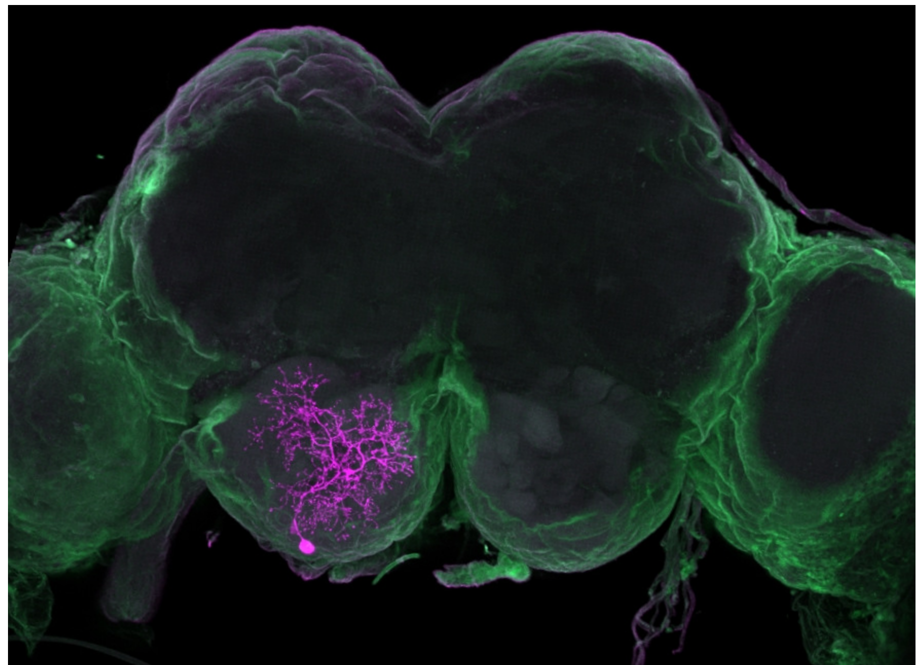
Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Social and Educational Sciences
Department of Psychology

Sara Holm

Electrophysiological properties of local interneurons in the primary olfactory center of the moth brain

Master's thesis in Psychology
Supervisor: Xi Chu
Co-supervisor: Jonas Hansen Kymre and Bente Gunnveig Berg
October 2021



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Preface and Acknowledgments

The work presented in this master thesis, both experimental and writing wise, has been conducted in the Chemosensory lab at the Department of Psychology (Norwegian University of Science and Technology; NTNU). In receiving the opportunity to carry out my master thesis in this lab, I finally got to study and gain insight into a scientific area which I have found intriguing for quite some time. This experience as well as the people working here has helped me gain knowledge surrounding both the general functions as well methodological practices required to conduct studies on the nervous system, for which I am extremely grateful. First and foremost, I would like to thank my main supervisor, Xi Chu, who has given me so much of her time. She has taught me how to perform, not only intracellular recording, but also calcium imaging, the procedure of dissection, confocal imaging as well as how to analyse and interpret electrophysiological data. With her immense amount of knowledge and ability to forward information in a suitable manner, she has provided guidance and support, of which I would call unequalled. Always with a positive and energetic presence. Apart from this, I have also greatly appreciated the information and support provided by my co-supervisor, Jonas Hansen Kymre, who gathered half of the data used in the statistical analyses.

Furthermore, my sincerest thanks goes out to my second co-supervisor, and leader of the chemosensory lab, Bente Gunnveig Berg. She has provided feedback and experience which has been very helpful. The environment she has created for developing and acquiring skills as well as understanding the nervous system, is unique. To Elena Ian, Pramod KC, and my fellow master students at the lab, Marte Schjetne and Line Aune, thank you for all the interesting and enjoyable conversations. And finally, my husband, Stian Thuen, who has always supported me in this journey, even though it has not been easy. You have had to put a lot of effort into making your work situation fit with this experience, moving from Oslo to Trondheim, all while providing a loving and safe environment for our son, Tørres. You have given me a chance to immerse myself in this study, without worry or guilt, which has made all the difference. It should also be mentioned that our son deserves some credit to, as he has been the most understanding and accepting little boy. Thank you for being the light of my life, giving me cause to continue in my academic pursuit, and reminding me that everything works out in the end.

Sara Holm

Abstract

Within the field of neuroscience, one of the ultimate goals is to obtain an understanding of the neural circuit, in particular general principles underlying local processing of signals. This type of comprehension, however, cannot be achieved without examining and identifying the fundamental mechanisms of such networks, preferably at the level of the single neuronal component. Because the neural circuits of humans usually appear immensely intricate, investigating and obtaining knowledge of this sort, has proven to be technically challenging. Therefore, it is ideal to utilizing modal organisms which possess comparable neural systems with that of mammals as well as being accessible for experimental studies. Insects, for example, have been favourable objects for exploring the olfactory system due to their advanced sense of smell and relatively simple brain. Furthermore, to understanding the processing of local signals, local interneurons (LNs) in the antennal lobe of male moths appear to be optimal experimental subjects, with emerging evidence showing that also in the moths, such neurons have diverse intrinsic signatures as well as various synaptic wiring patterns. Thus, we investigated the electrophysiological properties of morphologically characterized antennal lobe LNs. The comprehensive amount of precise data was obtained through a series of advanced approaches, combining sharp electrode intracellular recordings, iontophoretic staining and confocal microscopy. In half of the identified LNs, we found a distinct waveform of the spike which included a characteristic afterpotential, known as a depolarized afterpotential (DAP). This encouraged us to examine whether these untypical afterpotentials could reflect any difference in the spontaneous activity as well as response pattern of the neurons. Our results provided evidence that the waveform of the action potential might influence the intrinsic firing properties of these neurons. However, the impact of the waveform on the odour-evoked response seemed to be restricted.

Sammendrag

Et av de mest avgjørende målene innen nevrovitenskap er å oppnå en forståelse av nevrale kretser, spesielt de generelle prinsippene som ligger til grunn for hvordan signaler blir behandlet lokalt. Denne typen forståelse kan imidlertid ikke oppnås uten at de grunnleggende mekanismene til slike nevrale nettverk undersøkes og identifiseres, fortrinnsvis på nivået til den enkelte nevrale komponenten. Fordi menneskets nevrale kretser vanligvis fremstår som ekstremt intrikate, har det vist seg å være teknisk utfordrende å undersøke samt oppdrive kunnskap av denne typen. Av den grunn er det ideelt å benytte modale organismer som har sammenliknbare nevrale systemer som pattedyr, og som er tilgjengelige for eksperimentelle studier. Insekter har vist seg som gunstige objekter for å utforske luktsystemet på grunn av deres avanserte luktesans og relativt enkle hjerne. Videre, for å forstå behandlingen av lokale signaler, ser lokale interneuroner (LN) i antenneloben til hannmøll, ut til å være gunstige eksperimentelle emner, ettersom nye bevis peker mot at slike nevroner også i møll, har forskjellige iboende signaturer samt synaptiske forgreninger. Av den grunn har vi undersøkt de elektrofysiologiske egenskapene til morfologisk karakteriserte antennelobe-LN-er. Den omfattende mengden av presis data ble innhentet gjennom en rekke avanserte tilnærminger, som kombinerte skarp-elektrode intracellulære opptak, iontoporetisk farging og konfokal mikroskopi. I halvparten av de identifiserte LN-ene, ble en tydelig bølgeform av aksjonspotensialet funnet, hvilket inkluderte et karakteristisk etterpotensial, kjent som et depolarisert etterpotensial (DAP). Dette oppmuntret oss til å undersøke om slike utypiske etterpotensialer kan gjenspeile noen forskjell i den spontane aktiviteten så vel som responsmønsteret til nevronene. Resultatene våre ga inntrykk av at bølgeformen til aksjonspotensialet kan påvirke de iboende egenskapene disse nevronene har til å fyre. Imidlertid syntes bølgeformens innvirkning på luktfremkallende respons å være begrenset.

Abbreviations

AL	Antennal lobe
ALT	Antennal lobe tract
AVLP	Anterior ventro-lateral protocerebrum
Ca	Calyces of the mushroom bodies
CAN channels	Calcium activated non-selective cation channels
Cu	Cumulus
DAP	Depolarized afterpotential
dma	Dorsomedial anterior
dmp	Dorsomedial posterior unit
fMRI	Functional magnetic resonance imaging
GABA	γ-aminobutyric acid
GPCR	G-protein coupled receptor superfamily
HAP	Hyperpolarized afterpotential
ISI	Interspike interval
lALT	Lateral antennal lobe tract
LH	Lateral horn
LN	Local interneuron
LPOG	Labial-palp pit organ glomerulus
mALT	Medial antennal lobe tract
MB	Mushroom bodies
MGC	Macroglomerular complex
mlALT	Medio-lateral antennal lobe tract
OB	Olfactory bulb
OG	Ordinary glomeruli
OL	Optic lobe
OR	Olfactory receptors
OSN	Olfactory sensory neuron
PCx	Posterior complex
PN	Projection neuron
SIP	Superior intermediate protocerebrum
SLP	Superior lateral protocerebrum
SOG	Subesophageal ganglion
T3	Metathoracic ganglion

VPG	Ventroposterior glomeruli
VLP	Ventrolateral protocerebrum

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1. Introduction

As one of the oldest sensory faculties (Hoover, 2010), olfaction has evolved thus that different species may survive in their complex natural surroundings, by detecting and processing useful information from the chemical signals in the environment. The sense of smell thereby allows organisms to respond efficiently to food odours and conspecific as well as heterospecific signal compounds, enhancing the chance for both individual survival and population reproduction. Though such a description generally expresses the significant relevance chemo-sensation holds within the behaviour and neural functioning of non-human animals, a comparative regard can be transferred to humans as well.

The ways in which odour perception influence human experiences are miscellaneous, one classic instance is the important impact olfaction has on higher cognitive capacities such as memory and associative learning (Arshamian et al., 2013; Cahill et al., 1995; Haberly & Bower, 1989; Herz, 2004, 2016; Herz & Cupchik, 1995; Herz & Engen, 1996; Rochefort et al., 2002; Willander & Larsson, 2007; Wilson et al., 2004), which is so aptly illustrated by Marcel Proust`s anecdote about the madeleine cake (Proust & Scott-Moncrieff, 2002, p. 37). Olfaction has also been reported to influence social behaviours, such as supporting the initial bonding process between new-born infants and their mothers (Makin & Porter, 1989; Porter & Winberg, 1999; Russell, 1976; Soussignan et al., 1997; Sullivan & Toubas, 1998; Varendi & Porter, 2001), and aspects connected to mental health, e.g. the association between olfactory dysfunction/apathy and depression (Cramer et al., 2010; Smeets et al., 2009). In addition, olfaction effects appetite, food consumption and digestive processes which are fundamental in the act of selecting, identifying and rejecting foods (Stevenson, 2010). In light of the Covid-19 pandemic, the generally underestimated role of human olfaction can be said to have attracted new interest, as alterations in olfaction (or anosmia) is one of the most reported symptoms (Cetinkaya, 2020; Chiesa-Estomba et al., 2020; Gelardi et al., 2020; Hopkins et al., 2020; Lechien et al., 2020; Mastrangelo et al., 2021; Yan et al., 2020). Thus, understanding how chemical signals are processed by the neural circuit has indeed become a very timely issue.

Generally, encoding of sensory input is thought to be involved in computations of multiple neuronal components working together to dynamically transform peripheral input through connective synapses. However this is an area of scientific study that still holds a great deal of elusiveness in the empirical sense, also when it comes to olfaction (Ng et al., 2002). Therefore, increased knowledge about the neural principles of such a system and how it operates at a local level is required. This includes the neuronal specificities at different

synaptic levels as well as the wiring principles between different neurons. To this end, it seems prudent to explore such issues through the local interneuron (LNs) in the primary olfactory centre, as information about this neuron is still quite vague, compared to the other principle neurons within this circuit, the olfactory sensory neurons (OSNs) and projection neurons (PNs).

1.1 Similarities in olfactory processing in vertebrates and invertebrates

In studying signal detection, many organisms are found to use olfaction as one of the key senses to navigate within the environment, in order to find food or a mate. Such a navigation strategy includes adapting amidst diverse environments, managing salient and irrelevant stimuli accordingly, and arranging it thus that corresponding memories can be developed, evaluating the valency of an odour based on the conditional motivational state, as well as taking the noise ratio into account (Galizia, 2014). Interestingly, neural investigations of the sensory systems, have found the olfactory apparatus in particular to have retained a striking conservation during evolution i.e., the olfactory systems remains similar across a wide array of taxa. Thus, despite the phylogenetic distance, this means that vertebrates and invertebrates, share organisational features in respect to this system, which include the arrangement of sensory neurons in the periphery and the establishment of parallel and hierarchical processing along the central pathways (reviewed by Ache & Young, 2005; Hildebrand & Shepherd, 1997; Krieger & Breer, 1999; Lledo et al., 2005).

Figure 1 presents a schematic representation of the mouse and moth olfactory system, comprising three levels, with the peripheral level including OSNs targeting the brain via the primary olfactory centre, the antennal lobe (AL) in moths, and the olfactory bulb (OB) in mice. The structure of these neurons is uniform in both organisms at the peripheral level, in that they are small bipolar cells with a dendrite containing the receptor proteins and an unmyelinated axon conveying nerve impulses directly to the primary olfactory centre (Ache & Young, 2005; Hildebrand & Shepherd, 1997). In the AL and the OB, a striking similarity in the synaptic organization is apparent, as sensory terminals make contact with second-order neurons in characteristic spherical structures termed glomeruli (Shepherd, 1974). Moreover, LNs in both organisms have their dendrites/neurites spatially confined within the primary olfactory centre and process the incoming input before the signals are conveyed to the higher order brain centres via PNs. The prominent parallels also apply to the subsequent level of the olfactory pathway, which including the second order centres. At this level, the lateral protocerebrum in moths is a brain region that shares many anatomical and organizational

principles with the mammalian amygdala (Miyamichi et al., 2011; Sosulski et al., 2011), whereas the mushroom body calyx of the moth is analogue with the mammalian piriform cortex (Su et al., 2009), brain regions which are located only “one synapse” away from the external world in both systems. In addition, the strong ability of mammals as well as insects to establish odour memory, is linked to this distinct level of the olfactory pathway. Furthermore, the assembly of parallel tracts connecting the AL with the second olfactory centres in moths (Homberg et al., 1988) matches the mammalian olfactory tract targeting the cortical regions in the temporal lobe (Lledo et al., 2005). However, the similarities of the olfactory system in vertebrates and invertebrates apply not only to this kind of bottom-up processing, but also to top-down inputs from higher-order structures. This is made possible by the centrifugal neurons (CNs), found in both systems, which are hypothesized to shape the sensory representations, in mice for example, by involving internal states of the organism like hunger or arousal (Cansler et al., 2019), a function which is also expected to typify distinct CNs in moths (Zhao et al., 2013).

Other similarities applies to the local olfactory computation of the primary olfactory centre. Here, the synaptic density of each glomerulus is quite high, as peripheral input from OSNs converges onto LNs and PNs. In the OB, periglomerular cells together with granule cells (local interneuron in mammals) mediate lateral inhibition (Aungst et al., 2003; Shmuel et al., 2019), a function that is thought to be the same in the axon-less LNs in the AL. Moreover, olfactory computation in both vertebrates and invertebrates is organized in local networks where the synapses between LNs and PNs form microcircuit that integrates feed-forward and recurrent connections, thereby constitutes the functional subunits of the global network (Meyer et al., 2013).

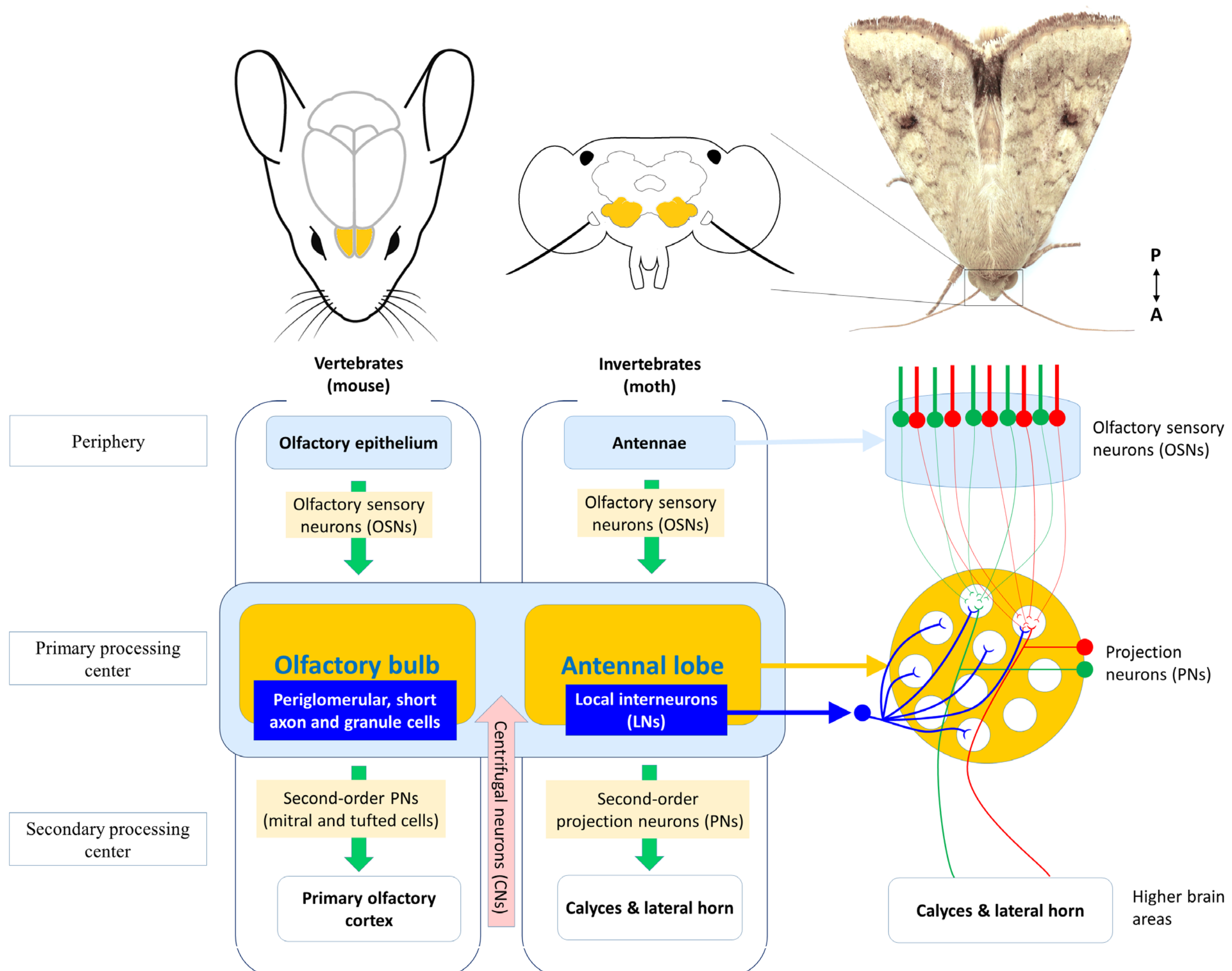


Figure 1. Schematic comparison of the olfactory systems of vertebrates (e.g., mouse) and invertebrates (e.g. moth). Both organisms detect odours through olfactory sensory neurons (OSNs) and transfer the odour input to the primary olfactory centre, where the signals are conveyed further on to higher brain areas via projection neurons (PNs) while the local interneurons (LNs) form local neuronal circuits. The feedback modulation from higher brain areas to the primary processing centres can be relayed by centrifugal neurons (CNs).

1.1.2 Insects as model for research on the functioning of local interneurons

Considering that the neuronal connectivity and architecture of insects, though in itself a complex constitution, appear to be more simplistic than that of mammals and more accessible in a neurobiological perspective, as well as the commonalities of the neuronal composition, described in the section above, insect models provide a good opportunity to increase the current comprehension of the morphological and functional properties of LNs.

Apart from this it is also important to address the relevant techniques which is often applied in studying the mammalian nervous system. One of the most common methods is using functional magnetic resonance imaging (fMRI), which has been widely used to reveal higher-order sensory processing. Yet, this approach is not possible to use for the study of LNs in the mammalian OB, as it is located on the inferior (bottom) side of the human central

hemispheres, and on the frontal part of the rodent brain. Unfortunately, this thereby makes the primary olfactory centre of the human brain inaccessible when using this method (Fournel et al., 2020), while studies of the rodent OB focus mainly on mapping at the level of glomerular resolution (e.g. Kida et al., 2002; Muir et al., 2019). Thus, to better understand the neural basis of olfaction, it is better to use an organism that shares neuro architectural similarities with humans, while the brain, at the same time, is easy to access for experimental investigations. The method of *in vivo* intracellular recording in the brain of intact insects, fits this research purpose well. Here, the neuron can be recorded while the neuronal connections are still complete, thereby being comparable to its "natural" state. Therefore, the validity of using such a method in studies focused on specific neuronal types, such as LNs, is quite high.

Furthermore, unlike the mammalian olfactory epithelium embedded deeply in the nasal cavity, the insect's organ for olfactory detection, the antennae, are a pair of protruding appendages on the head, that can be simply accessed for testing relevant odour stimulations. The antennae exhibit a discriminant sensitivity in relation to chemosensory inputs, whether it be odorants produced by plants, or insect produced chemicals that convey messages, also known as pheromones and intraspecific signals (Hansson, 1995; Karlson & Luscher, 1959).

1.2 Processing of olfactory information

In Lepidoptera (Noctuidae), recognition and processing of relevant chemical signals from the environment is quite interesting. Not only are their sensory neurons tuned to specific key components of plant odours, with the molecular receptive range seeming to display minimal overlap (Røstelien et al., 2005; Marit Stranden et al., 2003), they also possess an astounding ability to track minuscule amounts of pheromones, diluted with complex blends of other chemical components (Leal, 2005). In *Helicoverpa armigera*, the model organism of this thesis, pheromone compounds have also been found to have a double function, communicating information underlying attraction and sexual behaviour within species, to help males in locating conspecific females, as well as conveying heterospecific information to prevent attraction across species (reviewed by Berg et al., 2014; Mustaparta, 1996).

1.2.1 Detection and integration of olfactory cues at the peripheral level

The onset of signal detection is often defined by the contact between volatile odorants and fine-tuned neurons on the insect's olfactory organ, the antennae. Protruding from the cuticle of said antennas exoskeleton, are specialised hair-like structures called sensilla, and it is through pores in these structures that the odorants enter the olfactory organ. The objective

of this event being the subsequent interaction between the odorant molecules and the bipolar OSNs housed within. First however, the hydrophobic odorants finding themselves inside the aqueous enclosure of the sensillum lymph, bind to specialized odour-proteins. Importantly, the role of these odour-binding proteins is still elusive, though it is thought that they facilitate solubilization of the odorant molecules and transfer them to binding sites in the dendritic membrane of the OSN, where olfactory receptors (OR) are situated (reviewed by Hansson & Stensmyr, 2011; Sun et al., 2018). Generally, each of the OSNs are thought to expresses one type of receptor (Vosshall, 2000; Vosshall et al., 2000). Different insect species possess varying amounts of the OR genes. There are 62 in *Drosophila melanogaster* (fruit fly), 170 in *Apis melifera* (honeybee), and 66 in *Bombyx mori* (silk moth) (Touhara & Vosshall, 2009). Initially the insect ORs were thought to be a part of the G-protein coupled receptor superfamily (GPCRs) of vertebrates. However, it is now believed that they have adopted a different topology than the GPCRs, seemingly belonging to another transmembrane family, evolutionarily unrelated and expressed exclusively in insects (reviewed by Hansson & Stensmyr, 2011; Sato et al., 2008). In heliothine moths, the recognition of odorant signals by the sensory cells has been found to be more specialized than that of vertebrates and other insects (reviewed by Hansson & Stensmyr, 2011; Nara et al., 2011), in that the subsets of OSNs are seemingly tuned to one key plant odorant or sex pheromone. Though odours with similar molecular structures may also evoke a response, this is generally weaker (reviewed by Berg et al., 2014; Røstelien et al., 2000; Røstelien et al., 2005; M Stranden, I Liblikas, et al., 2003; M Stranden, T Røstelien, et al., 2003).

After the binding of odorants to the OSN receptors, a second messenger pathway is activated, where the chemical signal is transduced into graded electrical potentials within the OSN dendrite. A graded potential by itself is not always sufficient to activate an OSN, however, if it does, it may give rise to action potentials, traveling through the antennal nerve, directly into the AL, by way of the unmyelinated axon of the OSN (Hildebrand, 1996).

1.2.2 Glomerular assemblies in the antennal lobe and the three main antennal lobe neuron types

The primary centre for olfactory processing, the AL is located anteriorly in the insect brain (Berg et al., 2002; Hansson & Anton, 2000; reviewed by Homberg et al., 1989). This is a structure made up of three categories of central interneurons forming spherical neuropils called glomeruli, functioning as sites of convergence. These neuropils also include the afferent axon terminals of the OSNs (Anton & Homberg, 1999; Rø et al., 2007). Studies

on *D. melanogaster* have shown that all OSNs expressing the same type of receptor protein, converge onto one or two glomeruli in the AL (Su et al., 2009; Vosshall et al., 2000; Wilson & Mainen, 2006). It appears, therefore that insects have a specific representation of odours within definite glomeruli (Couto et al., 2005).

The number of glomeruli within distinct species differ, with the ALs of the *H. armigera* thought to encompass around 80 glomeruli each (Zhao, Ma, et al., 2016). Studies have also shown adaptions in the anatomical organisation of glomeruli in different insects' species (reviewed by Hansson & Stensmyr, 2011; Ignell et al., 2001; reviewed by Schachtner et al., 2005; Trona et al., 2010; Zube & Rössler, 2008), though within a species, the function and anatomical organisation appears to follow a specific standard. The AL of male moth for example, possesses a sexual dimorphism in the glomerular structures, as one of the glomerular complexes is found to exclusively encode information from OSNs sensitive to sex pheromone. This group of glomeruli is known as the macroglomerular complex (MGC), which in *H. armigera* constitutes three units, the largest one being the cumulus, while the two remaining ones are known as the dorsomedial anterior (dma) and the dorsomedial posterior (dmp) (reviewed by Berg et al., 2014; Christensen & Hildebrand, 1987; Hansson & Anton, 2000; Rospars & Hildebrand, 1992; Rospars, 1988; Wu et al., 2015). The remaining glomeruli within the AL of this species are sexually isomorphic, meaning that they are also present in female moths. The larger number encodes plant volatiles and are called ordinary glomeruli (OGs) (Berg et al., 2002; Christensen & Hildebrand, 2002; Galizia et al., 2000; Hansson & Anton, 2000; Hildebrand & Shepherd, 1997; reviewed by Homberg et al., 1989; Zhao et al., 2014). There is also the assembly of glomeruli called the posterior complex (PCx), its function however is not yet known. There is the labial palp-pit organ glomerulus (LPOG); which has been shown to be CO_2-responsive (reviewed by Guerenstein & Hildebrand, 2008; Zhao, Chen, et al., 2016), and finally the ventroposterior glomeruli (VPGs), an assembly of glomeruli mentioned in *H. armigera* (Kymre et al., 2020). This glomerular complex is located posteriorly to the LPOG and ventral to the AL hub. In other insects, findings have indicated a specificity in detection and processing of hygro- and thermo-sensation within glomeruli located in a corresponding position (Enjin et al., 2016; Frank et al., 2015; Kymre et al., 2020; Nishino et al., 2009; Nishino et al., 2003). Until recently however, some of these glomeruli were not thought to belong to the moths AL, resulting in knowledge abouts its functioning attributes being scarce (Skiri et al., 2005; Zhao, Chen, et al., 2016). Together, all of these glomerular assemblies form distinct sub-systems within the AL (Zhao, Chen, et al., 2016), though it is the synaptic interaction between the

neurons inhabiting these glomerular structures as well as the AL in general, that contribute to the occurrence of information processing within the central nervous system of insects. Generally, the OSN, the PNs, and the LNs make up an intrinsic network, where feedforward and feedback signalling are promoted through transmission within and between the glomerular structures. The chemical signal is transferred from the OSN through cholinergic, excitatory synapses to the principal output neurons of the AL, namely the PNs. As their name indicate, they function to forward the information onto higher brain areas (Wilson & Mainen, 2006). They arborize either uni- or multiglomerularly in the AL and primarily project to the lateral horn (LH) and the calyses of the mushroom bodies (Homberg et al., 1988; Ian, Zhao, et al., 2016). Before this, however, the LNs modulate the olfactory information as an intermediate, affecting both the presynaptic and postsynaptic processing of the chemical signal within the AL. Many LNs innervate most AL glomeruli (Anton & Homberg, 1999; Ng et al., 2002; Sachse & Galizia, 2002; Wilson & Mainen, 2006). In moths, the afferent projections of the PNs, unite via six parallel antennal-lobe tracts (ALTs) in each brain hemisphere. These tracts have been termed the medial, mediolateral, lateral, transverse, dorsomedial, and dorsal ALT (m-, ml-, l-, t- dm-, and d-ALT). The first three are considered the classical tracts, as these are the primary targets of the PNs (Homberg et al., 1988; Ian, Berg, et al., 2016).

1.2.3 Morphology and function of the local interneurons

The local interneuron of the AL is a neuronal group shown to exhibit functions comparable to that of local interneurons within the mammalian OB (Galizia, 2014; Lledo et al., 2008; Seki et al., 2010; Shepherd et al., 2004; Wachowiak & Shipley, 2006; Wilson, 2013). Generally, LNs within the AL are described as spatially confined neurons, synapsing specifically within this olfactory processing centre, with a relatively simple morphology. However, comprehensive studies have now shown that these neurons in effect are more diverse than earlier thought. The glomerular connectivity shows that some LNs innervate all or most glomeruli within the AL (Christensen et al., 1993; Galizia & Rössler, 2010; Seki & Kanzaki, 2008), while others are reported to target only a subset of the glomeruli (Christensen et al., 1993; Galizia & Rössler, 2010; Reisenman et al., 2011; Seki & Kanzaki, 2008). There also seems to be dissimilarities in the manner of intraglomerular arborization, manifested as either dense or sparse innervations, covering superficial parts of the glomerulus, its core, or both (Chou et al., 2010; Galizia & Rössler, 2010; Sachse et al., 2007; Seki et al., 2010). In addition, it may be that the glomeruli in which certain LNs receive input

can differ compared to the ones they form output in (Galizia & Rössler, 2010). Thus, there is morphological variation, as well as the intricacy in how LNs function to interconnect glomeruli and subsequently establish an internal circuitry within the AL (reviewed by Homberg et al., 1989; Martin et al., 2011).

Furthermore, the heterogeneous morphologies characterising LNs, have been supplemented with inquiries centring on their electrophysiological properties (Chou et al., 2010; Reisenman et al., 2011; Seki et al., 2010), leading to identification of LNs with bursty firing properties, as well as those that showed signs of tonic activity, often referred to as nonspiking (Fusca et al., 2013; Husch et al., 2009a, 2009b). The variation in spiking activity has been related to the influence of sodium and calcium as well as voltage dependant currents (Husch et al., 2009a).

Even the neurochemical properties of LNs seems to be more complicated than originally thought, as the general idea was that LNs were γ-aminobutyric acid (GABA)ergic neurons, regulating the local processing and subsequently shape the output to higher order brain areas through lateral inhibition of PNs (Martin et al., 2011). Though this is true for the greater part of LNs in moths (Berg et al., 2009; Reisenman et al., 2011; Seki & Kanzaki, 2008), as well as in other insects, molecules such as acetylcholine, glutamate, histamine and neuropeptides have also been found to take part in this process (Berg et al., 2009; Carlsson et al., 2010; Iwano & Kanzaki, 2005; Liu & Wilson, 2013; Nässel & Homberg, 2006; Sachse et al., 2006; Shang et al., 2007). In effect, this means that the formation of synapses is not only based on inhibition, but on excitatory connectivity as well, at times even occurring in the form of gap junctions (Huang et al., 2010; Yaksi & Wilson, 2010). Thereby, the complexity of this neuronal type has become apparent, all of which may be important for their ability to effectively modulate spike activity and contribute to gain control within the AL, through regulating input to as well as output across the other neuronal constituents (i.e. OSNs and PNs) (Galizia, 2014; Seki et al., 2010; Wilson, 2013).

1.2.4 Electrophysiological properties of local interneurons and signal coding

Within the study of sensory processing, one needs to examine how the sensory stimulus itself is encoded in the neuronal activity, as well as how the gradual transformation of this activity travels through the sensory processing stream. In such an attempt it is logical to examine the timing of nerve impulses, which are represented by spike trains. By analysing the timing of the single spikes, or precisely when the neuron fires relative to other neurons, knowledge about the temporal structure of the stimulus information can be extracted.

Moreover, insight into the cells ability to produce spikes, its presynaptic input and how these inputs are transformed into a postsynaptic output, may come to light (reviewed by Moore et al., 1966; Perkel et al., 1967; Stein et al., 2005). When it comes to the analyses of such spike data, computational techniques are generally applied. It is therefore important to remember the presence of unpredictability within the underlying process of action potentials, the variability and randomness of which is what yields the desired details about the neural code of the spike train.

The general regard of action potentials as an "all-or-none" principle, means that each of the individual spikes a neuron produces is viewed as indistinguishable from each other. In order to measure and distinguish the properties of neuron variability, the time instance of each individual spike as well as the time occurring between the subsequent spikes must be estimated and quantified during a constricted window of time. Processes of this type are commonly referred to as a stochastic point process, where information is coded in the timing, not the amplitude of the spikes. The time interval, which occurs between the action potentials, is what is known as the interspike interval (ISI), a commonly used estimate when studying the variability of spike trains (Perkel et al., 1967).

1.3 Aim of the thesis

As the knowledge of the synaptic circuitry in the primary olfactory centre is still quite ambiguous, understanding how information is processed within the neural network remains limited. To counteract this, it is crucial to identify specific elements within the synaptic structures of such systems. This study thus became concerned with the workings within the AL of the model *H. amigera*, with the main aim of investigating the local interneurons within these circuits. Additional information about these particular neurons is one of the most timely topics in neuroscience, as their properties and functional significance, to our knowledge, has only been studied to a limited extent (Chou et al., 2010; Christensen et al., 1993; Fusca et al., 2013; Husch et al., 2009b; Kymre et al., 2020; Lavialle-Defaix et al., 2015; Liu & Wilson, 2013; Matsumoto & Hildebrand, 1981; Ng et al., 2002; Reisenman et al., 2011; Rybak et al., 2016; Seki & Kanzaki, 2008; Seki et al., 2010; Shang et al., 2007; Tabuchi et al., 2015; Warren & Kloppenburg, 2014; Wilson & Laurent, 2005). We attempt to categorise and investigate putative differences in the electrophysiological properties of LNs, such as the waveform pattern, spontaneous firing activity as well as their response pattern, with the intent of gaining new insight into the functional characteristics of this neuronal type. In this regard, we will use sharp electrode intracellular recording and iontophoretic staining of individual

AL neurons to obtain new data, which will be examined and visualised using confocal microscopy. Hopefully, this can increase the attention surrounding this neuron type and further the comprehension of the local olfactory circuit, within the context of a larger network.

2. Methods and materials

2.1 Insects

Male moths, of the species *Helicoverpa armigera* (Lepidoptera; Noctuidae, Heliothinae) were used in this study. The pupae were obtained from Henan Jiyuan Baiyun industry Co, Ltd. (Henan, China), and kept in a climate chamber set to 24°C and with 60 % air humidity. The electrophysiological recording and staining were performed during daytime, and the climate chamber was therefore set to a reversed day-night cycle, where the lights were turned on at 18:00 and turned off at 8:00. After emergence, the moths were carefully transferred into a cylindrical Plexiglas container (20 cm high, and 12.5 cm in diameter), equipped with soft tissue lining. The moths were provided a 10% sucrose solution. The maximum number of male insects placed in each cylinder, were 8. Though the ethics of using Lepidoptera for experimental purposes is not established by formal restrictions in the Norwegian law of animal welfare (see www.lovdata.no/dokument/NL/lov/2009-06-19-97), the insects were always handled with care, to avoid unnecessary stress or harm.

2.2 Insect preparation

The moth was transferred from the climate chamber into a small plastic container and sedated in the refrigerator at 4 °C for about 5-10 minutes. Afterwards, it was gently placed into a narrow plastic tube without damaging the antennae. The exposed head capsule was then immobilized with dental wax (Kerr Corporation, Romulus, MI, USA). The following preparation were all done under a stereomicroscope (Leica DMC 4500). The antennae and the proboscis were carefully exposed and immobilized before the head was tilted into an optimal position. After gently removing the scales, the head capsule was opened with a razor-blade knife, and the cuticle and trachea were then carefully removed with forceps in order to expose the AL as well as protocerebral parts of the brain. The exposed area was continuously supplied with Ringer`s solution (in mM: 150 NaCl, 3 $CaCl_2$, 2 KCl, 25 sucrose, and 10 N-tris (hydroxymethyl)- methyl-2-amino-ethanesulfonic acid, pH 6.9) to provide nutrition and prevent dehydration of the brain.

2.3 Intracellular recording and odour stimulation

The moth was placed under a microscope (Leica MZ Apo), and a reference electrode coated with chloride was inserted into the preparation anteriorly to the mouth muscle, near the labial palps and proboscis. The tip of a glass microelectrode, pulled from borosilicate capillaries using a horizontal Flaming/Brown puller (P97; Sutter instruments, Novato, CA,

USA), was filled with a fluorescent dye, Micro-Ruby (Dextran tetramethylrhodamine-biotin). The whole microelectrode was filled with potassium acetate (0.2M). The microelectrode was then placed on a silver wire which was attached to a head-stage preamplifier (HS-2, Axon instruments, USA), and joined to an amplifier (Axoprobe-1A, Axon instruments, USA). The purpose of this connection is to enable the current circuit between the reference electrode and the recording electrode when submerged into the neural tissue, and to relay the fluctuations in voltage (neuronal spike signals) that occur within the brain circuit. As such, a micromanipulator (Leica) was used in order to lower the recording electrode into either of the ALs, with the purpose of obtaining stable contact with a neuron. Prior to entering the neuronal tissue, the resistance of the recording electrode was measured between 80 and 200 MΩ. The voltage fluctuations relayed to the Amplifier, i.e.the neuronal signal, were also displayed visually on an oscilloscope (Tektronix 5111A, Oregon, USA), as well as in an auditory manner on a loudspeaker (Monacor, MAB-30AK). The electrophysiological signals were also visualised on a computer, by use of the software, Spike2 (version 6.18, Cambridge Electronic Design Limited). As such, the spike activity was carefully monitored and assessed, though the final decision of whether neuronal contact was stable, as well as whether the neuron responded to odours, was subjective.

During recording, the antennal area of the moth was constantly ventilated with a steady stream of fresh air. During odour stimulation, a pulse of air from the continuous airstream (500ml/min) was diverted via a solenoid-activated valve (General Valve Corp., Fairfield, NJ, USA) through a glass cartridge bearing the odorant on a piece of filter paper. Up to five odours were tested in each recording experiment, each of which were repeated three times. The stimulation protocol was executed via the spike2 software, with the period of stimulation lasting 400 milliseconds, and the interstimulation interval lasting about 10 seconds (see Fig. 2). After testing all odour stimuli, the neuron was iontophoretically stained by applying 1.5–3 nA pulses via the amplifier, with a 200 milliseconds duration at 1 Hz for about 5–10 minutes. To secure anterograde transport of dye through the axonal processes of the neuron, the preparation was placed in a chamber holding 4°C and left there overnight.

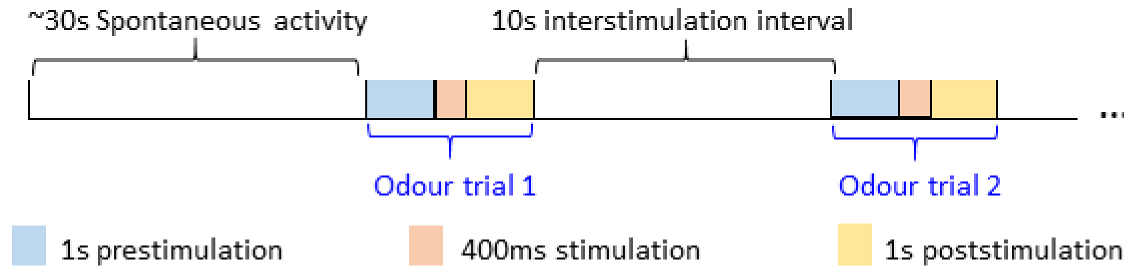


Figure 2. The procedure of odour stimulation. In obtaining a stable contact with the neuron, ~30 s spontaneous activity was collected prior to the stimulation application. Between every two trials there was a 10 s interval.

2.4 Odour stimulations

To examine the physiological properties of the local interneurons, we designed the following stimulation protocol where five stimuli were arbitrarily applied during the intracellular recordings. These included three insect-produced individual components, one plant odour mixture and a control. Each of the odour stimuli had a distinct behavioural significance for the insect. The conspecific female-produced primary pheromone, *cis-11-*hexadecenal (Z11-16:AL), serves as the main sex attractant. The secondary pheromone, *cis-9*-hexadecenal (Z9-16:AL), produced by conspecific females to form a species-specific ratio together with the primary pheromone (95:5), functions to identify the "correct" mate. This component is identical with the primary pheromone component of the coresidential and closely related species, *Helicoverpa assulta* (Berg et al., 2014), thus the secondary component alone should evoke an aversive reaction in male *H. armigera*. We also tested the behavioural antagonist, *cis-9*-tetradecenal (Z9-14:AL), which is another female-produced minor component, playing a dual role. At higher dosages, this component evokes inhibition of the attraction elicited by the primary pheromone, i.e. >5% (Gothilf et al., 1978; Kehat & Dunkelblum, 1990). At lower concentrations, however, it acts as an agonist, i.e. 0.3-5% (Wu et al., 2015; Zhang et al., 2012). All the insect-produced stimuli were diluted in hexane (99%, Sigma), and applied to a filter paper that was placed inside a 120 mm glass cartridge. The final amount per filter paper was 10 ng of the relevant stimulus. Two types of plant odour were used in this study, each of which was applied to one distinct group of insects. About half of the insects (N = 11) were exposed to the head space of a host plant (sunflower leaves), and the other half (N = 12) was exposed to a mixture of behaviourally relevant plant odours, previously shown to serve as an insect attractant. This mixture included 50µl Phenylacetaldehyde, 20µl of Salicylaldehyde, 10 µl of Methyl 2-methoxy benzoate, 10 µl of Linalool, and 10 µl (R)-(+)-Limonene. The two types of plant odour stimulations will from

this point on be referred to as one and the same, under the term 'plant odour'. To achieve a behaviourally relevant concentration, the stock solution of the last-mentioned plant odour stimulus was diluted in Hexane to a 10^4 concentration. The control stimulus was Hexane, which served as a solvent for all stimuli. The final amount of plant odours and control stimuli on each filter paper was 20 µl. When the stimuli were not in experimental use, they were sealed and stored in a freezer at –18 °C. The stimuli were renewed every 1-2 week.

2.5 Dissection, fixation, and dehydration

Following the overnight attempt at axonal transport of dye, the moth was put under a stereomicroscope (Leica, M60) for dissection. First the antennae were cut off using micro scissors. Then the eye capsules were removed with a razor knife and forceps, tailed by cutting the cuticle between the eye and brain. The proboscis was also removed before. The head was then removed from the body. The remaining cuticle and trachea were carefully removed in order to detach the brain. It was then placed in a 4 % paraformaldehyde solution (Roti-Histofix 4%, Carl Roth GmbH, Karlsruhe, Germany) in an Eppendorf-tube for 60 minutes at room temperature, or overnight in 4°C, in order to fixate the brain before dehydration. It was subsequently placed in a series of ethanol solutions (50%, 70%, 90%, 96%, 2 x 100%), each step lasting 10 minutes. The brain was then placed in methyl salicylate (methyl 2-hydroxybenzonate). Finally, the brain was placed in methyl salicylate in a perforated aluminium plate, covered by decker glass plates, and kept in the refrigerator until imaging.

2.6 Confocal microscopy

In order to visualise the stained neuron, the relevant brains were scanned by a confocal laser scanning microscope (LSM 800 Zeiss, Jena, Germany), situated at the Department of Biophysics and Medical Technology, NTNU. Equipped with a 10x (C-Apochromat 10x/0.45 NA) water objective and a 20x (Plan-Neofluar 20x/0.5 NA) objective, the confocal microscope scanned the brains using a Helium-Neon laser channel, operating at a wavelength of 553 nm, which excites the Micro-ruby staining. A 560 nm long-pass filter was utilized. In order to improve visualisation of all the relevant brain structures, a second channel, exiting endogenous fluorophores in the neural tissue, was included using an Argon laser at the wavelength of 493 nm in combination with a 505-550 nm band pass filter. The serial scans of optical sections were obtained with 1024 x 1024 pixel resolution, at 2-9 µm intervals depending on the objective used. The pinhole size was 1 airy unit. The confocal

images shown in this thesis were acquired and edited in ZEN 2.3 (blue edition, Carl Zeiss Microscopy GmbH, Jana, Germany).

2.7 Data analyses

Confocal stacks containing stained neurons were used to inspect the neuronal morphology and classify the neurons. Each preparation was carefully examined to see if they contained stained LNs. If a preparation was found to exhibit a stained AL PN, it was excluded from the following analyses. The criteria of morphological classification used here were based on the glomerular arborizations that were observed as being dense, sparse, or non-existent. In cases where more than one LN was stained, the homogeneity of the waveforms collected during the spontaneous activity was used to determine whether the preparation should be included in the physiological analyses. Spike 2.8 was used to assess the electrophysiological data of each LN and compare the average waveform templates of all the preparations. In instances where more than one template was generated, those with high compatibility were merged based on visual inspection. In registrations including several multiple waveforms, the LN sample was then omitted. Through the application of these preliminary investigations, a total of 23 morphologically identified LNs were finally included in the spike data analyses.

2.7.1 Spike sorting

The average spike waveforms of the spike trains from each included LN' spontaneous activity was generated. Every single action potential compatible with the waveform template was specified. The averaged waveform was then used as one of the extrinsic physiological features for each LN. Spike sorting was also employed in the pre-stimulation window, the stimulation window, and the post-stimulation window (Fig. 2). The time stamp of each specified spike collected here was then used to identify the firing properties during the spontaneous activity as well as each stimulation trail (see sections below).

2.7.2 Quantification of the electrophysiological data from the spontaneous activity

A relevant source for temporal fluctuation within the brain`s neural activity, is the spontaneous activity of neurons (Nobukawa et al., 2019). Thus, during each experiment, the spontaneous activity of the LN was collected prior to the stimulation application, with each sample lasting from 15 – 60 seconds. Based the time stamp identified during spike sorting, the interspike interval (ISI) of the spontaneous activity was also carefully quantified, which provided several descriptive physiological parameters such as the mean of ISIs, the mean firing rate, the median of ISIs, the minimum ISI, the maximum ISI and the ISI coefficient of variance (*cV*). These parameters were used to characterize the neuronal spontaneous firing profile, thus reflecting the synaptic connections of the neurons (reviewed by Luhmann et al., 2016).

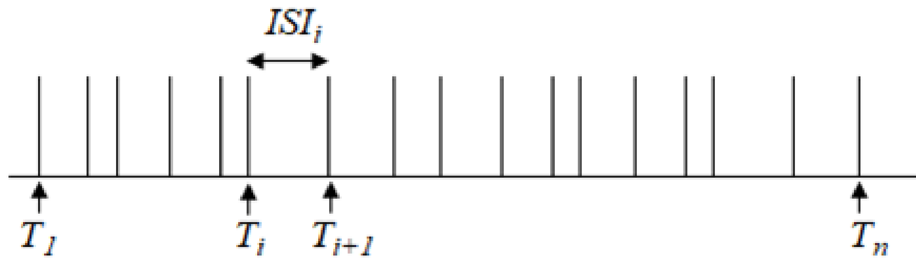


Figure 3. Segment of an artificial spike train, illustrating the computation of interspike interval (ISI).

Figure 3 explains how we calculated the ISI in an artificial spike train, where each vertical line represents a spike. Here, T_i represents the time stamp of the "*i*" spike and T_{i+1} represents the time stamp of the following spike. *n* signifies the total amount of spikes in a spike train. (see Fig. 3).

The mean ISI was computed from each ISI, which is the time duration between two subsequent spikes:

$$ISI_i = T_{i+1} - T_i$$

In order to quantify the mean ISI, one can apply the equation:

$$Mean\ (ISI) = \frac{T_n - T_1}{n - 1}$$

The mean firing rate was found by the equation:

$$FR = \frac{1}{Mean\ (ISI)}$$

The minimum ISI (ISI_{min}) and the maximum ISI (ISI_{max}) signify the range of the ISI within the spike train, while the burstiness is often described using the ISI coefficient of variance (cV). It refers to the random appearance of action potentials, and statistically describes the variation of the ISI. The standard procedure for computing the ISI cV, is by dividing the standard deviation of the neurons observed during the interspike intervals with the mean ISI (Nawrot, 2010).

The equation provided:

$$cV = \frac{Standard\ deviation\ (ISI)}{Mean(ISI)}$$

Assuming that that the firing activity of cortical neurons is highly irregular, a Poisson process is posited, where cV equals 1 ($cV = 1$) (Reviewed by Softky & Koch, 1993; van Vreeswijk, 2010). Small values close to 0 indicate regular firing, whereas large values close to or >1 indicate irregular firing distributions. A high ISI cV value may indicate bursty firing, though neurons with low firing rate and a high variability in ISI may also show low spiking as well as a high ISI cV value (reviewed by Softky & Koch, 1993).

2.7.3 Quantification of the odour-induced electrophysiological data

Following the recording of the spontaneous activity, each neuron was exposed to the stimulation protocol in a randomized order. The recorded odour response was used to quantify the electrophysiological activity of the LNs when stimulated. Each odour application within the stimulation trial comprised a time period of 2.5 seconds, this included a pre-stimulation window for baseline activity prior to the stimulus onset, lasting 1.1 seconds, a 0.4 second stimulation period, and a 1 second post-stimulation period (Fig. 2). To describe the temporal neuronal activity, the Z-scored instantaneous firing rates (ZIFR) of every 1 ms for each trial were computed. And the responses of the individual LNs where then measured through analysing the odour-evoked response properties. This was based on the mean ZIFR (MZIFR) across repetitive trials during application of the same stimulus. To examine the response amplitude for each stimulus, we first standardized the baseline activity by setting the MZIFR before the stimulation onset to zero. The response amplitude was then quantified as the average ΔMZIFR within the stimulation window.

2.8 The statistical analyses

The firing properties of each neuron were quantified, and 11 parameters were generated (See table 1): six parameters describing the spontaneous activity and five describing the response activity to the stimuli. Three investigations were performed based on electrophysiological features of the LNs. The first investigation was established upon the shape of the waveforms. According to the hyperpolarization status in the waveform, the 23 LNs were classified into two categories: LNs with afterhyperpolarized potential (HAP) (N = 12) and LNs with depolarized afterpotential (DAP) (N = 11). An independent sample t test was then performed to compare the mentioned parameters of spontaneous activity and response amplitudes between two categories of LNs.

	Parameters of the spontaneous activity	*Parameters of odour response*
1	Mean of ISIs	ΔMZIFR (Hexane)
2	Mean firing rate	ΔMZIFR (Plant odour mixture /sunflower)
3	Median of ISIs	ΔMZIFR (Primary pheromone component)
4	Minimum ISI	ΔMZIFR (Secondary pheromone component)
5	Maximum ISI	ΔMZIFR (Behavioural antagonist)
6	ISI coefficient of variance (cV)	

Table 1. Parameters in the data analyses

In the second investigation, two clustering analyses were performed on the basis of the spontaneous activities. First, we generated a hierarchical cluster analysis by using the centroid clustering method to obtain an overview of the linkage of all LNs. Then, a two-step cluster analysis (with Schwarz's Bayesian criterion and log likelihood distance) was conducted to categorize clusters according to the ideal parameter(s) illustrated by the hierarchical cluster test. To ensure the quality of the clustering, Pearson's correlation coefficients was also performed between every two of the six spontaneous activity parameters to determine the independence of each parameter. Highly correlated parameters were not included in the same cluster test simultaneously. The qualified clustering (greater than fair) was then used in an independent t-test to compare the response amplitude across different clusters. Lastly, we also performed a pilot two-step cluster analyses on the basis of the

response amplitudes to each stimulus, using independent *t*-tests to compare the difference between the spontaneous activity of the clusters.

All of the statistical analyses were performed in SPSS (Version 27.0), and as the majority of the spontaneous activity data was non-normally distributed (Shapiro-Wilk test, *p*s $< .01$) except the ISI *cV* (Shapiro-Wilk test, $p > .29$), we decided to conduct nonparametric tests when comparing these parameters. For the response data, on the other hand, all data were normally distributed (Shapiro-Wilk test, $p > .13$), thus parametric tests were used. For each independent sample *t*-test, the effect size Cohen's *d* was reported, where $d = .2$, $= .5$, and $= .8$ can be interpreted as small, medium and large effects, respectively (Cohen, 2013). The results were displayed in boxplots (interquartile range with 25th percentile and 75th percentile, median), and the descriptive statistics were illustrated in tables. All reported statistical data is two-tailed.

3. Results

Throughout the intracellular recording and iontophoretic staining data collection, we aimed to record from the central non-glomerular area of the AL, called the AL hub. This area is innervated mainly by branches of LNs, thus, these neurons were the most frequently encountered type. Altogether, 103 moths were prepared in this study, of which 72 were utilized in intracellular recording experiments. Among them, 35 preparations contained successfully stained neurons, resulting ~50% success rate in the method in general. However, brains holding stained LNs exclusively, included 13 preparations. Of these, 5 held a single strongly labelled LN, while 8 showed additional, weakly co-stained, LNs. Confocal images of all these stained LNs are presented in Fig. 4. To perform the statistical analysis, we included 11 LNs previously collected by Ph.d student Jonas Hansen Kymre. Out of the total number of 24 LNs, 21 innervated all AL glomeruli, including the MGC, PCx, LPOG and the VPG (AllG type), while 3 innervated a restricted number of glomeruli (non-AllG type). Examples of the type first mentioned, called AllG type LN, are shown in Fig. 4A. While most of these AllG type LNs innervated the AL glomeruli evenly, two neurons had rather dense arborizations in the LOPG and VPG in comparison with that in the MGC, PCx, and OG (Fig. 4A, LN_e and LN_l). Another AllG type LN had an untypical innervation pattern in the AL including relatively dense innervation in one of the anterior OG and spars innervation in the remaining AL glomeruli (Fig. 4A, LN_b). The other morphological type included the remaining 3 LNs that were termed non-AllG type. These LNs were categorised into two sub-types, and examples are shown in Fig. 4B - C. The first sub-type containing two LNs, appearing to be oligoglomerular, i.e., innervating only restricted regions in the AL. Both oliglomerular LNs evaded the MGC (÷MGC type) (see Fig. 4B). The second sub-type contained one LN that innervated all glomeruli except the LPOG and VPG (see Fig. 4C). Other neurons were also registered/stained during this experimental work. As they were not appropriate for the aims of this thesis, we decided to exclude them from the results. However, four examples of such preparations are presented in Appendix I-II.

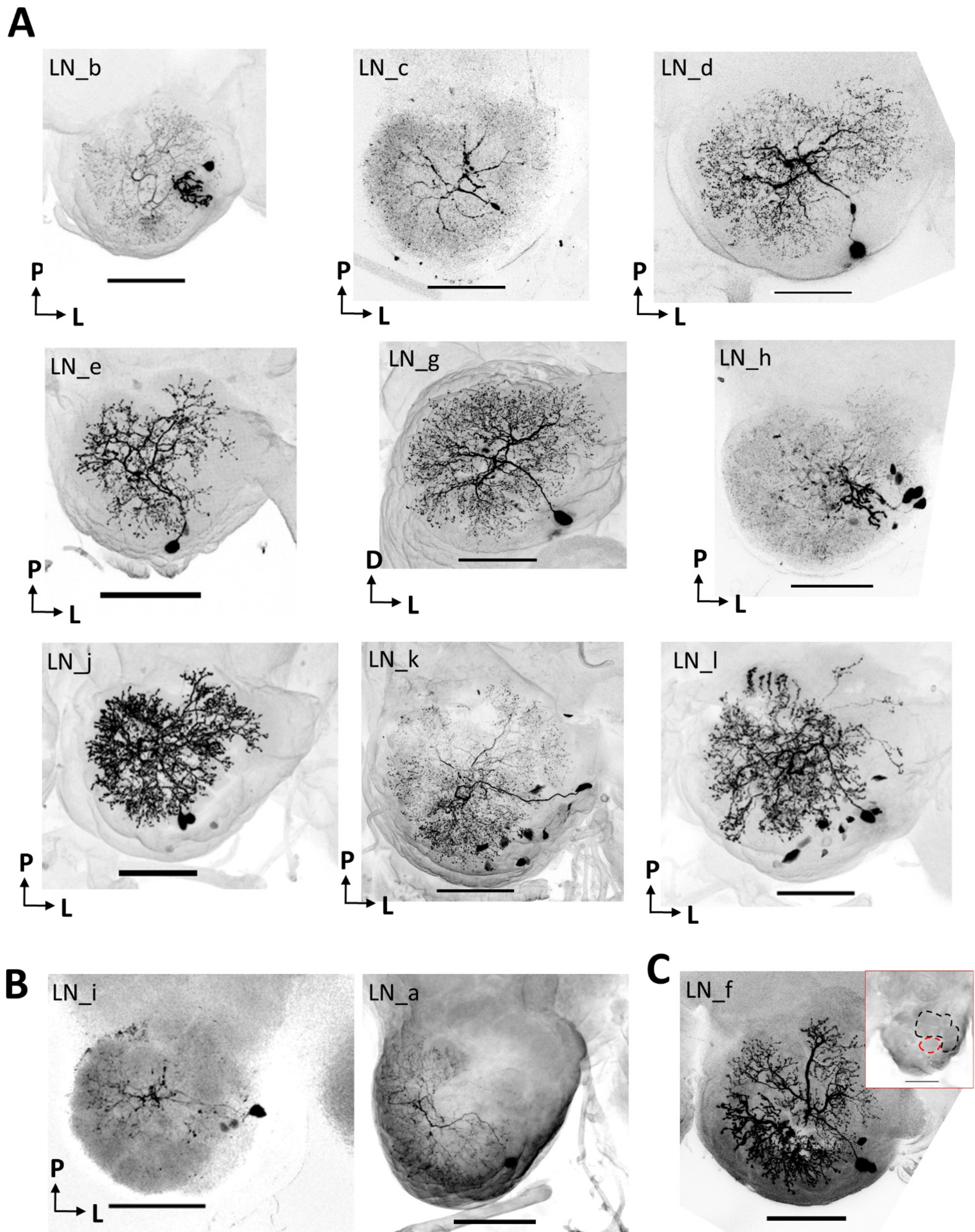


Figure 4. Confocal images of the 12 iontophoretically labeled antennal lobe (AL) local interneurons (LNs). (A) LNs innervating all AL glomeruli (AllG type) (ID: LN_b, LN_c, LN_d, LN_e, LN_g, LN_h, LN_j, LN_k, LN_l). Three of the AllG type LNs were atypical, the LN_e and LN_l innervated more densely within the LPOG and VPG than the other glomeruli, while the LN_b had a clearly denser innervation pattern within only one of the anterior ordinary glomeruli (OG). (B-C) Non-AllG type LNs. (B) LNs innervating all antennal lobe glomeruli except the MGC (÷MGC type) (ID: LN_i and LN_a). (C) The LN innervating all antennal lobe glomeruli apart from the labial palp-pit organ glomerulus (LPOG) (*red*) and the ventroposterior glomeruli (VPG) (*black*). Scale bars: 100 µm.

Because the odour response data was collected by two individuals, we tested whether there were any variabilities between the electrophysiological data collected by the two conductors. In doing this, a two-way repeated measures analysis was used, with one within factor (Stimulation: 5 stimuli), and one between factor (Conductor: Sara vs. Jonas). The result showed that different experimental conductors had no effect on the response amplitudes (between factors: $F_{1,13} = 1.00$, p =.34, Fig. 5).

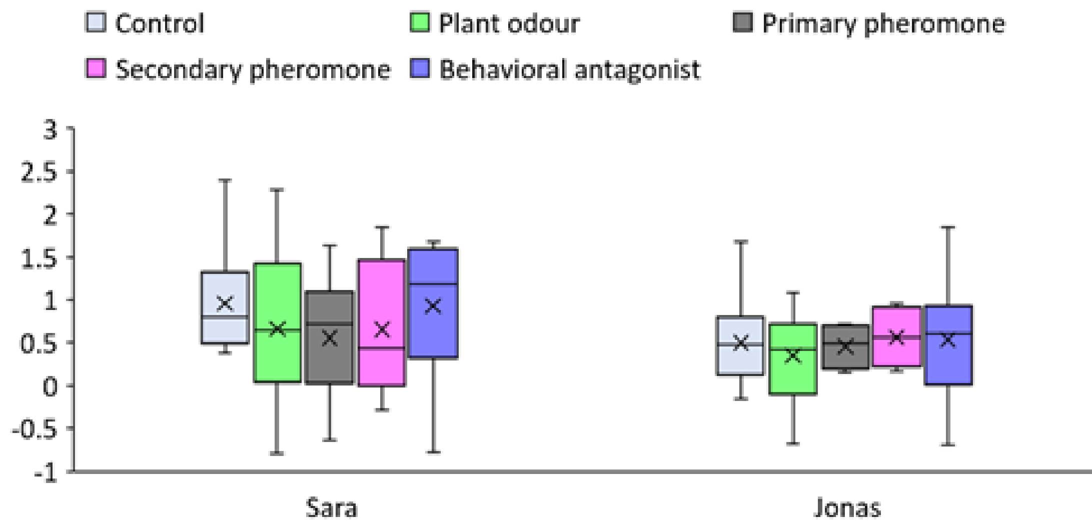
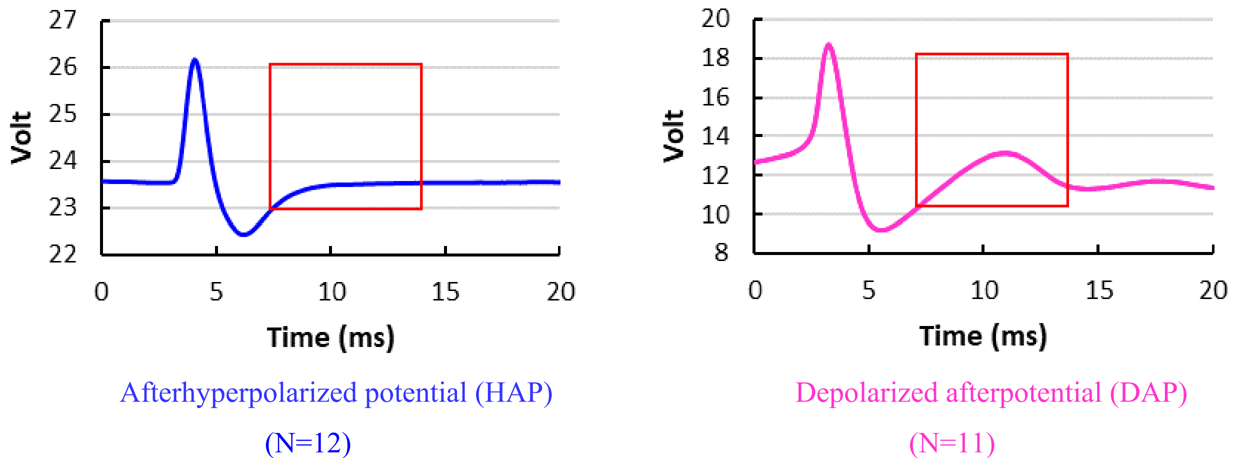


Figure 5. Inter-observer comparison between the two experimental conductors (Sara vs. Jonas). Repeated measures showed no difference between the two.

3.1 Electrophysiological characteristics and cluster analyses

3.1.1 Statistical analyses of waveform characteristics

Based on the criteria for waveform inspection (see section 2.7), one of the LNs was excluded from the statistical analyses as multiple waveforms were detected. In order to classify the firing pattern of the remaining 23 LNs, we first performed a quantitative analysis based on the average waveforms. By inspecting the spontaneous activity spike data, two different categories of waveforms were detected (examples of each category are shown in Fig. 6).



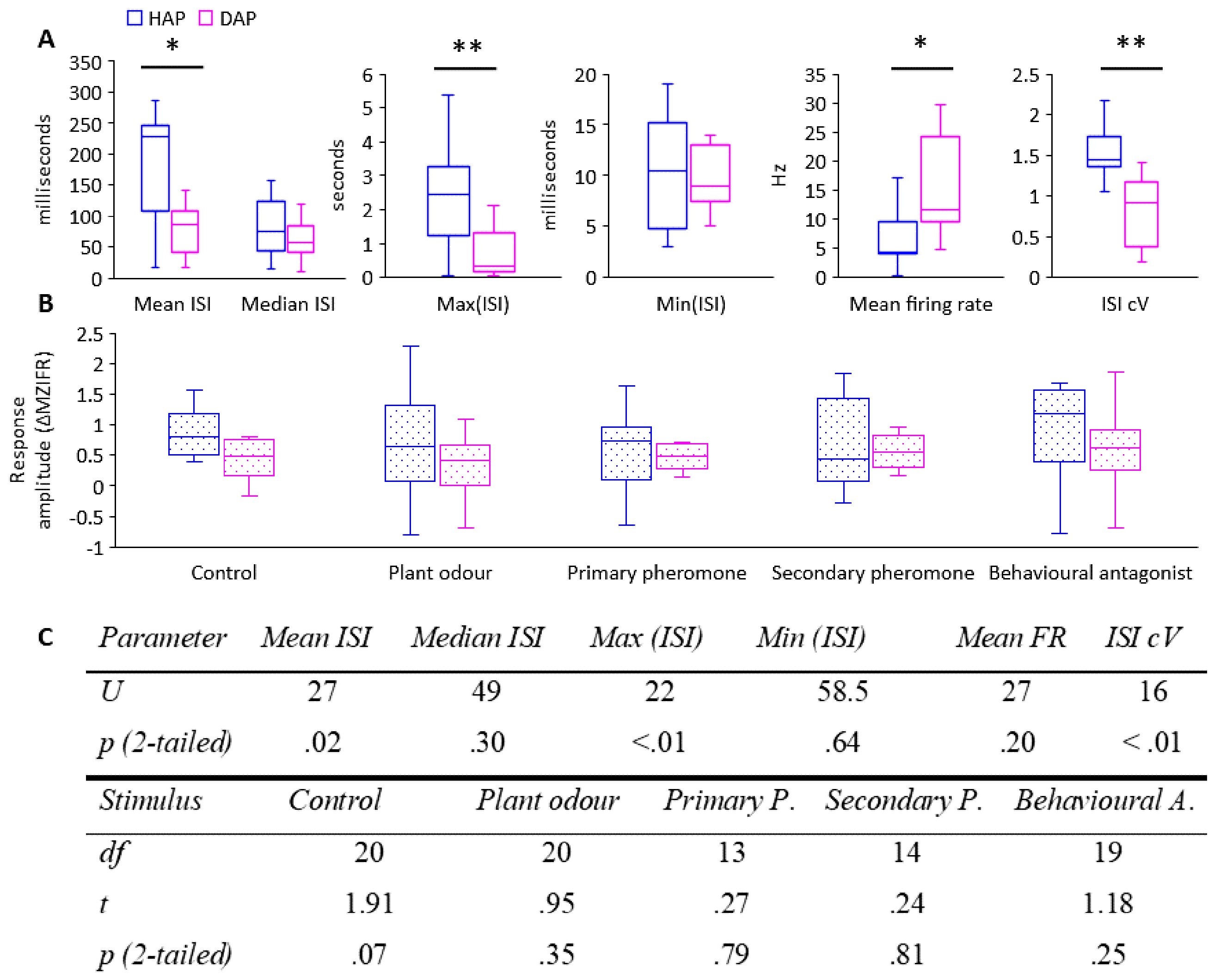
Afterhyperpolarized potential (HAP)
(N=12)

Depolarized afterpotential (DAP)
(N=11)

Figure 6. Illustration of the two types of afterpotentials following the action potential. In generating the mean spontaneous activity waveform of each local interneuron (*N*=23), the hyperpolarized afterpotential also termed HAP (left) and the depolarized afterpotential, termed DAP (right), became noticeable.

We then used Mann-Whitney *U* tests to compare the parameters of spontaneous activity (Fig. 7A and C), as well as independent *t*-tests to compare the parameters of the response amplitudes evoked by different stimuli between these two categories (Fig. 7B and C). The results showed that, in the HAP neurons, the mean interspike interval (ISI) and the maximum ISI were prolonged, while the mean firing rate was lower in comparison with the LNs in the DAP category. There was no difference between the two categories when we compared the median ISI and minimum ISI of the LNs. However, a comparison of the ISI *cV,* which reflects the spiking regularity of the neurons (Lei et al., 2011; Nawrot, 2010), showed that the neurons with HAP had a higher ISI *cV* than the neurons with DAP. This indicates that LNs with an afterhyperpolarized potential (HAP) waveform fire more irregularly and with more variability than the LNs having a depolarized afterpotential (DAP).

The DAPs were only observed during spontaneous activity, not during the period of stimulation. As the neurons in the two waveform categories exhibited a difference in their burstiness, we hypothesize that the disappearing of DAPs might affect their response profiles. The LNs' response amplitudes to odour stimuli were therefore compared between the two categories, which unexpectedly showed no difference in any of the given stimuli (Fig. 7B). Thus, indicating that the afterpolarized potential in LNs has a very limited impact on the response pattern.



Parameter	*Mean ISI*	*Median ISI*	*Max (ISI)*	*Min (ISI)*	*Mean FR*	*ISI cV*
U	27	49	22	58.5	27	16
p (2-tailed)	.02	.30	<.01	.64	.20	< .01

Stimulus	*Control*	*Plant odour*	*Primary P.*	*Secondary P.*	*Behavioural A.*
df	20	20	13	14	19
t	1.91	.95	.27	.24	1.18
p (2-tailed)	.07	.35	.79	.81	.25

Figure 7. Box plots of the parameters representing the spontaneous activity (A) and the response amplitude (ΔMZIFR) to the 5 odour stimuli during the entire 400 ms stimulation window (B), measured in neurons with the afterhyperpolarized potential waveform (HAP) and depolarized afterpotential waveform (DAP), respectively. (A)The neurons with DAP showed a different spontaneous activity from neurons with HAP. Mann-Whitney U tests were used, *, p < .05; **, p < .01. (B) Independent *t*-tests showed that the response amplitudes across stimuli did not show a difference between neurons with the different waveforms. (C) Statistic data of the comparisons shown in (A) and (B).

3.1.2 Spontaneous activity

Since the neuron's odour reaction did not seem to be dependent on its waveform, we next aimed to investigate whether the intrinsic firing properties during the spontaneous activity may play a role in the neuron's responding profile. First, a hierarchical cluster analysis was generated. The clustering result was based on the Mean ISI, maximum ISI, minimum ISI as well as ISI *cV*, and illustrated the linkage of the different LNs. The close distance within LN assemblies suggested the possibility of conducting a two-step cluster analysis, and by normalizing all parameters from 0 to 1 (Fig. 8), we found that the ISI *cV* was the most suited parameter for the further clustering.

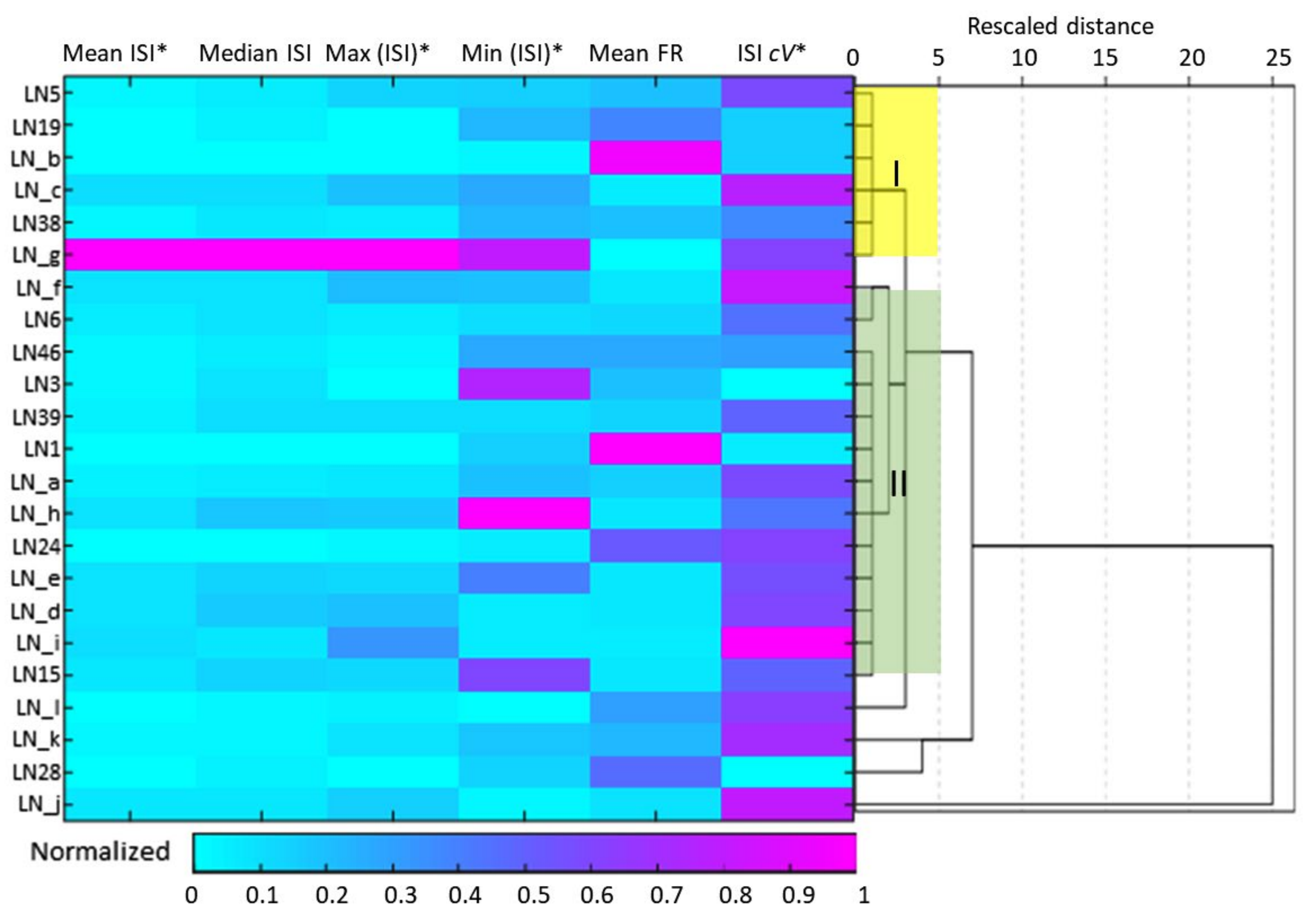
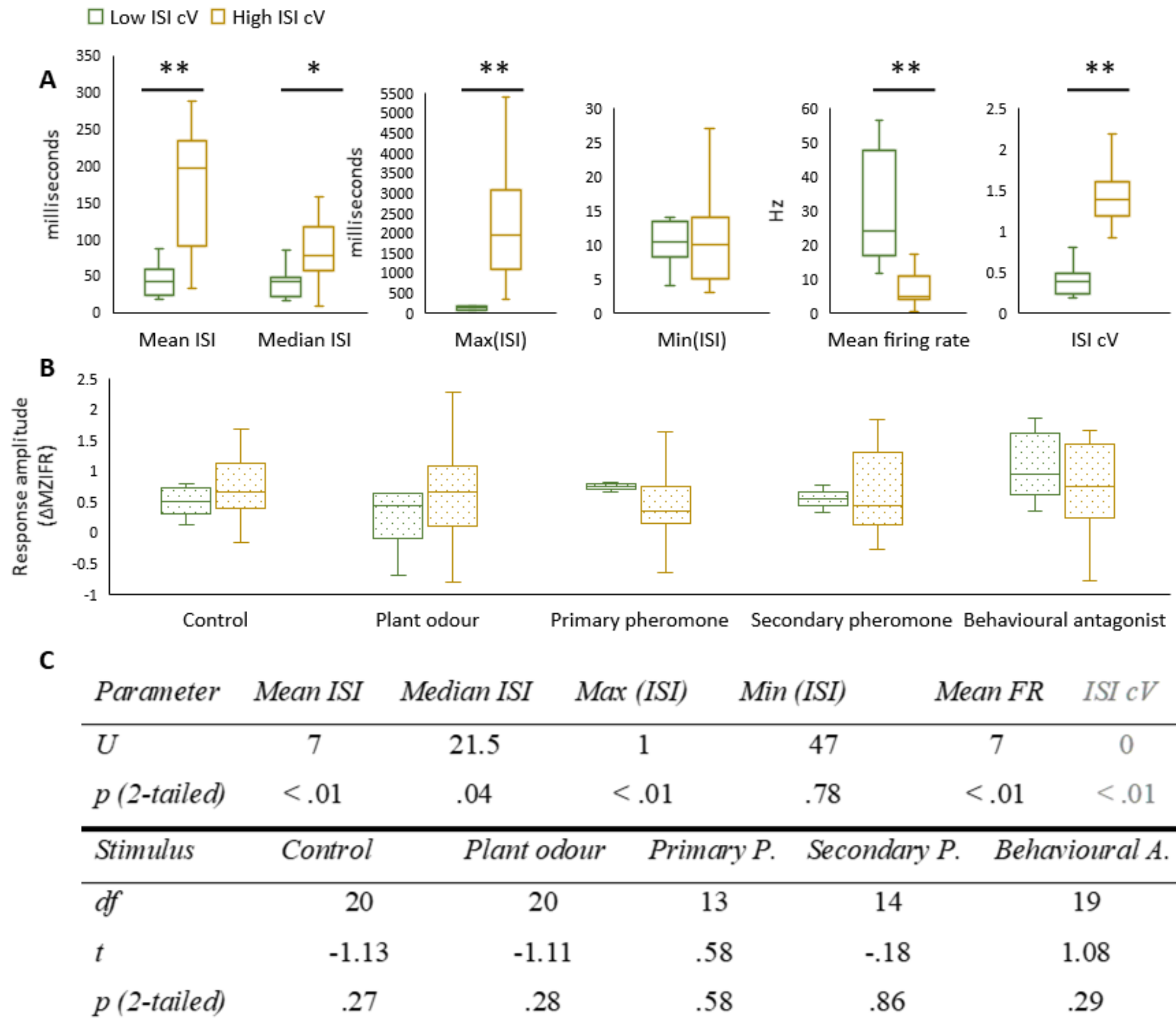


Figure 8. Intrinsic properties of the local interneurons (LNs). Spontaneous activity profiles (x-axis) of individual LNs (y-axis) are shown on the left. The classification dendrogram on the right was generated using the hierarchical clustering method, leading to two intrinsic types (I and II). Spontaneous activity parameters are normalized from 0 to 1 using linear model and colour coded for each neuron. *, Selected inputs for hierarchical clustering.

Two clusters (Low ISI *cV* and High ISI *cV,* see Fig. 9) were successfully identified by testing with an unsupervised two-step clustering analysis based on the parameter of ISI *cV*. The ratio between cluster sizes was 2.83, and the quality of cohesion and separation was "good" (average silhouette = 0.8). When comparing the clusters based on the ISI *cV* with the waveform, we found that 91.7% of the neurons in the HAP waveform category were clustered within the higher ISI *cV*, while only 45.4% of the neurons in the DAP waveform category were clustered into the low ISI *cV* cluster. This indicated that the neurons with a depolarized afterpotential (DAP) seem to have less predictability in their intrinsic firing properties (see Fig. 7A).

When comparing the spontaneous activity between the two clusters based on the ISI *cV*, the results of Mann-Whitney *U* tests showed the LNs in the high ISI *cV* cluster ($N = 6$) displayed a higher mean ISI, median ISI, maximum, and a lower mean firing rate than the LNs in the low ISI *cV* cluster ($N = 17$), while the minimum ISI from the two clusters exhibited comparable data. This indicated that the intrinsic firing variability of the LNs in the low ISI *cV* cluster was higher than that of the other cluster (Fig. 9, A and C). We also measured a putative response difference between the two clusters, though the independent sample *t*-test revealed that the response amplitude of the given stimulations across the two identified clusters suggested that such a difference was not present (Fig. 9, B and C). Thus, it appears as if the response profile of the individual LNs might not be related to the burstiness of the spontaneous activity.



Parameter	*Mean ISI*	*Median ISI*	*Max (ISI)*	*Min (ISI)*	*Mean FR*	*ISI cV*
U	7	21.5	1	47	7	0
p (2-tailed)	< .01	.04	< .01	.78	< .01	< .01

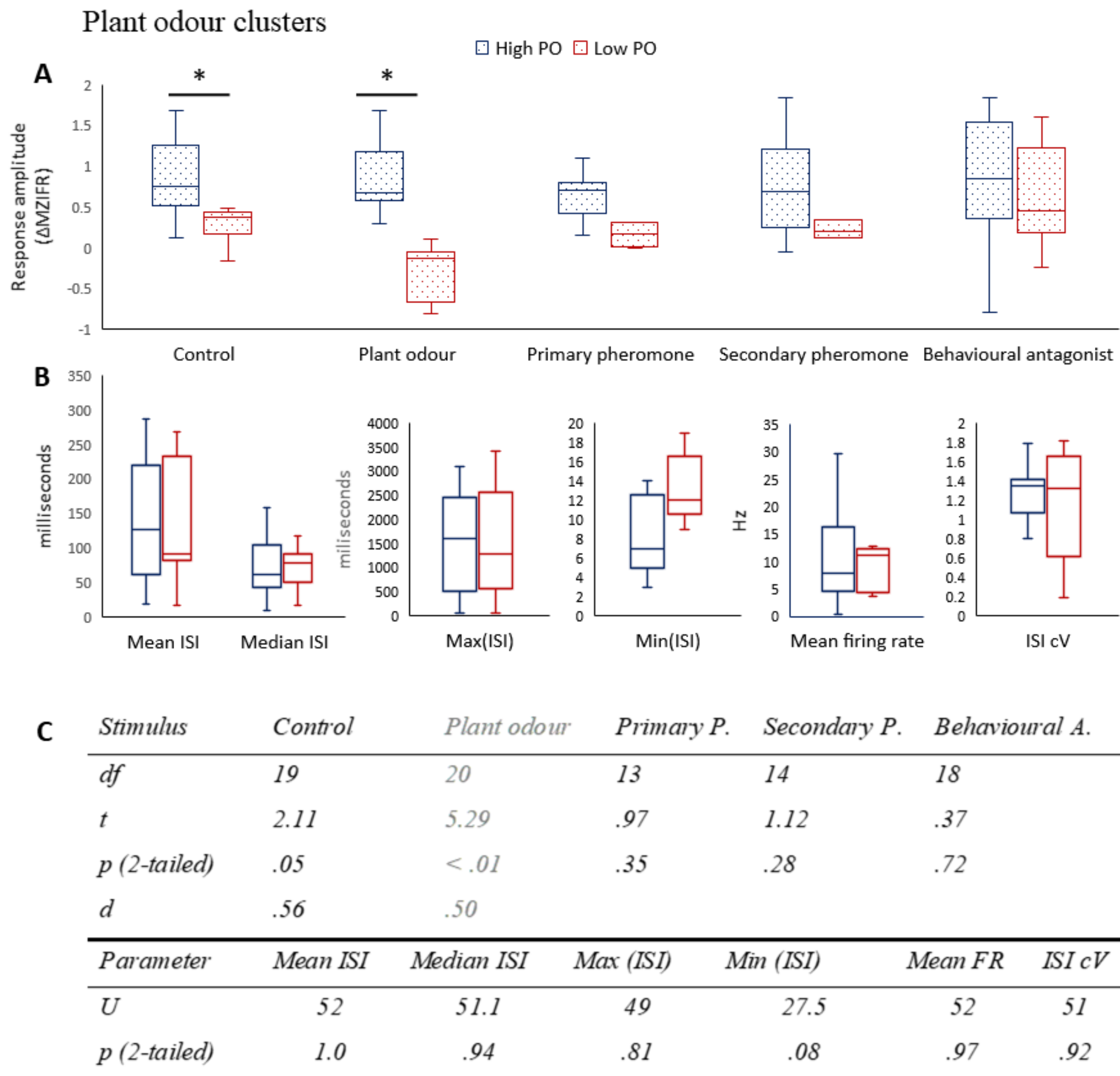
Stimulus	*Control*	*Plant odour*	*Primary P.*	*Secondary P.*	*Behavioural A.*
df	20	20	13	14	19
t	-1.13	-1.11	.58	-.18	1.08
p (2-tailed)	.27	.28	.58	.86	.29

Figure 9. Box plots of the parameters representing the spontaneous activity (A) and the response amplitude (ΔMZIFR) to the 5 odour stimuli during the entire 400 ms stimulation window (B), measured in neurons within the low ISI *cV* (*N*=17), and the high ISI *cV* clusters (*N*=6), respectively. When using the Mann-Whitney U tests, *, $p < .05$; **, $p < .01$, (A) the neurons within the low ISI *cV* cluster showed a different spontaneous activity from neurons within the high ISI *cV* cluster. (B) Independent *t*-tests showed that the response amplitudes across stimuli did not show any difference between clusters. (C) Statistic data of the comparisons shown in (A) and (B).

3.1.3 Response activity

As the ISI *cV* was the only parametric input suited for the spontaneous activity clustering analysis, and it showed restricted effect on the neuron's response pattern, we questioned whether other spontaneous activity parameters might play any role in shaping the response activity. Therefore, we designed a series of pilot clustering analyses in order to reverse examine the response profile of individual LNs to several behaviourally relevant odour stimuli (e.g., plant odour and sext pheromones), and investigate whether this might illustrate a putative association to the spontaneous firing properties.

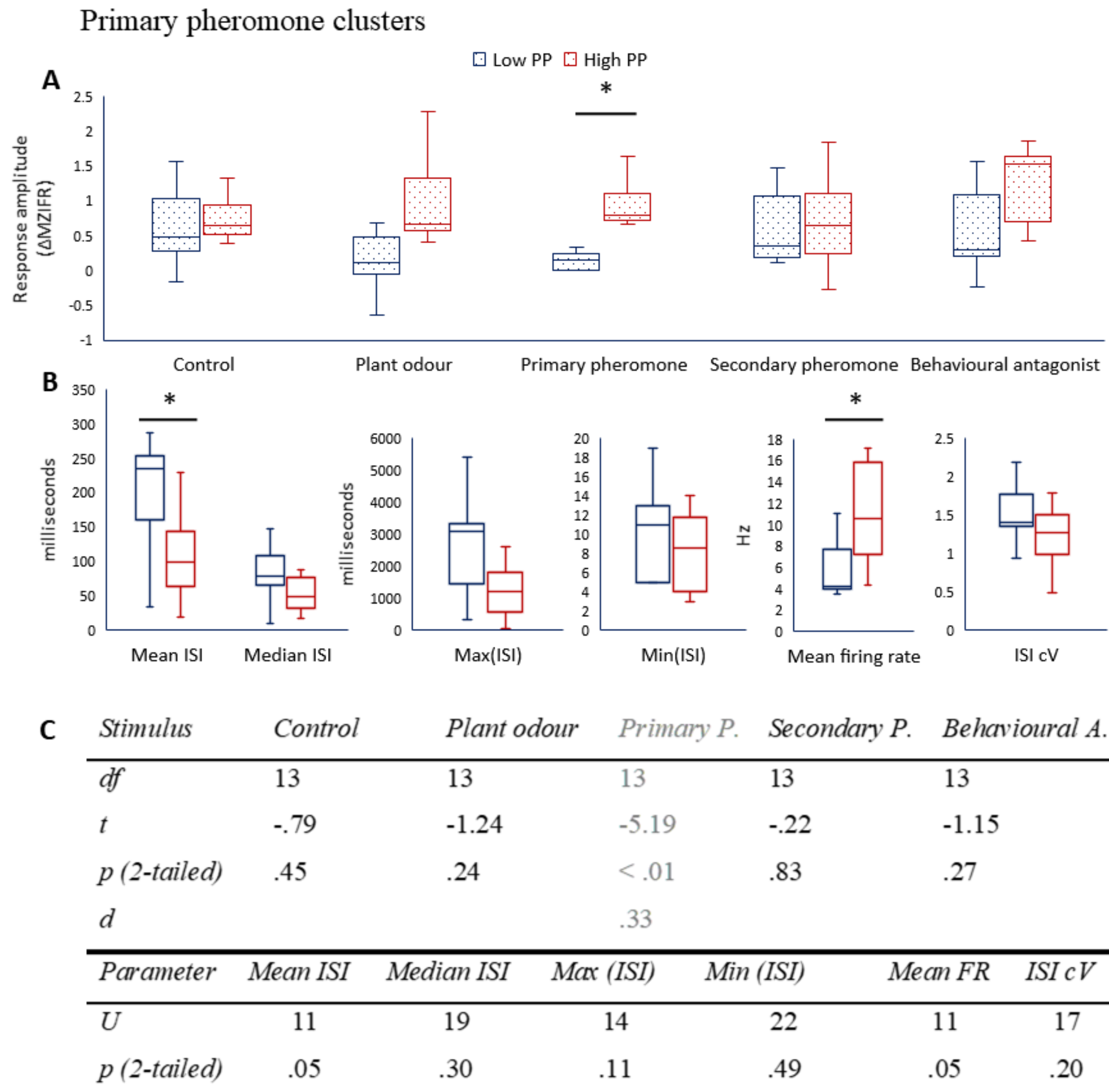
To conduct these tests, we first clustered the neurons based on the response amplitude of four of the five individual stimuli: plant odour, attractive primary pheromone, attractive secondary pheromone, and behavioural antagonist. By using the unsupervised two-step clustering analysis on the stimuli, two clusters were generated with a "good" quality of cohesion and separation (average silhouettes > 0.7). The Mann-Whitney *U* tests showed that the clustering based on plant odour, secondary pheromone and behaviour antagonist had no effect on the spontaneous activity parameters. Inexpertly though, the only difference in the spontaneous activity was across the clusters based on the response to the primary pheromone, where the LNs with a high primary pheromone response amplitude (High PP, $N = 8$) showed a lower mean ISI and a higher mean firing rate than the LNs in the other cluster (Low PP, $N = 7$, Fig. 10-13). The detailed response properties of all the recorded local interneurons during stimulus applications are shown in Appendix III.



Stimulus	*Control*	*Plant odour*	*Primary P.*	*Secondary P.*	*Behavioural A.*
df	*19*	*20*	*13*	*14*	*18*
t	*2.11*	*5.29*	*.97*	*1.12*	*.37*
p (2-tailed)	*.05*	*< .01*	*.35*	*.28*	*.72*
d	*.56*	*.50*			

Parameter	*Mean ISI*	*Median ISI*	*Max (ISI)*	*Min (ISI)*	*Mean FR*	*ISI cV*
U	*52*	*51.1*	*49*	*27.5*	*52*	*51*
p (2-tailed)	*1.0*	*.94*	*.81*	*.08*	*.97*	*.92*

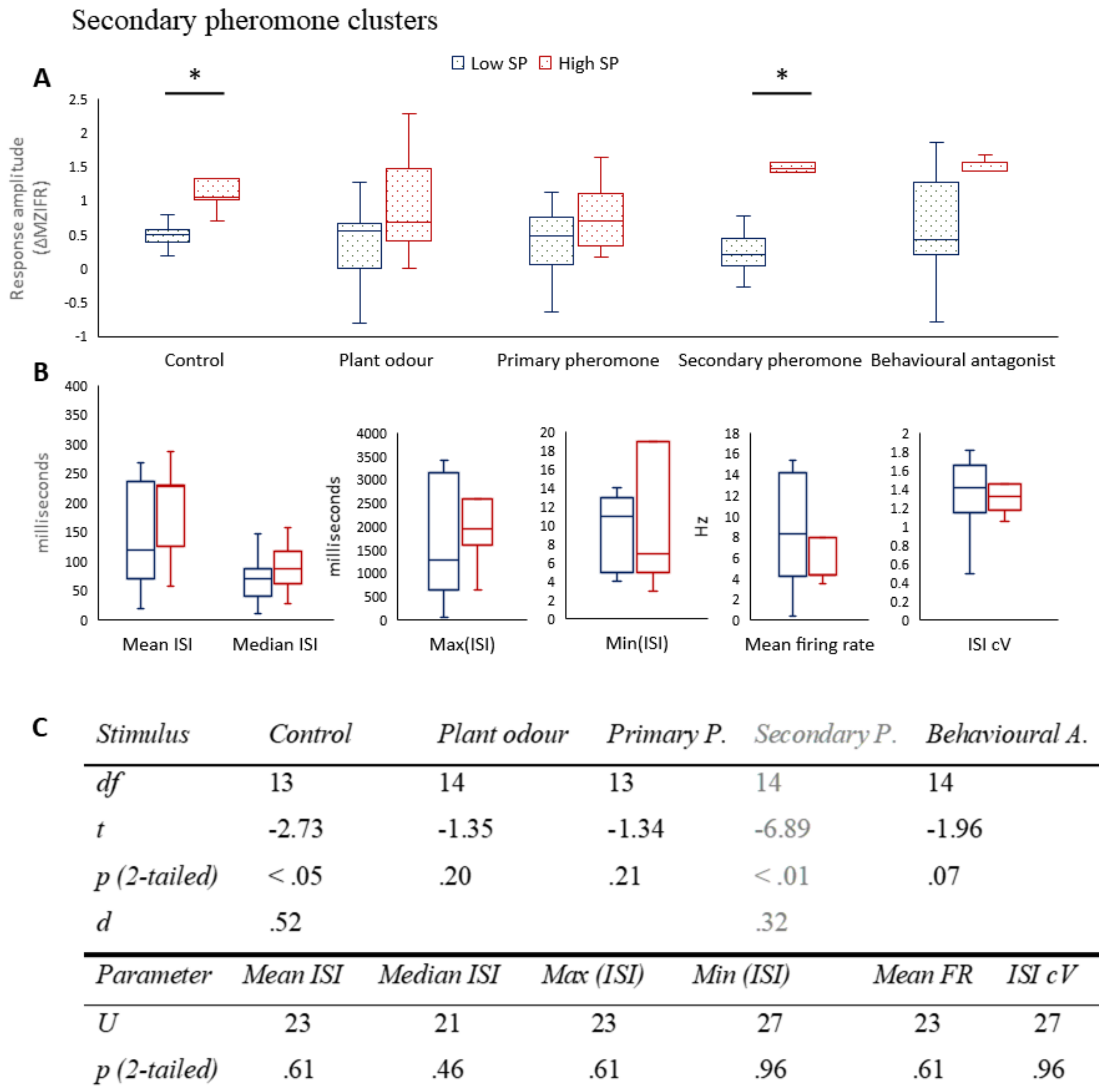
Figure 10. Box plots of the parameters representing the response amplitude (ΔMZIFR) to the 5 odour stimuli during the entire 400 ms stimulation window (A), and the spontaneous activity (B) measured in neurons within the high plant odour cluster (high PO) (N = 15) and the low plant odour cluster (low PO) (N =7). The independent t-test, *, p < .05; **, p < .01, showed that the neurons within the high PO cluster exhibited different response amplitudes across the stimulations, as opposed to the neurons within the low PO Cluster (A). The spontaneous activity did not show any difference across clusters. (C) Statistic data of the comparisons shown in (A) and (B).



Stimulus	*Control*	*Plant odour*	*Primary P.*	*Secondary P.*	*Behavioural A.*
df	13	13	13	13	13
t	-.79	-1.24	-5.19	-.22	-1.15
p (2-tailed)	.45	.24	< .01	.83	.27
d			.33		

Parameter	*Mean ISI*	*Median ISI*	*Max (ISI)*	*Min (ISI)*	*Mean FR*	*ISI cV*
U	11	19	14	22	11	17
p (2-tailed)	.05	.30	.11	.49	.05	.20

Figure 11. Box plots of the parameters representing the response amplitude (ΔMZIFR) to the 5 odour stimuli during the entire 400 ms stimulation window (A), and the spontaneous activity (B) measured in neurons within the low primary pheromone cluster (low PP) ($N = 7$) and the high primary pheromone cluster (high PP) ($N = 8$). The independent *t*-test, *, $p < .05$; **, $p < .01$, showed that the neurons within the low PP Cluster did not differ in response amplitudes across the stimulations, as opposed to the neurons within the high PP Cluster (A). Within the spontaneous activity, there was a difference across clusters. (C) Statistic data of the comparisons shown in (A) and (B).

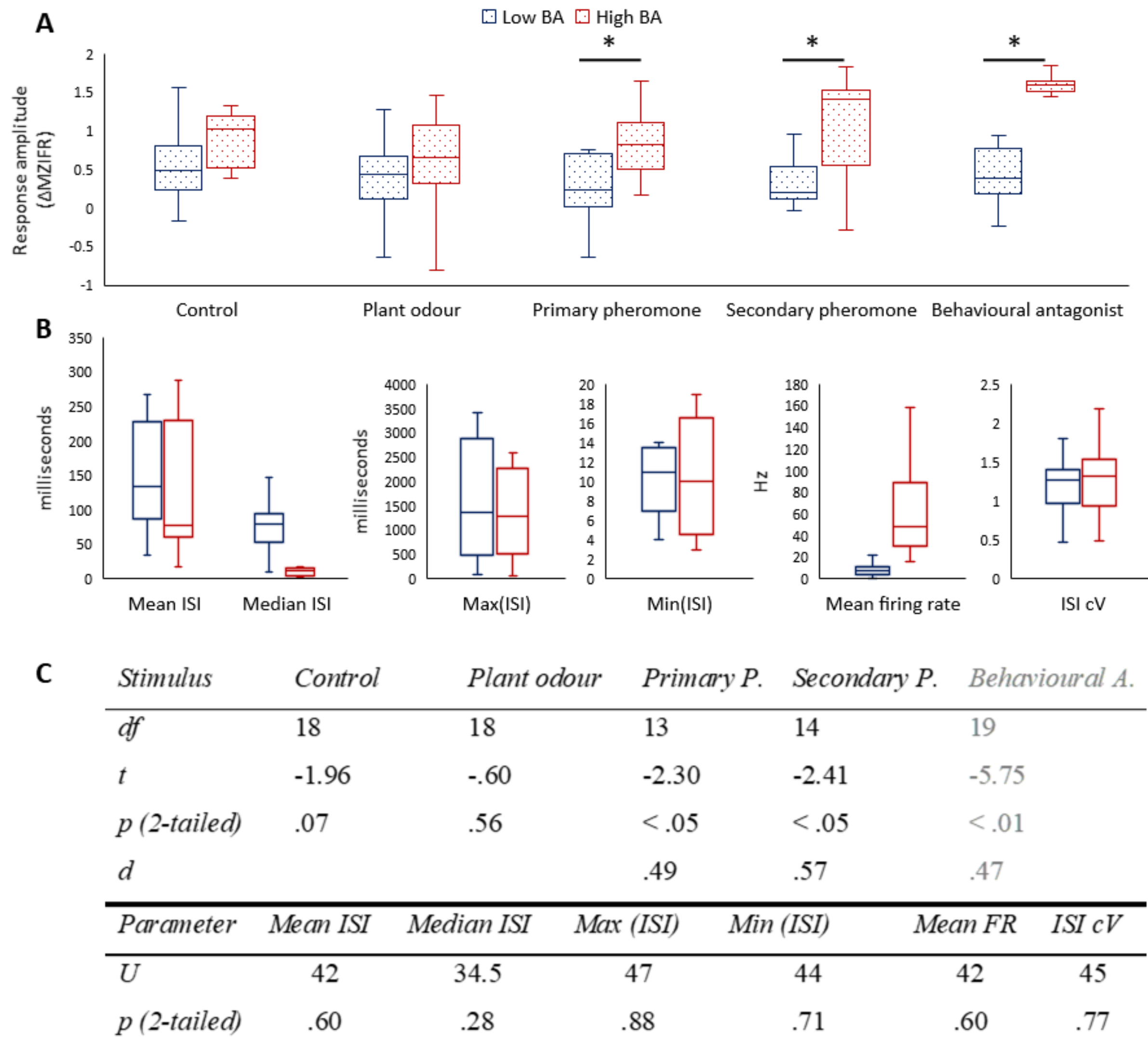


Stimulus	*Control*	*Plant odour*	*Primary P.*	*Secondary P.*	*Behavioural A.*
df	13	14	13	14	14
t	-2.73	-1.35	-1.34	-6.89	-1.96
p (2-tailed)	< .05	.20	.21	< .01	.07
d	.52			.32	

Parameter	*Mean ISI*	*Median ISI*	*Max (ISI)*	*Min (ISI)*	*Mean FR*	*ISI cV*
U	23	21	23	27	23	27
p (2-tailed)	.61	.46	.61	.96	.61	.96

Figure 12. Box plots of the parameters representing the response amplitude (ΔMZIFR) to the 5 odour stimuli during the entire 400 ms stimulation window (A), and the spontaneous activity (B) measured in neurons within the low secondary pheromone cluster (low SP) (N = 11) and the high secondary pheromone cluster (high SP) (N =5). The independent t-test, *, $p < .05$; **, $p < .01$, showed that the neurons within the low SP Cluster differed in response amplitudes across the stimulations, as opposed to the neurons within the high SP Cluster (A). There was no difference in the spontaneous activity across clusters. (C) Statistic data of the comparisons shown in (A) and (B).

Behavioural antagonist clusters



Stimulus	*Control*	*Plant odour*	*Primary P.*	*Secondary P.*	*Behavioural A.*
df	18	18	13	14	19
t	-1.96	-.60	-2.30	-2.41	-5.75
p (2-tailed)	.07	.56	< .05	< .05	< .01
d			.49	.57	.47

Parameter	*Mean ISI*	*Median ISI*	*Max (ISI)*	*Min (ISI)*	*Mean FR*	*ISI cV*
U	42	34.5	47	44	42	45
p (2-tailed)	.60	.28	.88	.71	.60	.77

Figure 13. Box plots of the parameters representing the response amplitude (ΔMZIFR) to the 5 odour stimuli during the entire 400 ms stimulation window (A), and the spontaneous activity (B) measured in neurons within the low behavioural antagonist cluster (low BA) (N = 14) and the high behavioural antagonist cluster (high BA) (N =7). The independent t-test, *, p < .05; **, p < .01, showed a difference in response amplitudes across the stimulations between the neurons within the low BA Cluster and thouse within the high BA Cluster (A). No difference was visible in the spontaneous activity across clusters. (C) Statistic data of the comparisons shown in (A) and (B).

Cross-correlation tests of the response amplitudes between every two stimuli is shown in Fig. 14. Among the given stimuli, plant odour, secondary pheromone and behavioural antagonist were correlated with the control (*p*s < .04), while the response amplitudes evoked by the primary pheromone was the only one that did not correlate with any of the other stimuli (*p*s > .26).

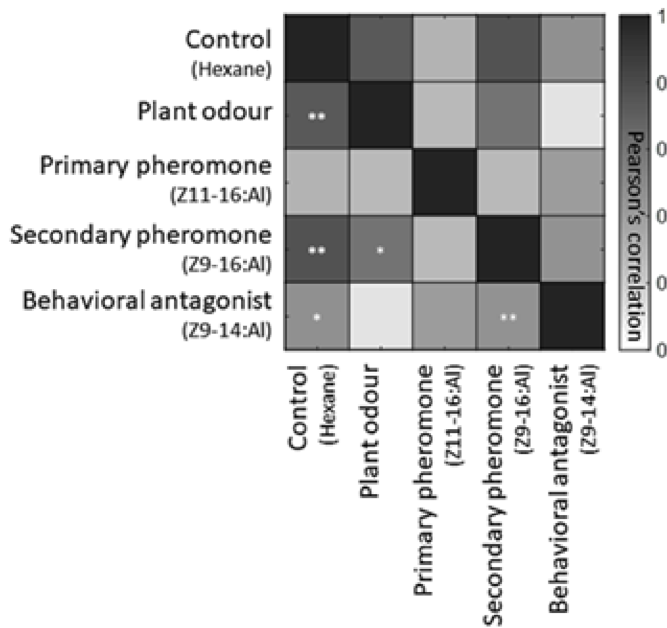


Figure 14. A cross-stimuli correlation plot of the LNs response amplitudes to the five presented stimuli (*N* = 23 LNs), using Pearson's correlation (*, p < .05, ** < .01). The correlation between the response amplitudes of the primary pheromone and that of the other pheromone stimuli is rather low.

4. Discussion

In this thesis, we investigated the electrophysiology of the local interneurons in the moth species, *Helicoverpa armigera*, as this type of neuron has illustrated a unique biological significance when it comes to odour coding in the olfactory local circuit. Both physiological and morphological properties of LNs within the primary olfactory centre were taken into consideration, grounded on intracellular recordings and iontophoretic staining. As these interneurons are highly diverse in many features, such as morphology, connectivity, molecular identity (e.g. neurotransmitters), as well as intrinsic electrophysiological properties (Kepecs & Fishell, 2014), such a task is not without its challenges. The proposition of this thesis was to examine possible classifications of LNs based on their electrophysiological properties and compare the response profiles between different classified groups. A topic for which the investigation of the moth pheromone response in these neurons, is particularly expedient, due to the advanced pheromone communication in this species, including odours with opposite behavioural valences (Kymre et al., 2021). The discussion section presented below, comprises four subsections, where the first two are centred around discussing the waveform impact and the possible connectivity between local interneurons and peripheral odour sensory neurons as well as the AL output neurons. The third section concerns comparisons between the moth and other model organisms, while the last subsection discusses other methodologies used in exploring similar topics, with different focus.

4.1 Mechanisms of depolarized afterpotential generation

During the initial examination of the 23 local interneurons we had obtained, the waveforms of these neurons illustrated a clear juxtaposition, resulting in two groups. One group ($N = 12$) showed an afterpotential similar to that of a hyperpolarized afterpotential, thus termed HAP neurons, while the second group of LNs ($N = 11$) exhibiting a depolarized afterpotential, resulting in the term DAP LNs. The categories were comparable in that both had a transient deflection of the membrane potential following the depolarizing current of the action potential below the resting potential. However, the hyperpolarization periods of the membrane potential in the two groups of LNs showed different trends.

Generally, the intrinsic membrane properties taking part in shaping a neurons waveform, result from activity-dependent conductance changes of ions moving across the membrane via several cation channels (Kandel et al., 2000, p. 157). The main contributing factor to the typical hyperpolarization (e.g., the HAP category in this thesis) is generally accepted to be Ca^{2+}- dependent K^{+} currents, carried by large conductance channels (e.g.,

voltage-dependent BK channels and A-type quick K^+ channels) (Bourque et al., 1985; Maícas Royo et al., 2016; Roper et al., 2003). The underlying ionic mechanisms for the depolarized afterpotential (DAP), however, are not yet fully understood. So far, most studies on this issue have been conducted in various cortical neurons in mammals, showing disparate results on the matter. A common idea is that influx of calcium in some way or other function as an underlying mechanism of DAPs (Andrew & Dudek, 1984; Greffrath et al., 1998; Jung et al., 2001; Kobayashi et al., 1997; Li & Hatton, 1997a, 1997b; Magee & Carruth, 1999; Metz et al., 2005; Stern & Armstrong, 1996; Su et al., 2002). Here, Ca^{2+}-dependent non-selective (CAN) cation channels are suggested to play a key role (Bourque, 1986; Ghamari-Langroudi & Bourque, 2002; Maícas Royo et al., 2016; Teruyama & Armstrong, 2007). Though, it has also been suggested that DAP generation in mammals may involve voltage-gated Na^+ channels, particularly in the neurons located in the medial entorhinal-cortex (Alessi et al., 2016).

In insect studies, DAP has been reported in the AL LNs in the Noctuid moth *Agrotis ipsilon*. The mechanism behind such depolarizations was assumed to be similar to that reported in the mammalian studies, involving the Ca^{2+}-dependent CAN cation channels as a basis for the DAP (Lavialle-Defaix et al., 2015). Therefore, we suggest that the results we obtained from the heliothine moth, showing that the LNs hyperpolarising in a DAP fashion were less bursty during the spontaneous activity than the HAP LNs, may also be valid for interneurons (e.g. periglomerular cells) in the primary mammalian olfactory centre, the olfactory bulb.

Furthermore, the depolarizing potential following the initial after-hyperpolarization has been proposed to be one of the contributing factors to enhance the probability for additional spikes, since a shorter interspike interval in neurons firing with DAPs would be expected (Alessi et al., 2016; Bourque, 1986; Csordás et al., 2020; Duménieu et al., 2015; Ghamari-Langroudi & Bourque, 2002; reviewed by Krahe & Gabbiani, 2004; Li & Hatton, 1997b; Teruyama & Armstrong, 2007). This corresponds with our data, as both the mean ISI and maximum ISI of LNs in the DAP category were shorter than those in the HAP category. It should be noted however, that the irregularity of spike firing, e.g. Poissonian firing, may not be indicated by the low mean ISI and high mean firing rate (Christodoulou & Bugmann, 2001). Thus, it was not surprising that the indicator for burstiness, the ISI c*V*, was shown to be lower within the DAP LNs. It therefore seems, that the depolarizing afterpotential may increase the mean firing rate and decrease the average ISI, all while inhibiting the emergence of bursts to some extent. This being in accordance with previously reported data, that DAPs

do not support bursting activity (Lavialle-Defaix et al., 2015), at least in insects. Though our data showed that the DAPs inhibited the spontaneous burstiness, this effect did not impact the responding period. One possible explanation could be that the depolarizing afterpotential disappeared during the stimulation window, which may be regulated via activated or inactivated Ca2+ channels.

4.2 The role of local interneurons as part of the antennal-lobe local circuitry

Within the antennal lobe, information is processed mainly through three neuronal types functioning together as a local network, the OSN, the LNs, and the PNs. The importance of OSNs lies in their role as conveyers of the odour stimulation from the periphery to the primary olfactory centre, thus initiating the process of information transmission and transformation within the AL (Hildebrand & Shepherd, 1997). Meanwhile, the PNs function as the AL output neurons, relaying the processed odour information to higher order areas (reviewed by Ache & Young, 2005; Homberg et al., 1988). A prominent feature of the local network within the AL glomeruli, is the feedforward relay of a larger number of OSNs onto a smaller number of PNs (Stocker, 2001; Wilson, 2011). However, within this sensory convergence of a higher amount of inputs onto a lower amount of outputs, the signals are not simply transferred between these neurons. Connections within and between the glomerular structures in the AL are facilitated by LNs, establishing synapses with axon terminals of OSNs (Ignell et al., 2009; Olsen & Wilson, 2008; Root et al., 2008) and reciprocal dendritic interactions with PN and other LNs (Huang et al., 2010; Wilson & Laurent, 2005; Yaksi & Wilson, 2010). Herein lies the functional significance of LNs; with their diverse innervation patterns within the glomeruli, these neurons constitute a global computational core (Ng et al., 2002), altering the input and output relationship within the AL (Chou et al., 2010; Reisenman et al., 2011).

The synaptic connections formed between these neurons can be quite diverse, ranging from feedforward interaction from OSN - LN - PN, or LN - LN - PN, to local feedback connections between a PN and a LN (Christensen et al., 1993; Distler & Boeckh, 1997; Distler et al., 1998; Olsen & Wilson, 2008; Root et al., 2008). This variety of connective organisation speaks to the complex intrinsic interplay occurring between LNs and the other two AL neuronal segments, whereby the LNs’ ability to assert gain control through presynaptic inhibition and/or excitation results in synchronisation and modulation of the PNs coding (Christensen et al., 1993; Ignell et al., 2009; Olsen & Wilson, 2008; Root et al., 2008; Seki et al., 2010; Shang et al., 2007).

Taking this into account, it is interesting that our results only showed a difference in the intrinsic response activity of the LNs during stimulation with the primary pheromone. These distinctions included neurons with a strong response revealing a relatively low mean ISI and a high mean firing rate, while LNs responding to the other stimuli did not display similar intrinsic reactions. It is also worth noting that our cross-correlation results showed that the response evoked by the primary pheromone was less correlated with the response evoked by other pheromone stimuli within the LNs (Fig. 14), a result that can be taken to be in agreement with previous reports on the output organization of pheromone signals. The general thought is that the individual glomeruli of the male-specific MGC unit are tuned to specific pheromone inputs, with the cumulus being related to the primary pheromone component, the dorsomedial posterior (dmp) primarily with the secondary pheromone component, and the dorsomedial anterior (dma) with the behavioural antagonist input (Wu et al., 2015). Additionally, there are reports indicating that the MGC PNs transmit to distinct output areas based on the behavioural valance of the signal they convey, with the primary pheromone PNs appearing to project to a segregated region in the protocerebrum, called the superior lateral protocerebrum (SLP), while the secondary pheromone and behavioural antagonist are represented in the lateral horn (LH) (See Fig.15 from Kymre et al., 2021), the center connected to both learned and innate behaviour (Schultzhaus et al., 2017). In line with this, our results showed that the Z11-16:AL-responding LNs with special properties, such as a higher intrinsic firing rate, may serve an important function within a unique neuronal circuit linked to attraction.

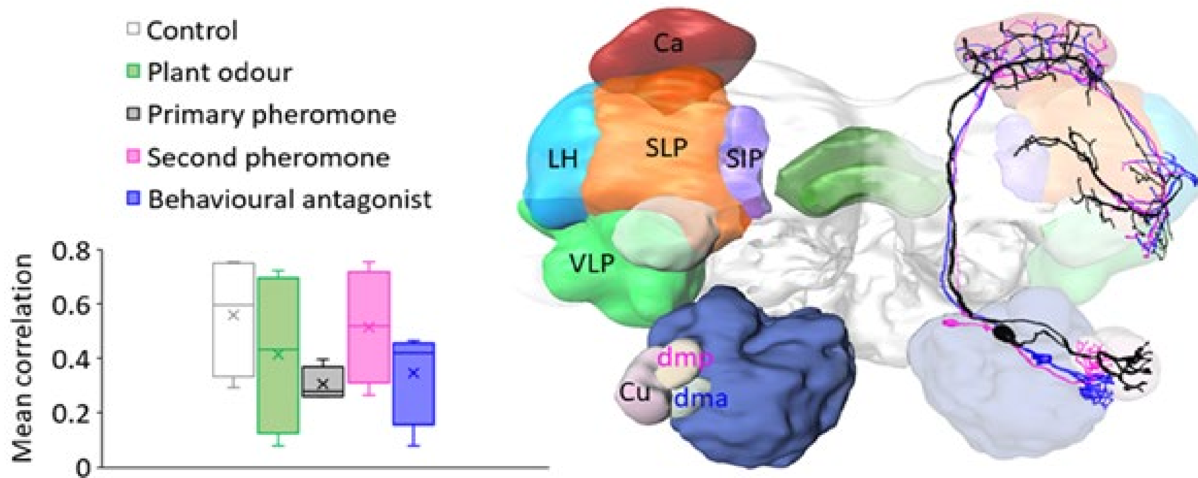


Figure 15. The putative connection between pheromone response pattern in LNs with the protocerebral projection organization of output neurons of the macroglomerular complex (MGC). (Left) The mean paired correlation of each given stimulus with the other odorant stimuli. The data was adapted from the Pearson's correlations in the Result section 3.1.3. (Right) Projection pattern of MGC output neurons in a representative brain in dorsal view, illustrating the brain neuropils targeted by the projection neurons (PNs) originating from cumulus is different from that innervated by the other two types of MGC-PNs, originating from the two smaller units. Ca, calyces of the mushroom bodies; LH, lateral horn; SLP, superior lateral protocerebrum; SIP, superior intermediate protocerebrum; VLP, ventrolateral protocerebrum; Cu, cumulus; dmp, dorsomedial posterior unit; dma, dorsomedial anterior. Adapted from Kymre et al. (2021).

Furthermore, the manner in which our LNs responded to the behavioural antagonist showed signs to be applicable to the primary and secondary pheromone components as well, which may be interpreted as a sign that the behavioural antagonist is one of the most represented pheromone stimuli within the MGC, thereby illustrating the general pattern of synaptic interactions taking place within this glomerular unit. Compared to the other two pheromone components, the behavioural antagonist has been found to possess a functional duality (so far only reported in *H. armigera*), acting to inhibit the pheromone attraction at higher dosages, and as an agonist at lower dosages (Chang et al., 2017; Gothilf et al., 1978; Kehat & Dunkelblum, 1990; Wu et al., 2015; Zhang et al., 2012). Therefore, our data could be interpreted thus, that signals related to the behavioural antagonist, though still segregated at the stage of peripheral input, evoke a unified response within LNs related to a more complex circuit within the MGC units unlike that of the primary and secondary component. Thus, the behavioural antagonist signals might not only be confined to the dma, explaining the simultaneous response to the other pheromones. Interestingly, recent studies in this species have found PNs from the cumulus to be excited not only by the primary pheromone but also by the behavioural antagonist (Chu et al., 2020; Kymre et al., 2021).

4.3 How the study of local circuits in an insect model is relevant for understanding odour processing in the human brain

Owing to their unique adaptations to distinct ecological niches and relatively simple nervous system, insects have become favoured subjects for studying chemosensory systems. As their olfactory system, in addition, is highly comparable with that of humans, insects are widely utilized for mapping signal pathways at an organizational level within neural systems, from the periphery to higher brain centres. Furthermore, the olfactory system of insects exhibits organizational simplifications as opposed to the human, one example being the number of glomeruli, which in humans is about ~5500 (Maresh et al., 2008) and less than 80 in moths (Zhao, Ma, et al., 2016). Fruit flies have less than 50 (Laissue et al., 1999). Notably, studies in humans have discovered that only a small proportion of the expressed olfactory receptors are functional (meaning that the functional glomeruli is also lessened)(Brunet et al., 1996; Buck & Axel, 1991; Buck, 1996; Gilad & Lancet, 2003; Goldman et al., 2005). Thus, to better understand the neuronal basis of olfaction, the ideal option is to use a suitable organism that shares similarities with the neural architecture of humans and at the same time has an easily accessible brain for performing relevant experimental investigations.

Hence, it is worth questioning whether the use of a moth species, such as the one we selected in this study, could report more information than other insect models, more specifically the fruit fly (*Drosophila melanogaster*), being the principal model species for the study of olfaction (Rybak et al., 2016). In recent years, studies on the OSNs (de Fouchier et al., 2017; He et al., 2021; Liu et al., 2013) and PNs (Chu et al., 2020; Kymre et al., 2020; Kymre et al., 2021) within the olfactory system of Lepidoptera, have shown that this is as an excellent model. Their stereotyped odour-guided behaviour based on chemosensory sensitivity and selectivity show a remarkable ability to track minute amounts of pheromones and female relevant odorants at a distance of more than one kilometre (reviewed by Kaissling, 2014; Leal, 2005), making tracking the neuronal response to this type of stimuli more reliable. Moreover, the unique manner of species-specific chemical communication in moths, reflected through the female moth producing a mixture of several pheromone components in a certain ratio, is different from that in *D. melanogaster*, where the study of innate attraction or aversion is mainly related to food/plant odours (Min et al., 2013; Ronderos et al., 2014; Ruebenbauer et al., 2008; Semmelhack & Wang, 2009). The valence of such odour responses, are highly dependent on the concentration, meaning that increasing an odour in this respect may activate olfactory receptors not only tuned to this specific odour, but also to other ligands (Semmelhack & Wang, 2009). In addition, experiments using food

odours are highly dependent on the internal status of the subject insect, as food deprivation enhances behavioural responses (Krashes et al., 2009; Root et al., 2011). In selecting sex pheromones as the odour stimuli however, the individual differences can be reduced, as tracing the source of a pheromone is related to a hardwired behaviour in comparison with that of foraging.

Another consideration is the number of glomerular units within the AL of male moths, with only three being pheromone sensitive (MGC) (Zhao, Chen, et al., 2016; Zhao, Ma, et al., 2016). In addition, the amount of OSNs expressing sex pheromone receptors is much larger than the number of OSNs expressing plant odours (Hansson & Anton, 2000; Hartlieb et al., 1997; reviewed by Homberg et al., 1989; Rospars, 1988), thereby resulting in a marked higher convergence in the MGC than that in the OG (Boeckh & Ernst, 1987; Root et al., 2007; reviewed by Stocker, 1994). Therefore, the output neurons from each glomerulus within the pheromone-specific region, MGC, display a remarkably low response threshold, being highly sensitive to pheromone stimulation (Jarriault et al., 2010; Jarriault et al., 2009; Rospars et al., 2014; van Drongelen et al., 1978). This may indicate a contrast in the response to pheromones versus food or plant odours including a relatively strong pheromone response in LNs innervating both subsystems.

4.4 Methodological considerations

In order to studying olfactory processing in *H. armigera*, we utilised the method of sharp electrode intracellular recording, so as to perform single cell recordings in vivo and access information about the dynamics of the membrane potential directly, such as the firing properties of a neuron as well as subthreshold synaptic activity. One of the limitations of this technique, however, is that it is carried out without being able to locate the exact site of the recording electrode (e.g. the recording site could be in the dendrites, the main axons or the soma), often generating substantial variation in the electrophysiological data. In comparison, intracellular recordings using a patch clamp technique would offer an opportunity to aim for the soma under visual guidance *in vivo*, thereby providing a greater targeted control during the high-resolution measurements (Cid & Liset, 2019; reviewed by Hill et al., 2020; Li et al., 2004). Other factors that may affect how successful sharp electrode measurements are, is the size of the neuron, the amounts of neurons within the specific area of recording (neuronal density), and mechanical instabilities, as highly stable conditions are required in order to obtain accurate data (Cid & Liset, 2019). Recordings using the sharp electrode technique are thus more likely to result from distant and elaborate dendritic trees.

In using insect models to obtain information about the single cell, this is often provided *in vivo*, whereas in humans as well as other mammals, such as rodents, it is retrieved *in vitro*. Recordings are then usually obtained from isolated brain slices, which allows greater onset control in terms of pharmacological manipulation than *in vivo* measurements, retaining the local architecture and synaptic morphology of the cell (Hill et al., 2020). However, the sharp electrode technique can preserve the high integrity of the brain, something which is less possible if applying a patch clamp method of the slice technique. In order to explore the general brain function in humans, it is also possible to use functional magnetic resonance imaging (fMRI). An advantageous procedure, as it is non-invasive, while at the same time producing relatively good spatiotemporal resolution of an entire network within engaged brain areas by measures of the haemodynamic changes after enhanced neural activity. However, the limitations of such a method lies in that only neuronal mass activity is reflected, which cannot yield the same amount of information at the electrophysiological level as that of intracellular recordings (Logothetis, 2008).

Although, fMRI has been widely used to reveal higher-order olfactory processing in humans, this approach is unsuited for studying specific components of the olfactory bulb, such as LNs. Also, as the primary olfactory centre is located on the inferior (bottom) side of the cerebral hemispheres, it is unfortunately inaccessible to fMRI (Fournel et al., 2020). Thus, in the attempt to better understand the neural basis of olfaction, we believe this thesis used a suitable option of measuring electrophysiological data, provided by an ideal organism, sharing neuro architectural similarities with humans, while at the same time having an easily accessible brain for performing relevant experimental investigations.

For the statistical analyses our sample consisted of 23 LNs, a size that can be considered to yield low statistical power, heightening the probability for introducing false positives and false negatives (type I and type II errors) during testing (reviewed by Button et al., 2013). In addition to the small sample size, some of the parameters were not normally distributed, a condition which made us question whether the use of the parametric model was the best for our analyses (Siegel, 1957).

Furthermore, some of the recorded data from the response activity of our LNs were missing during the experiment due to the short window of stable neuronal contact, something which must be taken into consideration while interpreting some of the findings. For example, the notion that that the spontaneous activity parameters only affected the response pattern of the LNs reacting to the primary pheromone stimulation.

5. Conclusion

In trying to provide information on the physiological properties of LNs, a distinct dissimilarity in the pattern of the neurons' waveforms were discovered. The two characteristic kinds of afterpotentials were categorized into the hyperpolarized afterpotential (HAP) and the depolarized afterpotential (DAP). The two types of LNs displaying each of these afterpotentials were then examined with respect to the firing activity. The results revealed that the DAP LNs impacted the intrinsic firing rate but not the response activity, leading us to question whether the contribution of such an afterpotential might be essential for signal transduction and integration within the local neural network. The answer to which, may only be found through additional studies on the topic. In accordance with previous reports, the specific spontaneous firing activity of the primary pheromone pathway LNs suggests that these neurons are involved in a unique neuronal circuit linked to attraction. We also noticed that the behavioural antagonist signals seemed to evoke a more unified response within the local circuit of the MGC, which can signify that the LNs activated by this stimulus contribute to a more complex circuitry within this glomerular assembly as opposed to those responding to the primary and secondary pheromone component.

In discovering the depolarized afterpotential, and its effect on the LNs intrinsic activity, the similarity between the olfactory system of insects and mammals become relevant yet again, as it provides cause to speculating whether parallels might also be echoed in the intrinsic properties, such as the ion channelling of the LNs. In order to determine the conductance mechanisms that underlie the DAP in LNs, it would be interesting to further examine the endogenous properties of these neurons, in addition to examine whether the morphological characteristics of the LNs in some way might be related to such distinct waveforms.

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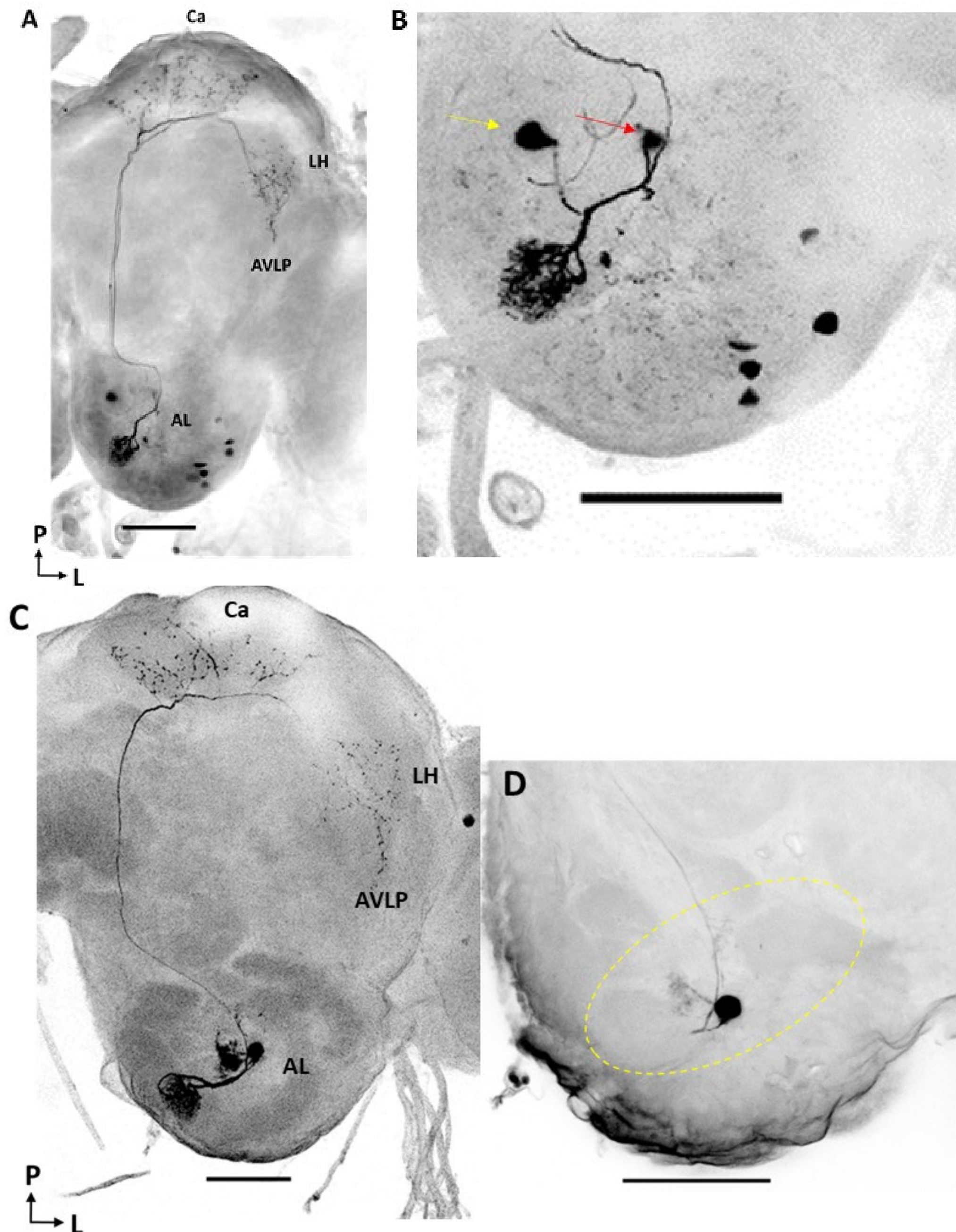
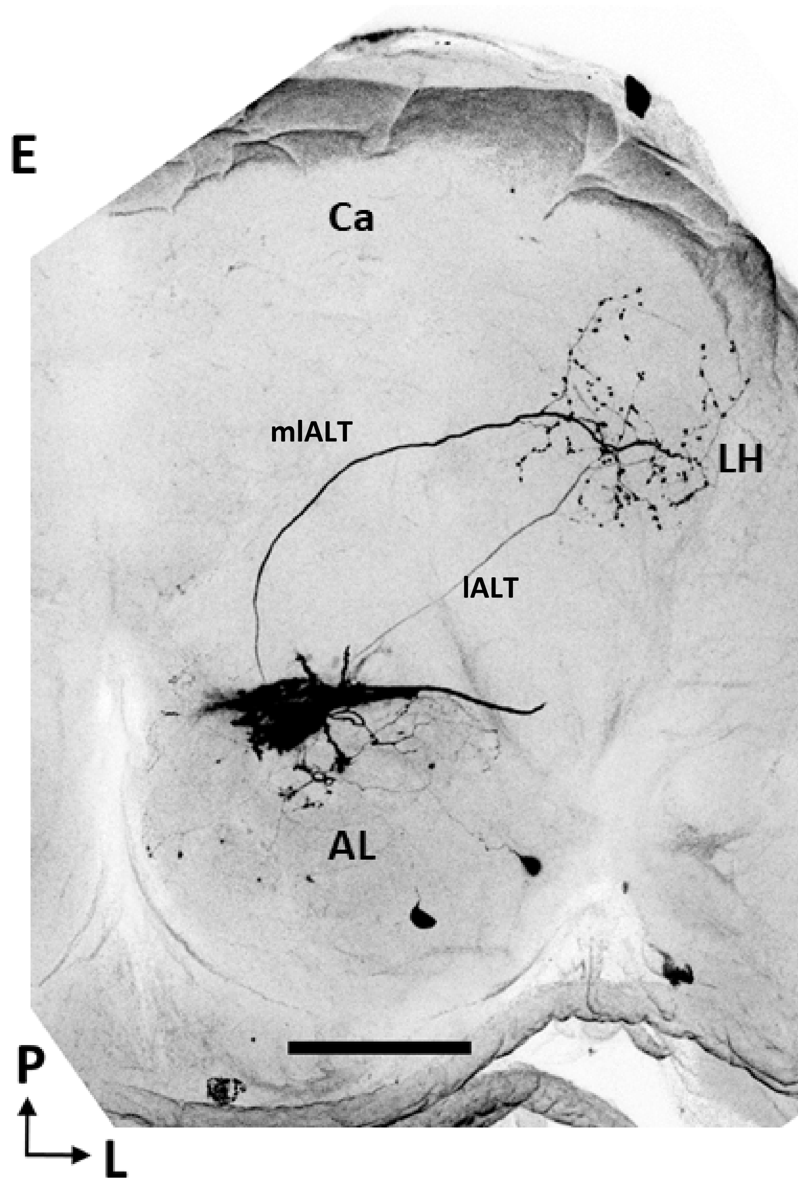
Appendix I

Medial tract projection neurons, mediolateral tract projection neuron and lateral tract projection neuron



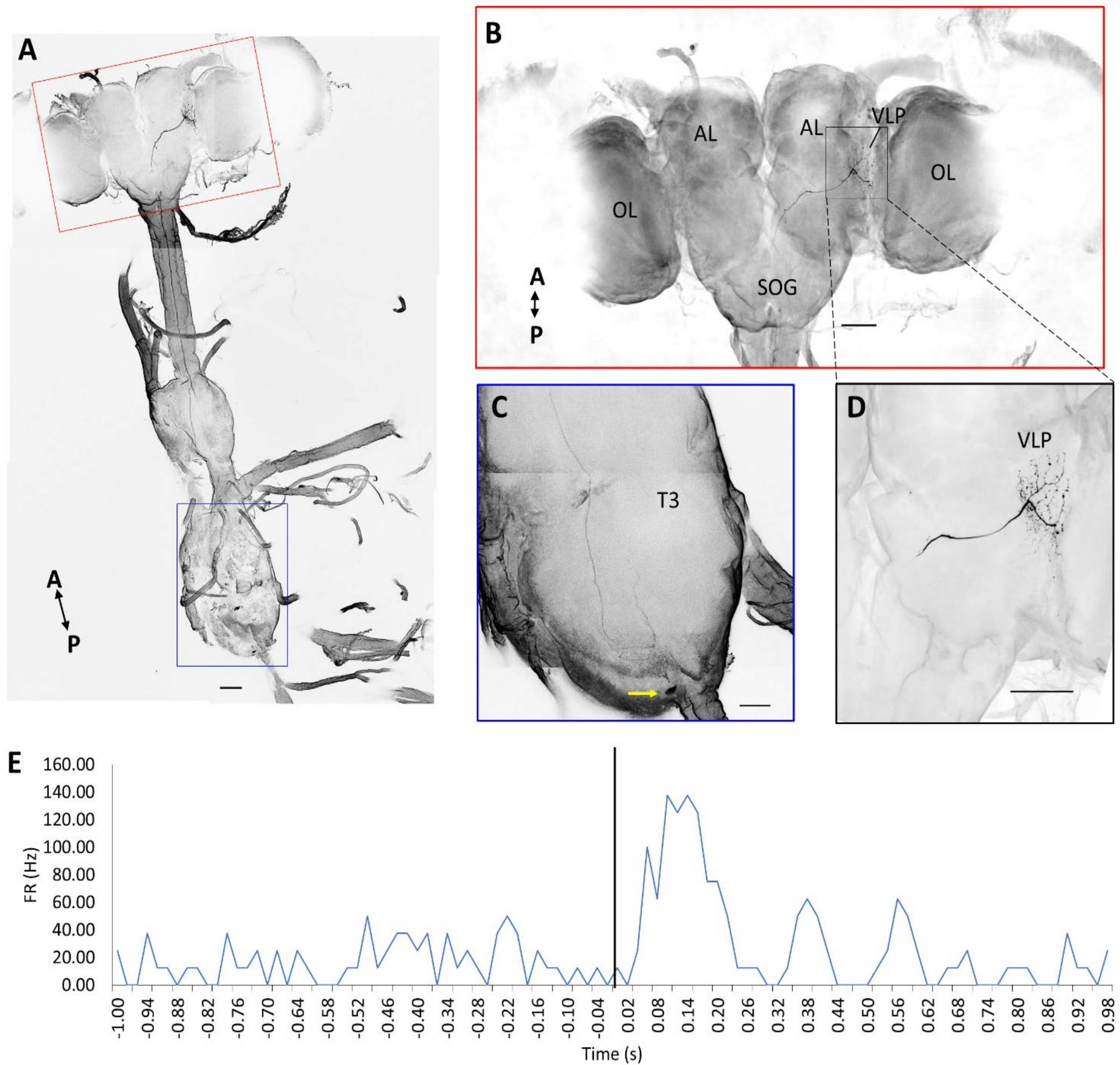
Appendix I, Figure 1. Confocal image of two co-stainings, containing three projection neurons (PNs) confined to the medial track (mALT), seen from dorsal view. (A) One neuron is uniglomerular, the other is multiglomerular, both with axonal innervations found in the calyces (Ca), the lateral horn (LH) and partly the anterior ventro-lateral protocerebrum (AVLP). (B) The soma of these two neurons are located at the medial cell cluster (*red* for the uniglomerular PN and *yellow* for the multiglomerular PN) of the antennal lobe (AL), coincides with the previously reported mALT PNs anatomy (Ian, Zhao, et al., 2016; Kymre et al., 2021). (C) A multiglomerular mALT PN with innervations within the AL is located to two glomeruli, the projections residing in the calyces (Ca), the lateral horn (LH) and the anterior ventro-lateral protocerebrum (AVLP). (D) The cell body is located in the lateral cell cluster (*yellow* dash circle). Scale bars: 100 µm.



*Appendix I, Figure 2. A c*onfocal image of a co-staining, containing two PNs, seen from dorsal view. (E) One mediolateral antennal lobe tract (mlALT) PN and one lateral antennal lobe tract (lALT) PN, both innervating the lateral horn (LH) exclusively. The Somata are located at the lateral cell cluster. Scale bars: 100 µm

Appendix II

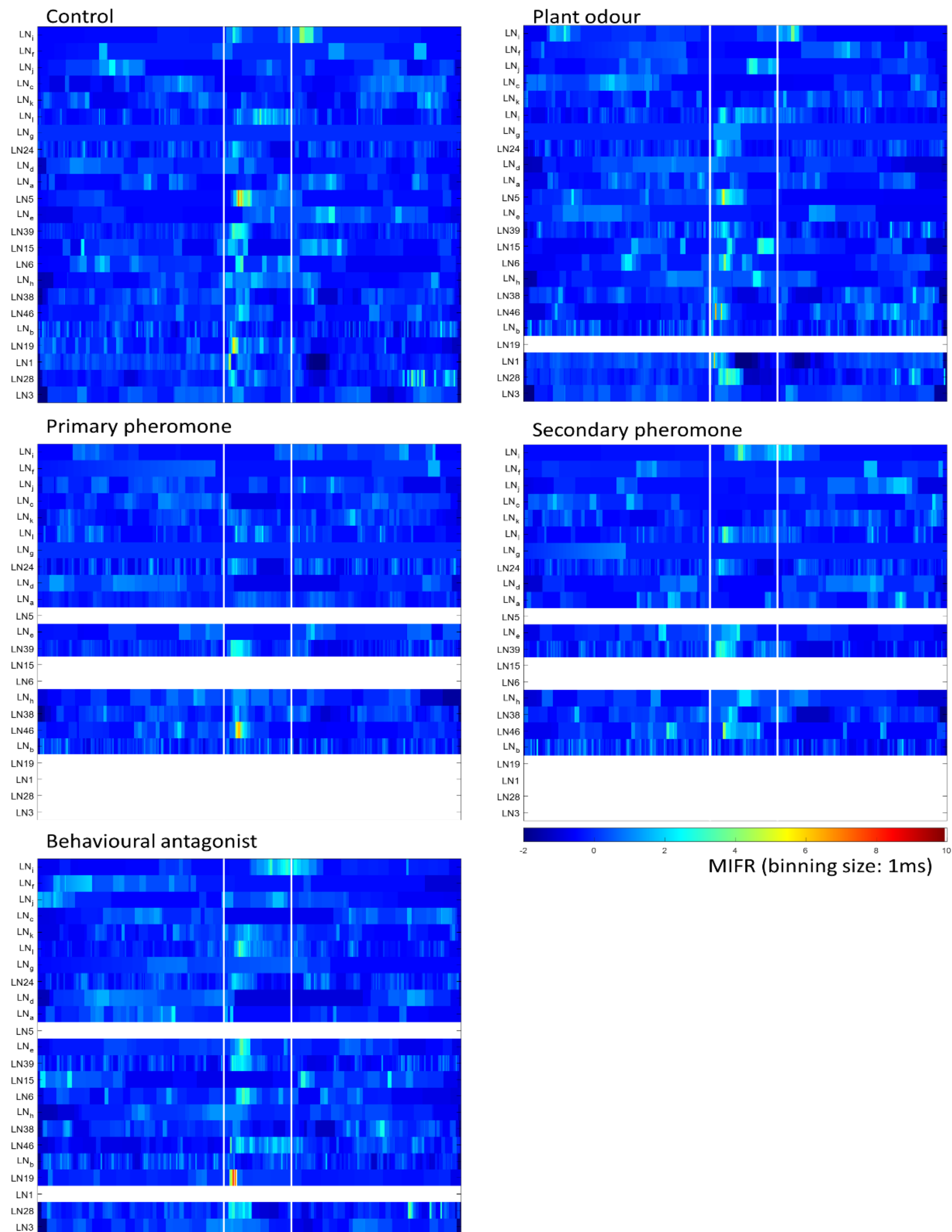
Morphology and physiology of a sound sensitive ascending neuron projecting to the SOG and VLP.



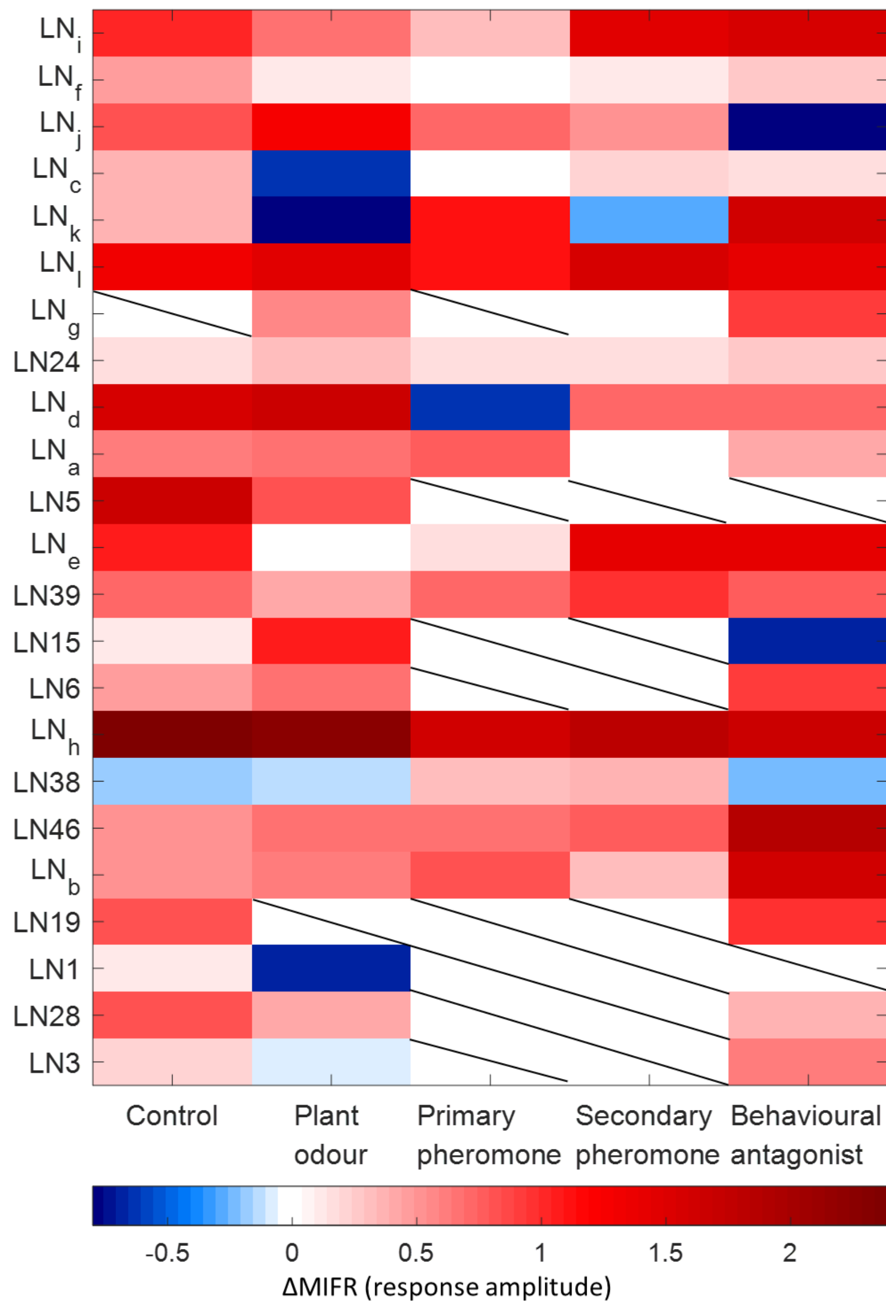
Appendix II, Figure 4. Morphological and physiological characteristics of the sound sensitive neuron. (A) Confocal image of a sound neuron in frontal orientation. (B) Neural terminals in the ventrolateral protocerebrum (VLP) and short neural branches in the subesophageal ganglion (SOG) (*red* square). (C) The soma (*yellow* arrow*, in blue square*) is located at the metathoracic ganglion (T3). (D) A higher magnification image of the *black* square in (B) shows the detailed neural processes in the VLP in a frontal view. (E) Mean response trace of the neuron firing rate showing excitatory responses to the sound of hand clapping. The vertical line illustrated the onset of the clap. The temporal resolution of the excitation of the neuron showed a tonic pattern. Unlike the previously reported sound neuron, which responded strongly to ultrasound (Pfuhl et al., 2014), this neuron appeared response to general sounds as well. OL, optic lobe. Scale bars: 100 µm.

Appendix III

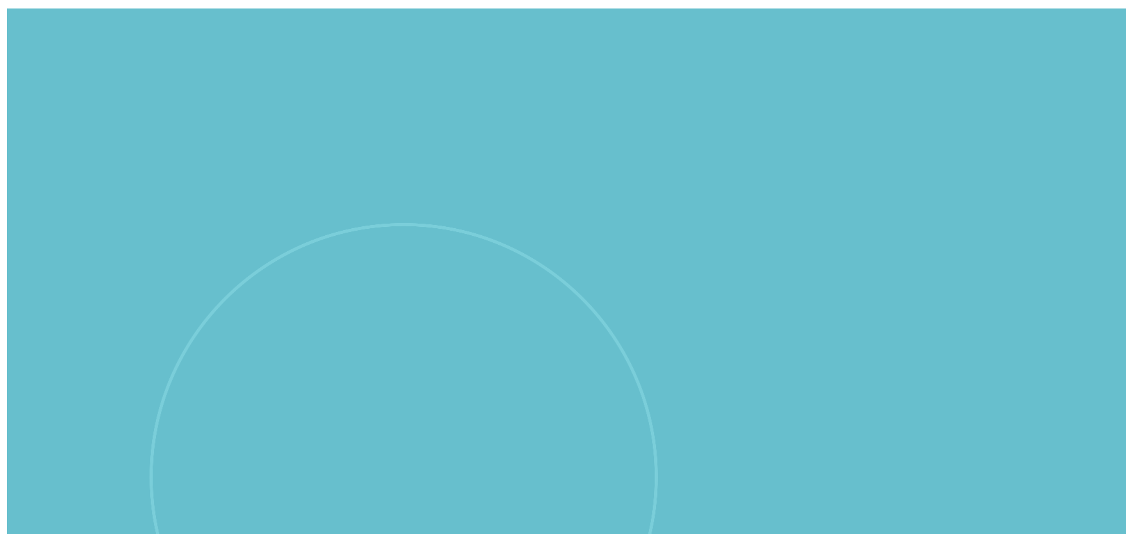
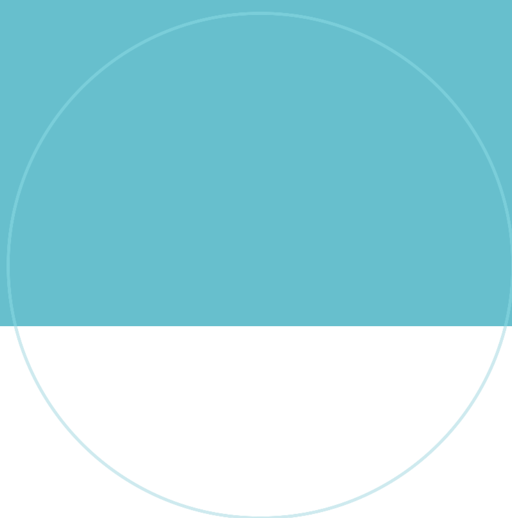
Heat maps of the response properties of all the recorded local interneurons during stimulus applications. Neurons in the following figures were sorted in the same random sequence.



Appendix III, Figure 5. An overview of temporal response properties of the recorded LNs. The heat map showed the neuronal activity around the period of odour stimulation (1s prestimulation window, 0.4s stimulation window and 1s poststimulation window). Each row shows the mean Z-scored instantaneous firing rate (MZIFR) of every single neuron to the same stimulus. The empty rows indicate missing data.



Appendix III, Figure 6. Overview of the response amplitude and pure response of each recorded LN. Each row shows the response amplitude during stimulations of control, plant odour and three insect-produced odours. Black lines indicate the missing data during experiments. MZIFR; mean Z-scored instantaneous firing rate.

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