inhibitory response is logical, taken into consideration the role of the cumulus in sexual behavior. Unless the current neuron received inhibitory information from the OSNs innervating the cumulus, which is highly unlikely, the inhibition was probably mediated via GABAergic LNs. Multiglomerular LNs, as the ones found in the present study, innervate multiple, if not all glomeruli, often including glomeruli in the MGC (Seki and Kanzaki, 2008).

It is difficult to imagine that a neuron innervating the entire cumulus does not have any synapses with OSNs conveying information about the major pheromone component. Possible explanations could be that the OSNs were damaged or that the major pheromone substance used for stimulation was no longer potent. However, two separate stimuli cartages containing the major pheromone component failed to give any response. Concerning the possibility of damaged OSNs, this explanation seems unlikely as responses to particular substances actually *were* recorded.

Combinatorial responses of uniglomerular MGC PNs have previously been reported in heliothine moths, including both synergy and inhibition (Berg et al., 1998; Christensen et al., 1995; Vickers et al., 1998). Inhibition by other pheromones, or by interspecific substances in PNs innervating the glomeruli receiving information about the major pheromone, has been reported before both for *H. virescens* and *B. mori* (Christensen et al., 1995; Kanzaki et al., 2003). Uniglomerular PNs in the MGC showing only inhibition have previously been reported in M. sexta (Christensen and Hildebrand, 1987), however, the neuron described in their study on does not morphologically resemble the neuron found in the present study. Also, the activity change was in response to mechanical stimulation of the antennal nerve, and not odor stimuli. However, the result by Christensen and Hildebrand (1987) and the findings in the present study indicate the possibility of a population of PNs innervating the MGC that only respond by inhibition.

Physiology of PNs innervating ordinary glomeruli

Relatively few of the PNs innervating ordinary glomeruli showed any clear responses (table 4, appendix I). One recording originating from preparation BV, which contained two stained neurons (one multiglomerular PN projecting in the mALT and the other in the lALT) was unique as compared to the pure excitatory or inhibitory responses usually reported in AL PNs. As shown in figure 6, various stimuli including mixtures of pheromones and plant odors, induced an inhibitory response followed by an excitatory phase. Similar responses were observed when stimulating with single pheromones and plant odors. However, repeated stimulations induced a response to the pheromone mixture only. Because two neurons were stained, it is not possible to determine which of the neurons the complex response occurred from. Though, it can be concluded that it originated from a multiglomerular PN. Interestingly, Namiki and Kanzaki (2011) have also reported multiglomerular PNs showing complex responses of similar type.

In addition to the unusual response pattern in the current recording, the long duration of the spikes as well as their curve path during the repolarizing phase was also quite special. Whether the special curve path is a true characteristic of the current neuron or an artifact caused by the electrical setup is, however, an open question.

Study remarks and future aspects

Because of the small size of the preparation it was generally difficult to maintain a stable contact with the neuron during the intracellular recordings. This means that a limited amount of time was available for testing the stimuli, and therefore, not all neurons were tested with all odors.

To verify the different target regions of functionally different PNs in the LH, including the male-specific PN and the PNs associated with ordinary glomeruli, the individual neurons should be reconstructed and integrated in the standard brain atlas of the *H. virescens* male. The same should be done to verify the overlapping terminals from multiglomerular PNs in medial and the lateral ALT. Also, it would be interesting to further investigate the terminations of the uni- and multiglomerular PNs in the mALT by means of reconstruction.

Because of the lack of comparable physiological data obtained, no statistical tests were carried out in the present study. However, if statistics provides a meaningful tool to investigate the properties of the different PNs, this should be utilized.

Conclusion

Based on the findings in the present study, the following conclusions were made:

1. The PNs, originating in the MGC and the ordinary glomeruli, respectively, target different regions in the LH.
2. The response pattern of the male-specific PN indicates that the individual MGC glomeruli are connected via LNs.
3. A morphologically new type of multiglomerular PN confined to the mALT was identified in the current study.
4. Multiglomerular PNs passing in the medial and lateral ALTs may carry information that is integrated in third order neurons since they target seemingly overlapping areas in the LH.
5. Some LNs seem to innervate both the ordinary glomeruli.

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Abbreviations

AL	antennal lobe
GABA	Gamma amino butyric acid
mALT	medial antennal lobe tract
MGC	macroglomerular complex
mlALT	medio lateral ALT
lALT	lateral antennal lobe tract
LH	lateral horn
LN	local interneuron
OBP	olfactory binding protein
OSN	olfactory sensory neuron
OR	olfactory receptor
PN	projection neuron

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Appendix I

The results of all the recordings are found in table 4 under. In general, few responses were found. The MGC PN in BI was inhibited by the two interspecific substances. None of the uniglomerular PNs innervating ordinary showed any clear responses; BII responded inhibitory to a number of stimuli, however, the date from this preparation is unreliable. BIII and BIV did not respond to any of the tested odorants. One of the two multiglomerular neurons in preparation BV responded in a complex manner to all tested odorants except for ylang ylang. The complex response was also observed to some degree when stimulating with the control, but the response was more prominent when stimulating with odor substances. The response pattern was not repeated except in the case of the mixture of the two pheromone components. One of the two stained local interneurons in BVI did not respond to any of the tested stimuli except for a small reduction in spike amplitude when testing with the two single pheromone components.

Table 4: Responses to different stimuli in all the preparations. The responses were classified as no response (0), inhibitory (-), excitatory (+) or complex (C). Not all the stimuli were tested in all the preparations, not tested (nt).

Preparation nr	**BI**	**BII**	**BIII**	**BIV**	**BV**	**BVI**
Control	0	-	-	0	C	0
Z11-16:ALD + Z9-14:ALD	0	0	0	0	C	0
Z11-16:ALD	0	0	0	0	C	0/-
Z9-14:ALD	0	-	0	0	C	0/-
Z11-16:OH	-	-	0	0	C	0
Z11-16:AC	-	-	0	0	C	0
Z11-16:ALD + B10	0	-	0	nt	C	0
Z11-16:ALD + Linalool	0	-	0	0	C	0
B10	nt	0	0	nt	C	0
Linalool	nt	-	0	nt	C	0
Geraniol germacrene d	nt	nt	0	nt	C	nt
Farnesene	nt	nt	0	nt	C	nt
2-phenylethanol	nt	nt	0	nt	C	nt
3Z-hexenyl acetate	nt	nt	0	nt	C	nt
Ocimene	nt	nt	0	nt	C	nt
Ylang-ylang	nt	nt	0	nt	0	nt

Appendix II

Response from neuron in preparation BII is found in figure 8 below

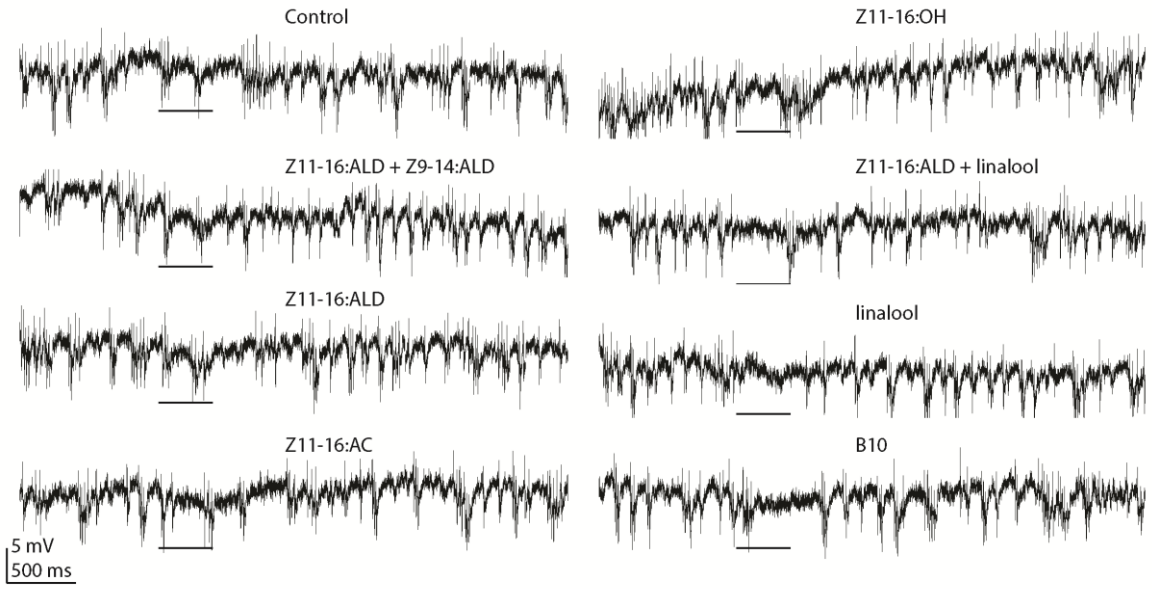

Figure 8: the response profile from preparation BII. Most of the stimuli gave an inhibition, however, the quality of the response is not reliable because there seems to be an unstable connection with the neuron. This made it difficult to predict what spikes should be considered part of the response and not.

Appendix III

In preparation BIII, a neuron confined to the protocerebrum was stained. It had projections in close proximity of the AL and calyces, and seemed to project contralateral.

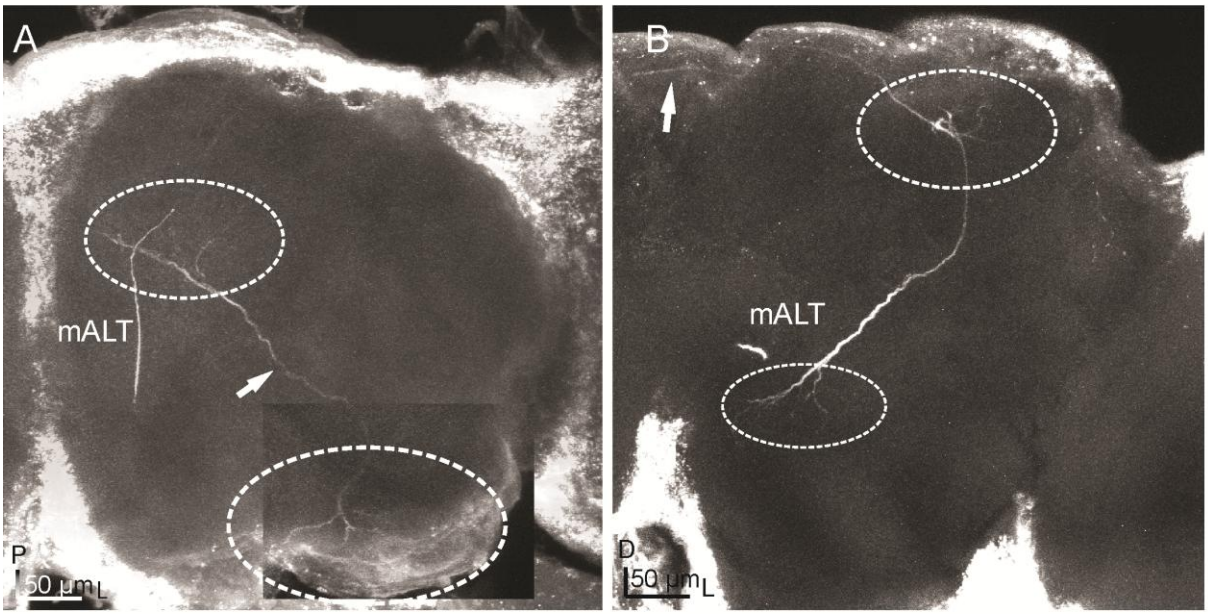

Figure 9: the neuron from preparation BIII confined to the protocerebrum. The two circles mark the arborization areas of the neuron in both A and B. The medial antennal libe tract (mALT) neuron is also marked in both images. **A:** Dorsal view confocal image of the neuron. **B:** frontal view of the neuron. The arrow points towards the projection to the contralateral side of the brain.