Master's thesis

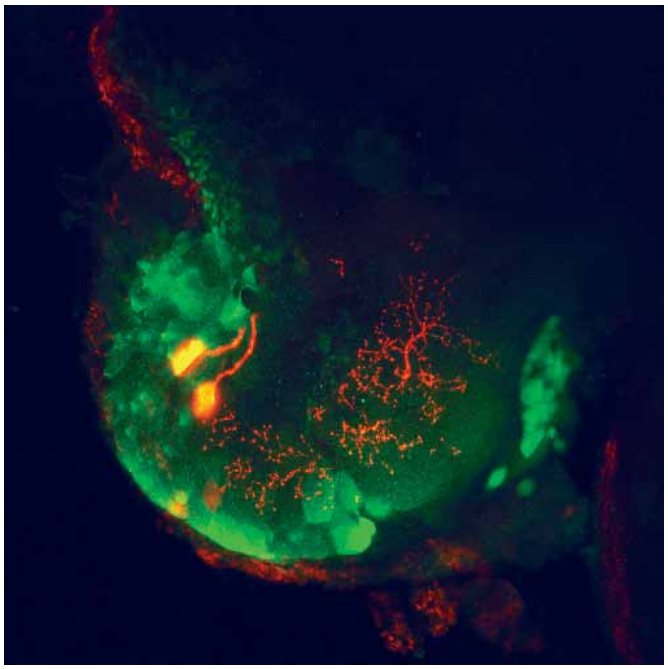
NTNU
Norwegian University of
Science and Technology
Faculty of Social Sciences
and Technology Management
Department of Psychology

Jens Marius Halvorsen

GABA immunostaining in the central olfactory pathway of the moth brain - visualization of single neurons and neural populations

Master thesis in psychology

Trondheim, autumn 2010



NTNU – Trondheim
Norwegian University of
Science and Technology

Forord

Masteroppgaven i biologisk og kognitiv psykologi ble utført hos gruppe for nevrofag, medisinsk teknisk forskningssenter, NTNU.

Jeg vil benytte anledningen til å takke min veileder førsteamanuensis Bente Berg for at jeg fikk være med på et spennende prosjekt. Det har vært svært lærerikt, mye takket være tett oppfølging og mye god veiledning. Å ha en veileder som alltid gjør seg tilgjengelig for spørsmål og diskusjon har vært veldig viktig og inspirerende. Jeg vil takke bi veileder Dr. Xin-Cheng Zhao for tålmodig opplæring i og hjelp med de praktiske sidene ved laboratoriearbeidet samt for mange gode tips underveis. Det har også vært veldig hyggelig å bli kjent med den flotte og hjelpsomme gjengen med ansatte og studenter som har vært på laben i prosjekt perioden.

Grunnforsking på et modellobjekt er på mange måter et ytterpunkt for psykologi faget. Jeg er takknemmelig for muligheten jeg har hatt til å fordype meg på et så avgrenset område som nattsvermer hjernen. Det har vært svært inspirerende å få et innblikk i helt grunnleggende biologiske elementer. Selv det tilsynelatende enkle er ofte svært komplekst og sammensatt.

Det ble et forholdsvis langvarig prosjekt, mye på grunn av at min kjære Helena kom til verden. Jeg støtte også på en del praktiske problemer underveis og jeg vil takke de som gjorde det mulig å få gjennomført prosjektet etter at jeg flyttet tilbake til Oslo. Einar Otto Stangvik, Eirik, Arnhild og Bjørn Hassel, tusen takk!

Jens Marius Halvorsen

Abstract

γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system of most living organisms. A large number of neurons that contain GABA has been found in the primary olfactory centers of both humans and insects. The first integration center in the insect olfactory pathway is the antennal lobe. The antennal lobe is well suited for studying the basic principles that underlie processing of olfactory information. The moth is well suited model candidate because of the relative simplicity of the olfactory system. Only four types of neurons constitute the system: Sensory neurons, projection neurons which carry signal information to higher integration centers in the protocerebrum, local interneurons who communicate within the antennal lobe, and centrifugal neurons which modulate the information stream by sending projections from the protocerebrum into the antennal lobe. Most of the antennal-lobe local interneurons is presumed to be GABAergic. The distribution of GABA was investigated through application of a GABA aniserum. Both whole brain preperations and viberatome sections were scanned with a confocal laser microscope. In addition to pure GABA stainings, double labelings of pre-stained single neurons and antnnocerebral tracts were carried out. The results were finally analysed and presented through imaging software. The results were largely consistent with earlier reports. In the noctuid moth *Heliothis virescens* and *Helicoverpa assulta* the antennal lobe local interneurons are all located in the lateral cellcluster. In addition a few GABAergic projection neuron somata is also located in this cellcluster. Comprehensive immune-reactivity has been found in parts of the antenno-cerebral tracts that are constituted by projection neuron axons. The possible function of GABAergic neural networks in the olfactory system is addressed in the discussion.

1.0 Introduction

The ability of enjoying food depends on our two chemical senses, smell and taste. In fact, olfaction constitutes a considerable portion of what we usually perceive as taste. In addition to providing information about nutrition, the olfactory sense enables us to smell flowers and perfumes, and it may even warn us of potential hazards like poisonous fumes, gases, or fires. Compared to other sensory systems, like vision and hearing, olfaction might seem insignificant for humans. The repertoire of obviously "odor-driven" behaviours expressed by a number of animal species has definitively been reduced in humans. The olfactory pathways are strongly associated with brain areas considered distant in terms of phylogenetic development (Bear, Connors and Paradisio, 2007; Smith, 2008). This might further indicate a less important role of the olfactory system in humans today. Shepherd (2006), however, challenges us to rethink this assumption. One of his arguments is that olfactory information is closely integrated with gustatory information in the human brain. This integration which takes place in neo-cortical regions has to be envisaged to fully understand the significance of human olfaction (Figure 1).

Despite of species specific arrangements of the olfactory pathways, in particular as concerns higher synaptic levels, a number of striking similarities both as concerns structure and function have been found between distantly related species, as for instance vertebrates and invertebrates (Hildebrand & Sheperd, 1997). It seems as though nature has conserved, or alternatively reinvented particular neural arrangements along different evolutionary lines in order to deal with the task of processing olfactory information. One such universal principle is the organization of neuropil areas found in the primary olfactory centre of most living organisms, where terminals of olfactory receptor neurons are nested together and surrounded by glia cells to form anatomical subunits of what is termed glomeruli. The glomerular structures are fundamental for the neural network in this particular brain region and involve principally the same functional classes of neurons across different species (Shepherd, 1972; Shepherd & Greer, 1990; Anton & Homberg, 1999).

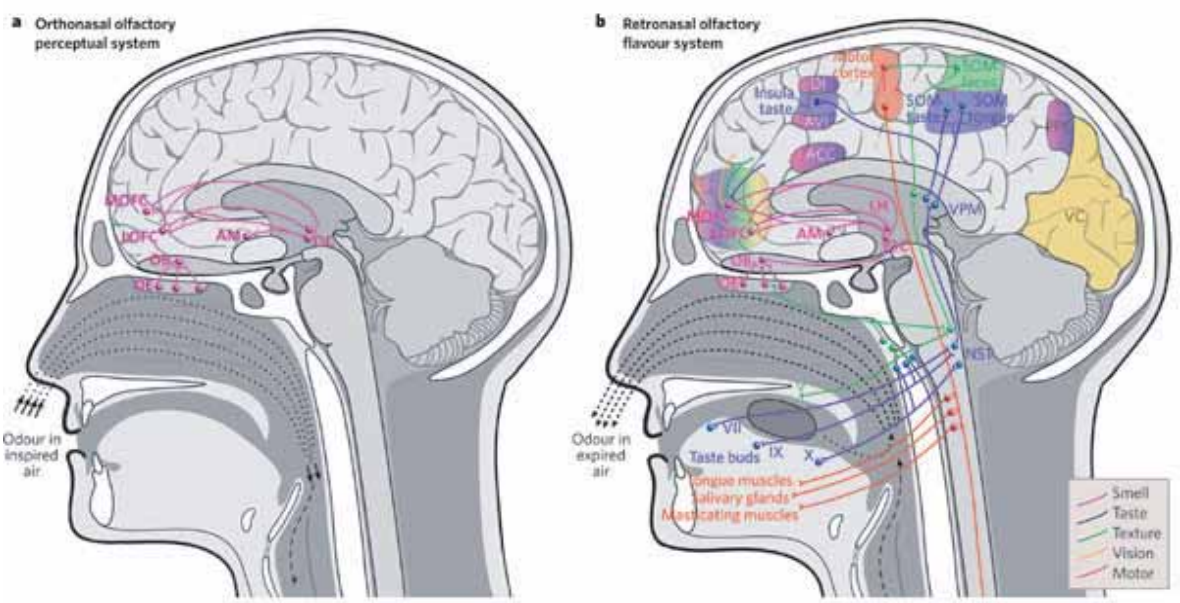


Figure 1: A model from G.Shepherd (2006) visualizing the complex processing underlying olfactory perception. **A:** A conventional description of the olfactory pathway, including the projections from the thalamus towards the orbito frontal neocortex. **B:** A model of the integrated processing streams that combine olfaction and taste during food ingestion and constitute our perception of flavour.

1.1 The moth as a model organism

The fact that the olfactory system to a large extent show striking similarities across species makes the use of model organisms beneficial for investigation of general neural principles characterizing this system highly relevant. Due to a well developed sense of smell and an easily accessible nervous system, insects have served as important models to explore the logic of chemosensory systems, as well as principles of neurotransmission in general. The pioneering discovery of an odorant containing communicative properties was the identification of *bombykol* produced by the silkworm moth *Bombyx mori* (Butenandt, 1959).This was the first odor molecule classified as a pheromone (Karlson & Lüscher, 1959). The identification and availability of biologically relevant odor stimuli is one of the great advantages of studying moths. In particular, a number of sexual pheromones and plant odor substances have been identified as ligands for olfactory sensory neurons in moth species of the subfamily *Heliothinea* (Insecta; Lepidoptera; Noctuidae), (Almaas & Mustaparta, 1990,1991; Berg, Almaas, Bjalie & Mustaparta, 1998; Stranden et al., 2003). Developing standardized olfactory tests for humans face several challenges. It has not been possible to determine what constitute the most relevant odours to include in such standardized tests nor

do we know the stimulation thresholds in detail. According to Wilson and Mainen (2006) it seems unlikely that such tests can be developed based on our current knowledge.

Another argument for the moth as a model object is the relative simplicity of the nervous system. The primary olfactory center of insects contains for instance a more restricted number of glomeruli than that of mammals; a fact that makes possible the identification of individual glomeruli and their function. Moths usually have about 60-70 glomeruli (Anton & Homberg, 1999) whereas rats have 2400-4200 glomeruli (Royet, Distel, Hudson & Gervais, 1998). A final argument for utilizing moths for experimental research is the accessibility of their nervous system. Both the peripheral and central parts of the olfactory pathway allow detailed investigation of basic neural arrangements. The inhibitory network of local interneurons in the primary olfactory center of the insect brain, which has been throroughly studied, is a good example (McLeod & Laurent, 1996; Laurent, 2002).

A total number of around 365 heliothine are distributed all over the world in hot and tropical areas (Cho, Mitchell, Mitter, Regier, Matthews and Roberts 2008). Several of the species are notoriously injurious crop pests and the historical interest in studying *Heliothinea* can be explained partly by this fact (Todd, 1978).

1.2 Anatomical organization of the insect olfactory pathway

1.2.1 The olfactory receptor neurons are located on the antennae

The airborne odorants are detected by olfactory receptor neurons housed in hair-like structures, sensilla, which cover the antennae. The receptor neurons are small bipolar neurons with a dendrite pointing to the apical tip of the sensillum and an axon projecting directly to the brain. Their cell bodies are located in the antenna. The membrane of the dendrite contains the odor receptors. Olfactory chemical recognition involves the interaction between odor molecules and suitable receptor proteins in the chemosensory membrane (reviewed by Galizia & Rössler, 2010). The molecular structure identity of olfactory receptors has been investigated in several vertebrates as well as insects. Until recently it was assumed that olfactory sensory neurons of insects have G-protein coupled receptors (GPCRs) corresponding to those identified in mammals. The mammalian odor receptors, which are encoded by genes belonging to the largest gene family identified, are of the metabotropic type (Buck & Axel, 1991; Mombaerts, 1999). This means that they activate particular ion channels

through a second messenger system. The molecule that acts as a second messenger in olfactory reception has been identified as cyclic AMP (cAMP), (Breer, 1994.).

Two recent studies do however challenge the classical understanding of insect olfactory receptors, and might shed light on an evolutionary unique route of the insect olfactory system. Based on molecular studies performed on the fruit fly *Drosophila,* the silk moth *B. mori,* and the malaria mosquito *Anopheles gambiae*, a new class of odor receptors are proposed: These are heteromeric ligand-gated ion channels comprising two metabotropic receptors, one variable odorant-binding subunit and one constant so-called Or83b family subunit (Sato et al, 2008.,Wicher et al, 2008; Nakagawa & Vosshall, 2009). The fast response kinetics indicate that the odor receptor acts as a directly coupled ion channel (Sato et al, 2008). A consensus model has been proposed including two transduction pathways, one fast based on a directly coupled ion channel and one slow based on a second messenger system (Nakagawa & Vosshall, 2009).

1.2.2 The antennal lobe, the primary olfactory center of the moth brain

The axons of the numerous antennal receptor neurons, which make up the antennal nerve, enter the primary olfactory center of the brain, the so-called antennal lobe (Figure 2). The relative As mentioned above, the terminals of the sensory axons make synapses with antennal-lobe neurons in characteristic structures termed glomeruli. In the majority of species studied, each sensory neuron targets one single glomerulus (Carlsson & Hansson, 2003). Three categories of antennal lobe neurons are included in the glomerular network: 1) projection neurons, 2) local inteneurons, and 3) centrifugal neurons (Anton & Homberg, 1999).

As mentioned above, moths usually have a number of glomeruli ranging from 60-65 (Figure 2). The relative size of a glomerulus is believed to correspond to the number of incoming afferents (Homberg, Christensen & Hildebrand, 1989; Anton & Homberg, 1999). The number of glomeruli seems to be quite consistent across various lepidopteran species and not determined by the actual size of the organism (Masente-Roca, Gadenne and Anton, 2005; Berg et al, 2002). A relatively large number of so-called ordinary glomeruli, which receive input from receptor neurons detecting plant odors, are present in both males and females. The macroglomerular complex (MGC) is a male-specific structure located dorso-laterally in the

antennal lobe, at the entrance of the antennal nerve. This structure, consisting of a few enlarged glomeruli, is responsible for processing information about sex pheromones produced by the female. The MGC of *H. virescens* contains four glomeruli, two large units receiving pheromone information and two smaller units receiving interspecific signal information (Berg et al, 1998).

One particular glomerulus, easily recognized due to its position and size, is the so-called labial pit organ, an isomorphic glomerulus located in the most ventral part of the antennal lobe (Kent, Harrow, Quatararo and Hildebrand, 1986). The labial pit organ receives input from the labial palps. Studies have shown its involvement in processing information about CO_2, a task believed to be vital in searching for food (Guerstein, Christensen & Hildebrand, 2004). Two enlarged female specific glomeruli located at the entrance of the antennal nerve has been reported in *H. virescens* (Berg et al, 2002). Similar structures have also been described in *M. sexta,* where physiological data indicate their involvement in processing host plant odor information (King, Christensen & Hildebrand, 2002).

1.2.3 Neuronal elements of the antennal lobe

The projection neurons receive input from local interneurons and receptor neurons and carry olfactory information from the antennal lobe to higher integration areas of the protocerebrum. Projection neurons are often referred to as output neurons (Homberg et al., 1989). Morphological classifications of projection neurons have been made in several species based on whether their dendritic arborisation pattern is multiglomerular or uniglomerular. The most common output elements in the moth antennal lobe, as in the antennal lobe of most other insect species, belong to the category of uniglomerular projection neurons (Hansson and Anton, 2000). In *H. virescens*, eight subtypes of antennal-lobe projection neuron have been idetified based on dendritic ramification patterns (Rø, Müller & Mustaparta, 2007).

Local interneurons are anaxonal elements residing within the antennal lobe. Distinctions can be made between at least three types of local neurons: 1. multiglomerular neurons with hetrogenous arborizations,2. multiglomerular neurons with asymmetrical arborizations, and 3. oligoglomerular neurons innervating only a few glomeruli (Hansson and Anton, 2000). Thus, the neurons differ in their general glomerular branching patterns. It is likely that some local neurons have output in one glomerulus and receive input from several other glomeruli

(Galizia, 2010). Most local neurons use GABA as a neurotransmitter and form inhibitory neural networks (Hansson and Anton, 2000 and Galizia & Rössler, 2010).

The third category of antennal lobe neurons, the centrifugal neurons, comprises a relatively few neurons receiving synaptic input from different brain areas and having their output synapses in the antennal lobe. The functions of these neurons are believed to be modulatory. One suggested modulatory function is the increase or decrease of receptor neuron sensitivity (Carlsson & Hansson, 2003; Galizia, 2008). One class of such feedback neurons are serotonin-immunoreactive and was first discovered in the sphinx moth *M. sexta* (Kent et al. 1986). Similar neurons have been found in several insect species (reviewed in Schactner, Schmidt & Homberg, 2005). Recently, this neuron type was identified in a heliothine moth. This neuron has a large somata located in the lateral cell cluster. Its axonal arborisations stretch across the brain to the contra lateral antennal lobe where it innervates all glomeruli, including the MGC structures (Zhao and Berg, 2009)

1.2.4 Cell clusters of antennal-lobe neurons

The somata of the projection neurons and local interneurons form three cell clusters that encircle parts of the glomerular neuropil (Figure 2). A large ventro-lateral cluster (referred to as the lateral cell cluster) includes the somata of all local interneurons and some projection neurons, whereas a smaller medial and a tiny anterior cell cluster (referred to as the medial and the anterior cell cluster respectively) contain somata of projection neurons only (Anton & Homberg, 1999)**.** Centrifugal neurons usually have their cell bodies outside the antennal lobe (Homberg et. al, 1989, Hansson & Anton, 2000).

1.2.5 Antennocerebral tracts connecting the antennal lobe with the protocerebrum

The axons of the projection neurons in insects form three main pathways connecting the antennal lobe to specific regions in the protocerebrum, 1) the inner antenno-cerebral tract, 2) the middle antenno-cerebral tract, 3) and the outer antenno-cerebral tract (Schactner, Schmidt & Homberg, 2005). A “typical” projection neuron has uniglomerular dendritic ramifications in the antennal lobe and an axonal projection through the inner tract targeting the calyces and then the lateral protocerebrum. The projection neurons that do not follow this pattern and project through the middle or the outer tract principally seem to have multiglomerular arborizations in the antennal lobe. The antennal-lobe projection neurons address two main

areas in the protocerebrum: the calyces, the lateral protocerebrum. The calyces are part of anatomical structures called the mushroom bodies, a prominent structure well preserved across different insect species (Fahrbach, 2006, Farris, 2004, 2008b).

The inner tract, being the largest, exits the antennal lobe dorsomedially and it runs posteriorly in the protocerebrum where it bypasses the central body on its lateral edge. The inner tract takes a lateral turn before it reaches its main target area, the calyces. Most inner tract branches continue from the calyces to the lateral parts if the median protocerebrum. The middle antenno-cerebral tract runs together with the inner tract as it exits the antennal lobe. It then bends laterally and project directly to slightly different areas of the lateral protocerebrum. The exit point of the outer tract is located ventrally to those of the inner and middle tract. It turns laterally, like the middle tract and target areas in the lateral parts protocerebrum. From the lateral horn a few outer tract branches make a dorsomedial turn and target the calyses (Rø, Müller & Mustaparta, 2007). Two additional tracts are referred to as the dorsal antennocerebral tract and the dorsomedial antennocerebral tract (Homberg, Montague and Hildebrand, 1988; Berg, Schachtner & Homberg, 2009).

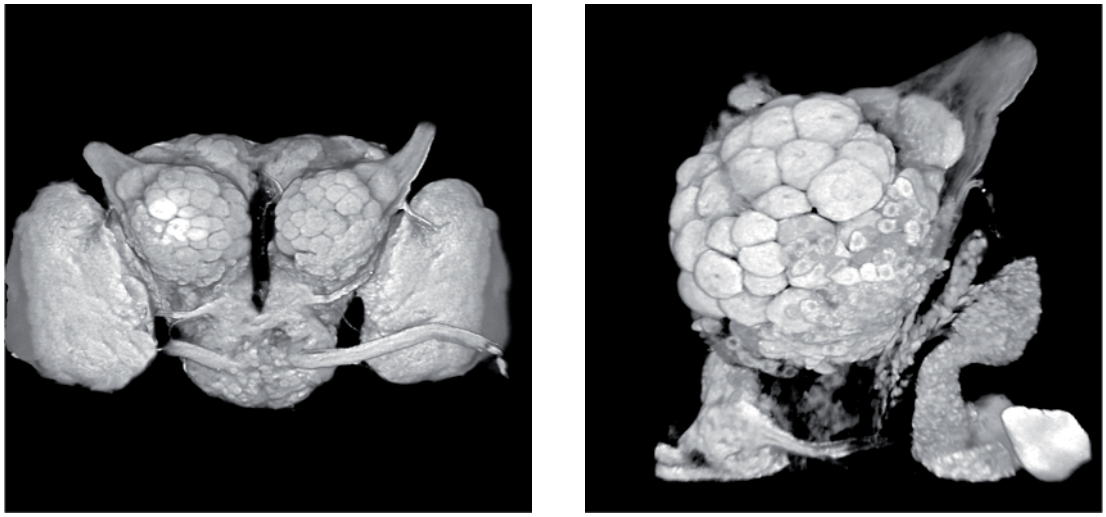


Figure 2: Images are confocal reconstructions from the *H. virescens* and *H.assulta* 3d brain atlas (Berg et al., 2002). **Left:** Whole brain showing the two large optic lobes on each side and the prominent antennal lobes. The primary olfactory centers distinct glomerular organization appears. **Right:** A close look on the left antennal lobe visualizing the glomeruli structures, the large lateral cellcluster and the entrancepoint of the antennal nerve.

1.3 Classes of neurotransmitters identified in the insect olfactory system

In order to fully comprehend the logic of information processing in a particular network it is essential, in addition to mapping the anatomical arrangement, to achieve knowledge about the neuro-chemical organization. Pharmacological investigation of insect neural pathways can be traced back to the 1940s (Kerkut, Pitman & Walker, 1969). The criterion for classifying a signal molecule as a transmitter is that the neuron actually produces the substance, stores it in terminals, and releases it when the cell is depolarized. The neuroactive substances found in insects strikingly resemble those found in other species although the functional roles of the substances may vary between the different nervous systems (Osborne, 1996 ; Caveney & Donly, 2002; Brodal, 2007).

Several neurotransmitter candidates that have been found in insects belong to the molecularly simple “classical” transmitters. This family of small-molecular neurotransmitters include acethylcholin, which is believed to be the primary transmitter of insect olfactory receptor neurons (Homberg & Müller, 1999). Comparative studies of different insect species do however indicate that some olfactory receptor neurons make use of other transmitters and that currently unknown substances play a key role in the signal transduction of these neurons (reviewed in Galizia, 2008). Acetylcholine is, by the way, the most widespread excitatory transmitter in the insect nervous system (Homberg & Müller, 1999). In honeybees projection neurons with uniglomerular ramification patterns have been found to use acethylcoline as a transmitter (reviewed Galizia & Rössler, 2010).

Biogenic amines like serotononin, dopamine, and octopamine also categorize as classical transmitters. Centrifugal neurons seem to rely on biogenic amines learned but only few examples have been described in detail (Galizia, 2008). Zhao and Berg (2009) have characterized the morphology and physiology of a single serotonin immunoreactive neuron in the olfactory system of the moth *Helicoverpa asssulta*. This modulatory neuron is believed to be involved in regulating the sensitivity of antennal lobe interneurons. Another well described centrifugal neuron is the octopaminergic VuMmx1 neuron in the honeybee, presumably serving a vital role in associative reward learning (Hammer, 1997).

The final group of classical transmitters found in the olfactory system of insects are amino acids. Amino acids include GABA, glutamate, and glycine. Most local neurons of the antennal lobe are GABAergic and this is supported by data from physiological data showing that local neurons are predominantly inhibitory (Carlsson & Hansson, 2003). The function of

these inhibitory local neurons and the networks they constitute will be discussed in greater detail.Whereas the signalling substances used by antennal lobe projection neurons are in large part unknown, subcategories of projection neurons have proved to be GABAergic. Findings in moth and a few other species indicate these are multiglomerular neurons ihibiting spiking activity (reviewed in Hansson and Anton, 2000, Galizia, 2008). These GABAergic output neurons project through the middle antennocerebral tract (Berg et al. 2009; Hoskins, Homberg, Kingan, Christensen & Hildebrand, 1986).

In addition to the classical neurotransmitters mentioned above, neuropeptides constitute a second family of transmittersubstances. These are considerably larger molecules as one peptide consists of 5-30 amino acids. Among those identified in insects are allatostatin, proctolin, allatotropin, FMRFamide, tachykinins, and leucokinin. (Osborne, 1996; Caveney & Donly, 2002; Brodal, 2007). Neuropeptides are often found co-localized with classical transmitters. An example is the co-localization of GABA and various neuropeptides in local neurons of the antennal lobe often contain various neuropeptides (Berg et al. 2009).

1.4 GABA in olfactory pathways of the moth brain

1.4.1. Distribution of GABA in the olfactory pathways

The presence of γ-aminobutric acid (GABA) in the brain was discovered more than 50 years ago in mouse tissue by Eugene Roberts and J. Awapara. Its characteristics as a neurotransmitter was first generally accepted after numerous physiological investigations in the 1960's (Roberts, 2000). The inhibitory properties of GABA were originally discovered in an invertebrate, namely from studies of the lobster nervous system (Otsuka, Iversen, Hall & Kravitz, 1966). GABA has since then been recognized as the major inhibitory neurotransmitter in the mammalian nervous system, as well as in insects and simpler organisms (Brodal, 2007). The GABA receptors found in insects are usually divided into two main categories based on functional properties, one $GABA_A$ and $GABA_B$ type (Jorgensen, 2005).and similar distinction is made in vertebrate nervous systems (Brodal, 2007).

Most places the effect of GABA is direct opening of CL^- channels causing a brief hyperpolarization in the postsynaptic membrane. The GABA receptor chloride channel is termed $GABA_{A.}$ GABA may also cause a longer lasting hyperpolarization by *indirect* opening of K^+ channels or blocking of Ca^{2+} . The receptors involved in this signaling pathway are termed $GABA_B$ receptors. This class of receptors is *metabotrope* receptors and they work via

G-proteins. Generally, $GABA_A$ receptors outnumber $GABA_B$ receptors in most nervous systems. The different receptor types show that GABA is capable of both fast and slow inhibition (Brodal, 2007). Insect $GABA_A$ receptors are often distinguished from the corresponding type in vertebrates by their insensitivity to bicuculline and baclofen (Jorgensen, 2005).

In the *H.virescens,* previous reports have demonstrated extensive GABA immunostaining in all glomeruli of the antennal lobe (Berg, Shachtner & Homberg, 2009). The GABA immunoreactivity also included the calyces of the mushroom bodies and the lateral protocerebrum. Of the main antennocerebral tracts the middle tract contained a considerable number of GABAergic fibers. The prominent inner tract was immunonegative and only a few fibers in the outer tract contained stained axons. However, the smaller dorsal tract showed strong GABA immunoreactivity (Berg, Shachtner & Homberg, 2009).

1.4.2 The function of inhibitory GABA mediated networks in the insect olfactory system

The vast presence of GABA in the olfactory system indicates that inhibition plays a vital role in the olfactory signal processing. It is generally accepted that inhibitory networks is involved in olfactory coding and odor recognition. Research has indicated these codes might have both spatial and temporal qualities (Galizia & Szyszka, 2008; Wilson & Laurent, 2005). The precise nature of the principles underlying these codes are however still unclear. The subject will be further addressed in the discussion.

1.5 Main aim of the thesis:

To establish a method for general GABA immunolabeling of the moth brain for the purpose of mapping, by the double-fluorescence technique, single inhibitory neurons and inhibitory neuron populations in the central olfactory pathway.

Spesific goals:

1. To establish an optimal working protocol for GABA immunolabeling with the antiserum used here.
2. To characterize single pre-stained antennal-lobe neurons, that means iontophoretically labeled projection neurons and local interneurons, according to their GABA-immunoreactive properties.
3. To point out inhibitory fibers in the antennocerebral tracts and to map their cell bodies in the lateral cell cluster by utilizing anterogradely and retrogradely pre-stained preparations.

2.0 Materials and method

2.1 Ethics

All experiments were be carried out according to Norwegian law (lov om dyrevern §1). This law states that invertebrates like the insects used in the experiments for this thesis is not included by the law's restriction. However, it is a general practice at the laboratory that the insects are treated well, given food and kept in as natural light and temperature surroundings as practically possible not to evoke unnecessary stress or pain.

2.2 Insects

Adult male moths of the species *Heliothis virescense* and *Helicoverpa assulta* were used in the present study. *Heliothis virescense* originates from laboratory cultures at Syngenta; Basel, Switzerland and the *Helicoverpa assulta* from laboratory cultures at the Chinese Academy of science, Beijing, Peoples republic of China. The moths are delivered by mail as pupea and are sorted by gender on arrival. Each day, moths that emerged were placed in separate containers marked with date and gender and fed on a solution of sucrose and water. In this state they remain until used for experimentation.

2.3 Antiserum

A GABA antiserum from Sigma (A202 Sigma anti-GABA) was used in the experiments. The antibody, developed in rabbit using GABA-BSA as the immunogen, has been purified through antigen specific affinity isolation. In this way, essentially all rabbit serum proteins which do not specifically bind to GABA, including immunoglobulins, have been removed. A minimum working dilution of 1:10.000 has been determined by a dot plot immunoassay (Sigma, product information). In order to establish an optimal procedure for use of this antiserum on *H. virescense*, different procedural variations and chemical concentrations were tried out.

2.4 Immunostaining

A total of 80-90 male and female moths were utilized in the experiments. This number includes insects used for learning the experimental procedure and preparations that were damaged or otherwise not deemed fit for confocal microscopy. A total of 32 brains were scanned, including both whole mount preparations and free-floating vibratome sections.

In order to dissect the brain from the head capsule, the insect was fastened by dental wax inside a plastic tube with the head exposed. The dissection was carried out under a microscope (Leica MC6 and WILD type 36970). The antennae, cuticula, and underlying muscles were removed by fine tools (scissors, a razor blade, and forceps) so that the brain was exposed. After opening the head capsule the brain was kept wet using insect Ringer solution (in mM: 150$NaC1_2$,3$CaC1_2$, 3KC1 and 10*N*-tris (hydroxymethyl) – methyl 2 amino-ethabesulfonic acid , Ph 6.9). Finally, the dissected brain was fixed in a 4% paraformaldehyde solution for one hour at room temperature or overnight in a refrigerator before further processing.

2.4.1 Free-floating vibratom sections

After fixation the preparations were rinsed in phosphate buffered saline (PBS-in mM: 684NaC1, 13KC1, 50.7Na_2HPO_4, 5KH_2PO_4, pH 7.4) prior to being imbedded in frontal or dorsal orientation in a gelatine/albumin solution. The embedded brains were stored in a post fixative 4% formaldehyde buffer for 18 hours before being sectioned in 40 µm thick slices using a vibrating blade microtome (Leica VT 1000S; Leica, Nussloch, Germany). After thorough washing in PBS containing 0.3% Triton X-100 the brain sections were preincubated for one hour at room temperature in PBS containing 0.5% TrX and 5% normal goat-serum (NGS) for reduction of background staining. The primary antibody, rabbit anti-GABA was then applied at concentrations of 1:2000 and 1.3000 in 500 µl solutions (1:2000 being the preferred concentration). The preparations remained in this state overnight (24 hours). Incubation at room temperature and at 4 °C was tried out with no consistent difference observed in staining quality. In some trials NGS was added along with the primary antibody without any significant advantage observed. After incubation in the primary antibody, the sections were washed before application of the secondary antibody, Cy3 anti-rabbit. From this stage the preparations were treated in darkness. Thus, the preparations were kept in a cold and dark room for two hours before being washed, and finally mounted on glass coverslips for scanning under a confocal laser microscope. To make the sections transparent, the

preparations were dehydrated in a series of increasing alcoholconsentrations from 50% to 100%, and finally cleared in Xylene.

2.4.2 Whole mount preparations

Dissection of brains for wholemount preparation were carried out like described above for the ones used for vibratom sections After fixation in 4% paraformaldehyde, the preparations were washed in PBS before incubation with NGS for 3 hours at room temperature. The preferred concentration of the primary antibody was 1:1000 and the incubation time was 3 days. Incubation time with the second antibody, Cy3 anti-rabbit was 24 hours. The whole mounts were finally submerged in methylsalicylecalate and mounted on a coverslip for confocal laser scanning.

2.4.3 Double-labeling experiments

In addition to the GABA immunostaings described above, double labelling experiments were performed on 16 brains. These preparations included previously stained neurons, acquired by the single-cell recording and staining technique or the mass-filling technique. The previously stained preparations that were stored in methylsalicylate were rehydrated in a descending alcohol series, 2x10 minutes in 100% and 5 minutes each in 96%,90%,70%, and 50%. After rehydration the double labelled preparations followed the experimental procedure described for whole mount preparations.

2.5 Confocal microscopy

Serial optical images were acquired by using a confocal laser scanning microscope (LSM 510, META Zeiss, Jena, Germany) with a 40x objective (C-Achroplan 40·/0.8W; C-Apochromat 10x·/0.45W) and a 20x objective (plan-Neofluar/0.5 I).
The thickness of each image section was from 2-6 µm. The pinhole size was 1 for preparations scanned with one channel and 2 for preparations scanned with two channels: The resolution was 1024 · 1024 pixels. For the exciting the fluorescence of Cy3/rhodamine (Ex_{max} 550nm, Em_{max} 570nm), the 543nm line of a HeN1 laser was used. Cy5 (Ex_{max} 682nm, Em_{max} 647nm) was excited by the 633-nm line of a HeNe2 laser.

2.6 Image processing

Optical sections from the confocal stacks were analyzed using the visualization software LSM 510. By use of a special tool in the software, 3D-reconstructions of several sections were obtained. Image brightness and contrast were adjusted in Adobe Photoshop CS3 (Adobe System, San Jose, CA) before the final image layout was edited in Adobe Illustrator CS3.

3.0 Results

Application of antiserum against GABA resulted in a distinct staining pattern of the moth brain. Several brain regions including the antennal lobes, the optic lobes, the central body, and the calyces of the mushroom bodies were stained. The anatomical organization of these areas was to some extent revealed, as shown in figure 3. The general staining included deeper parts of the brain and visualized the antennocerebral tracts in some preparations. The inner tract appeared in shape of its immunonegativity whereas the middle tract was weakly immunoreactive.

The staining pattern of the whole brain preparations usually displayed a stronger immunolabeling in the peripheral parts of the brain than in the deeper parts and the fluorescence was often seen as a layer on the surface of the preparations. In general, the antibody seems to provide some unspecific staining in addition to the specific. An example is staining of the antennal nerve, as shown in figure 4. The data also imply that there is some cross reaction between the antibody and the dye used for intracellular staining.

3.1 GABA immunostaining of the antennal lobe

Comprehensive staining was demonstrated in seemingly all glomeruli of the antennal lobe (Fig. 4). A large number of antennal-lobe somata were also labeled, all localized in the lateral cell cluster. Staining of both whole brain preparations and vibratome sections proved to be useful for visualizing structures of interest in the antennal lobe. And even thought the GABA labeling becomes slightly weaker from peripheral to central parts of the brain, all glomeruli including those most posteriorly located were visible.

Thus, GABA-immunolabeling visualized the distinct glomerular organization of the antennal lobe. As shown in figure 4, an arrangement of glomeruli covering the peripheral part of the whole antennal lobe, whith the central neuropil lacking glomeruli, was revealed. The antibody staining allowed for identification of hallmark glomeruli as the male-specific macroglomerular complex (MGC) located dorsally in the antennal lobe, by the antennal nerve entrance (Fig. 4G, and the isomorphic labial pit organ (LPO) positioned at the most ventral part of the antennal lobe (Fig. 4F). The numerous ordinary glomeruli could be distinguished from one another based on their size and position as shown in figure 4 and 5.

In addition to visualizing the glomeruli as spherical structures separated from one another, the GABA- antiserum labeled somata in the lateral cell cluster strongly. Figure 5 shows a large

number of stained somata intermingled among unstained somata in this large cell group. In some preparations, the primary neurites of the lateral cell cluster somata appeared as a bundle of fibers ascending from the cell bodies towards the core of the antennal lobe (Fig. 5A). The labeled somata showed variation in size, some being larger and some smaller than the average. In several preparations a few prominent somata, being considerably larger than the rest, appeared. Differently sized somata are demonstrated in fig 4 and 5.

As shown in figure 5B, the medial cell cluster included no immunoreactive somata. (The string of small cell bodies showing strong immunolabeling in figure 5B is located outside the antennal lobe).

3.2 Double-labeling experiments

Double-labeling experiments were carried out on previously stained preparations using both vibratome sections and whole brains. All the double-stained preparations presented in the figures originate from whole brains as these provided the best results. Altogether the double-labeling experiments include preparations containing pre-stained neurons of both local interneurons and projection neurons, as well as pre-stained preparations including mass-stainings that visualize the antennocerebral tracts and the projection-neuron somata in the lateral cell cluster of the antennal lobe.

In general, the strong immunolabeling of the antennal lobes allowed for identification of GABA immunoreactive somata of the iontophoretically stained single neurons. The pre-stained neurons were categorized as local neurons or projection neurons based on their total morphology. Many pre-stained preparations contained more than one labeled neuron and some contained both local neurons and projection neurons.

3.2.1 Immuno-labeling of preparations containing pre-stained local interneurons

Figure 6 shows three pre-stained cell bodies of local interneurons. The differently sized cell bodies are located in the lateral cell cluster and the neurons have multiglomerlar branches providing connections to most glomeruli. The double-labelings demonstrate that they are all GABA-ergic.

A second preparation showed one large double-labeled soma in the lateral cell cluster, also belonging to a local neuron. As shown in figure 7, the neuron connects to several glomeruli

throughout the antennal lobe. The double-labeling of the cell body and of neural branches in the core of the antennal lobe proves that the neuron is GABA-ergic.

One of the previously stained local interneurons did not display any immunoreactivity against the GABA antiserum. Figure 8 A presents a iontophoretically stained local neuron with its cell body in the lateral cell cluster. The pre-stained cell body is, however, not included in the collection of immunoreactive somata shown in figure 8A, B, and C and the branches of the neuron in the central core of the antennal lobe does not appear to have been double stained. The size of the GABA immunonegative local neuron cell body was relatively small as compared to most of the immunoreactive somata.

3.2.2Immuno-labeling of preparations containing pre-stained projection neurons

In figure 9, a total of 4 neurons are stained, one of which is a projection neuronThe dense innervation pattern in one glomerulus, an axon passing in the inner antennocerebral tract, and axon terminals in the calyces of the mushroom bodies and the lateral protocerebrum, demonstrate the presence of a uniglomerular projection neuron (Figure 9A-A''). One of the small pre-stained somata is clearly immunonegative and is likely the cellbody of the stained projection neuron (Figure 9C-C''). A weak immunostaining of the axon of the iontophoretically stained projection neurons appears (Figure 9B-B'). The neuronal branches of these 4 neurons connect to seemingly all glomeruli. This preparation shows that parts of the intracellularly stained neural branches have been stained by the GABA antibody. This is probably due to a cross-reaction between the dextrane used for the intracellular staining and the GABA antiserum and will be addressed in the discussion.

The two largest of the pre-stained cell-bodies are GABA ergic and most likely belong to multiglomerular local neurons (Fig 9C-C'). The double-stained neuronal branches in the core of the antennal lobe are likely parts of these local interneurons. The fourth pre-stained cellbody, located in the periphery of the lateral cell cluster, could not be categorized according to GABA immunoreactivity due to the general immune-staining of the brain surface (Figure 9C-C'').

One double-labeled preparation contained two pre-stained projection neurons of the uni-glomerular type, one with soma in the medial cell cluster (Fig. 10A) and one with soma in the lateral cell cluster (Fig. 10B). As demonstrated in figure 8, none of the somata contained

GABA. The preparation was slightly damaged and a 20x scan of the brain is therefore added to help visualize the brain position and anatomical structures relative to the location of the stained neurons.

3.2.3 Immuno-labeling of preparations wherein the antennocerebral tracts were pre-stained

The experiments included both anterogradely and retrogradely stained preparations, which means preparations with dye applied to the antennal lobe and the calyces, respectively. Figure 11 shows a preparation with anterogradely pre-stained antennocerebral tracts. As demonstrated in the figure, GABA immunoreactive fibers were present in the medial antennocerebral tract. The preparation also showed strong GABA immunoreactivity in the calyces. The inner antennocerebral tract shows no GABA immunoreactivity, neither does the outer tract.

Images of the lateral cell cluster, obtained from a preparation with retrogradely pre-stained antennocerebral tracts, are seen in figure 12. The pre-stained somata, which are all somata of projection neurons, include both GABA immuno-negative and GABA immuno-positive elements; the GABA immuno-negative somata are distributed throughout the cell cluster whereas the immuno-positive somata are more grouped and located in a ventral part. The last-mentioned category, the GABAergic somata, is also smaller than the category not containing GABA.

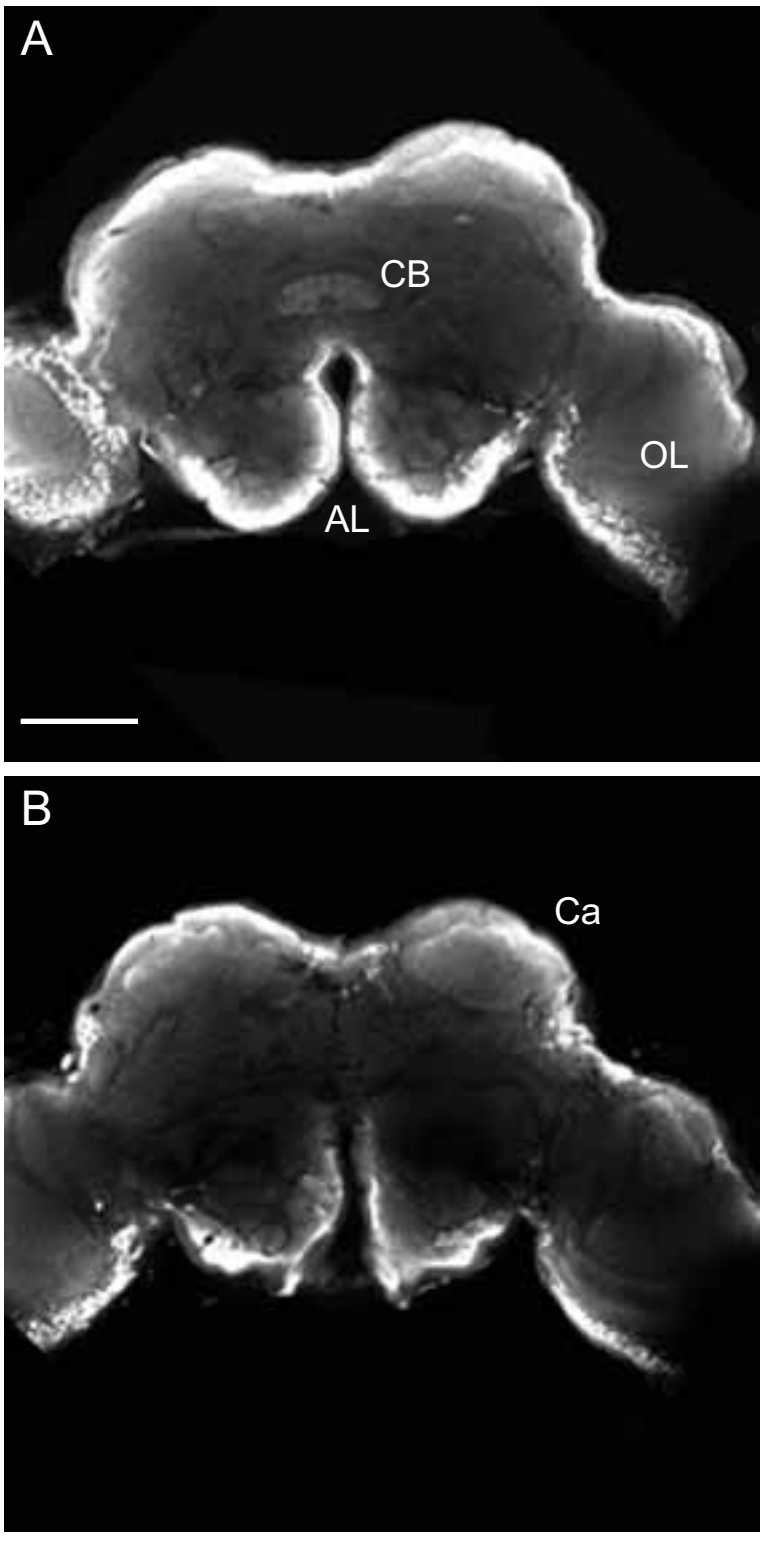


Figure 3: Confocal images from a dorsally oriented whole-brain preparation stained with GABA antiserum (scanned with a 10x objective; the antiserum concentration was 1:1000). **A**: One section showing immunoreactivity in the antennal lobe (AL), the optic lobe (OL), and the central body (CB). The AL contains an arrangement of distinct glomerular structures encircled by stained somata (parts of the strong labeling in the periphery is probably due to technical conditions). Staining of the CB includes mainly the lower part and indicates the characteristic organization of this structure. Immunolabeling of the OL visualizes the sub-units of this structure, also here surrounded by strongly labeled cell bodies. **B:** One section, 60 µm dorsally of that shown in figure 3A, showing immunoreactivity in the Calyses (Ca). Scalebar: 200 µm.

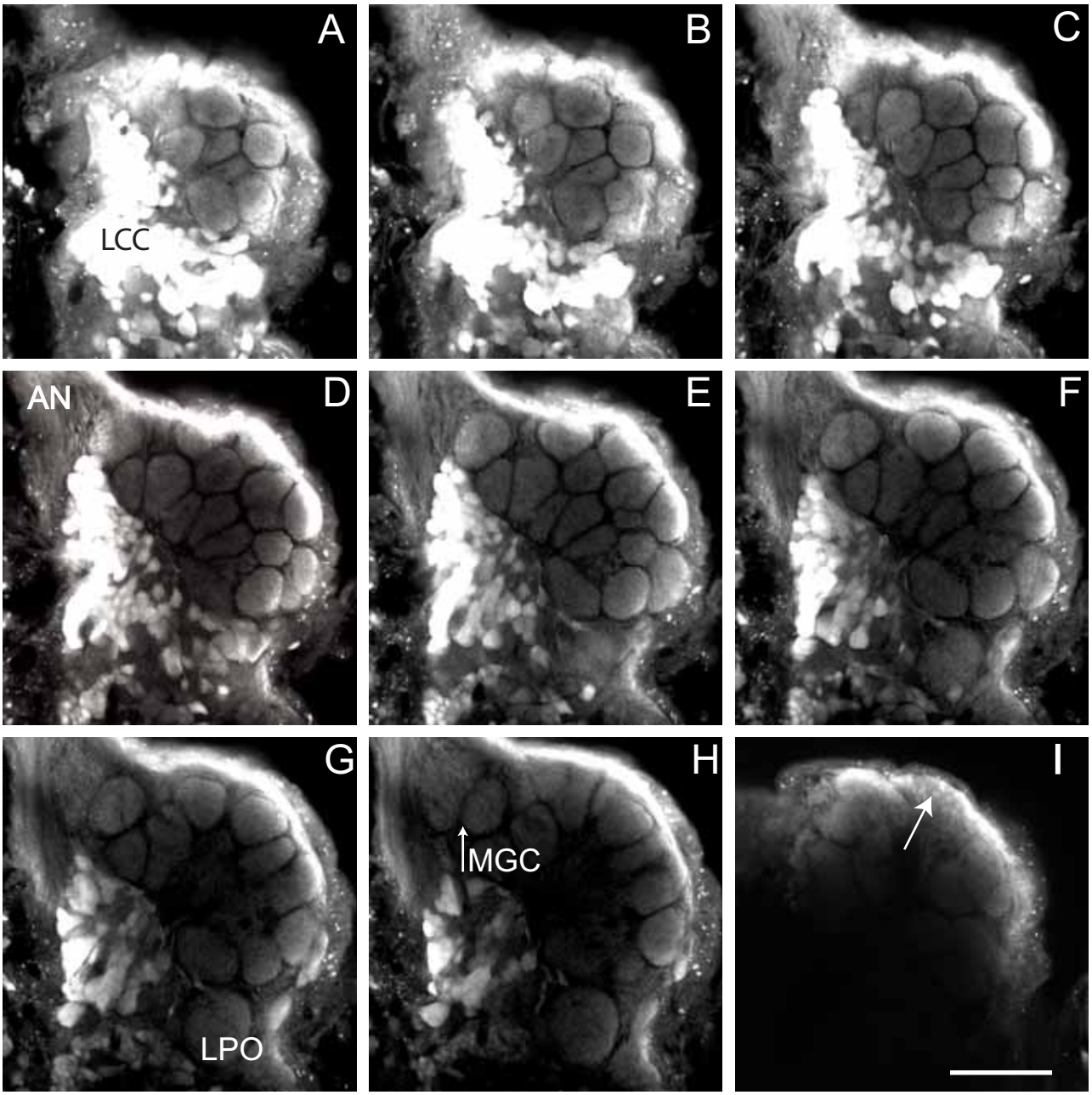


Figure 4: Confocal image sections of the antennal lobe in a frontal view, from anterior to posterior, showing GABA immunoreactivity (whole brain preparation scanned with a 40x objective). Strong immunolabeling of numerous somata located in the lateral cell cluster (LCC) is demonstrated **(A-H)**. In addition, distinct glomerular structures located at the periphery of the antennal lobe are visible. The macro glomerular complex (MGC) and the labial pit organ (LPO) were identified and are marked **(G)**. GABA-immunonegative somata in the medial cell cluster are indicated by an arrow in I. Due to a general background staining, the antennal nerve (AN) is visble. Scalebar: 100 µm.

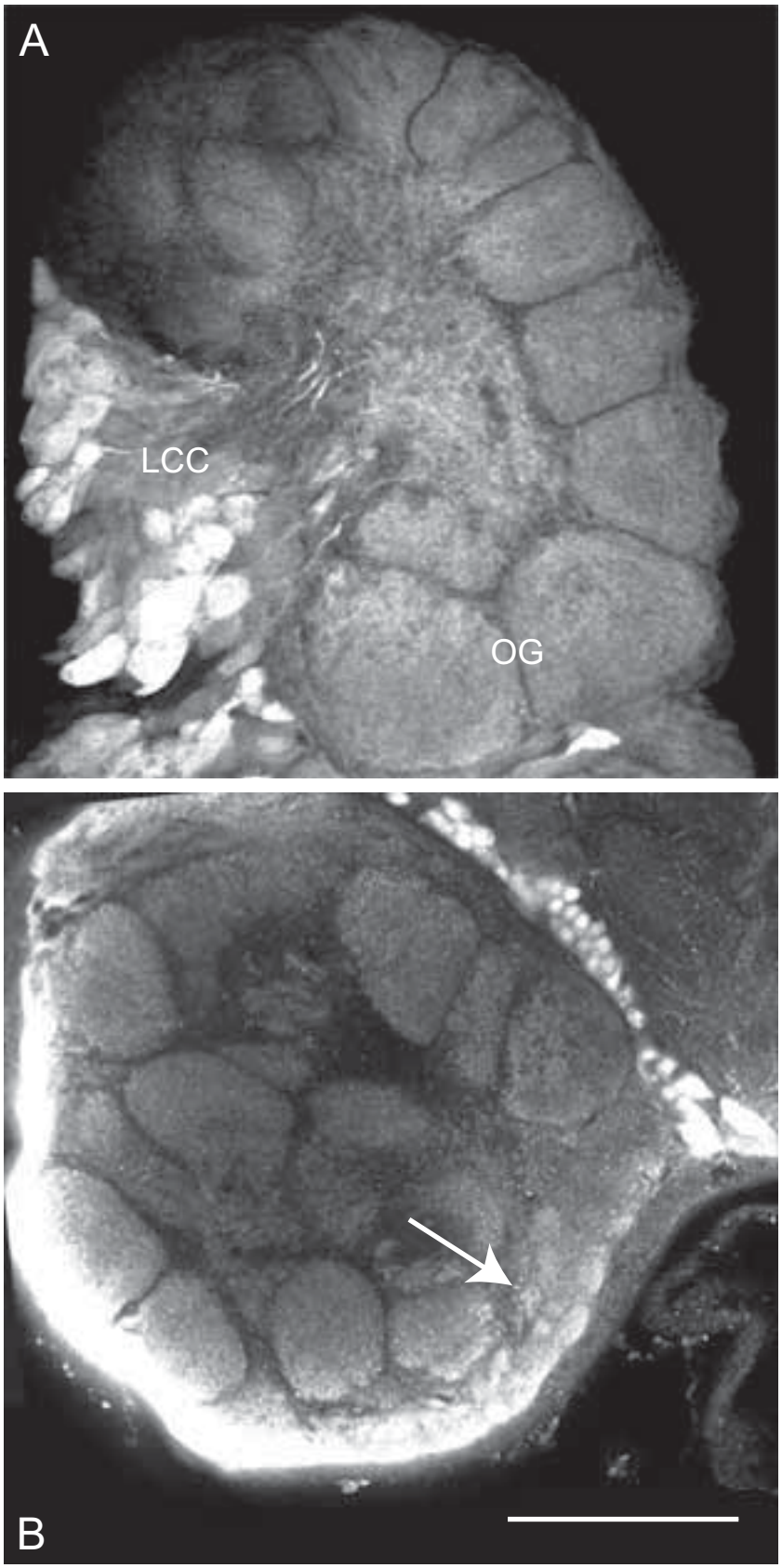


Figure 5: Confocal images from two brain preparations, one sectioned by a vibratome **(A)** and one as a whole brain **(B)**, showing GABA-staining in the antennal lobe (scanned with a 40x objective). **A:** Frontally orientated vibratome section showing GABA staining of several distinct glomeruli, including . two large ordinary glomeruli (OG) located ventrally in the antennal lobe. As shown, the glomerular units surround the center of the antennal lobe which contains unorganized labeled branches. Some of these branches are constituted by primary neurites connected to immunoreactive somata in the lateral cell cluster (LCC). **B:** A dorsally oriented whole brain preparation showing the lack of GABA-labeld somata in the medial cell cluster (arrow). Many small GABA immunoreactive somata are present outside the antennal lobe. Scalebar: 100 µm.

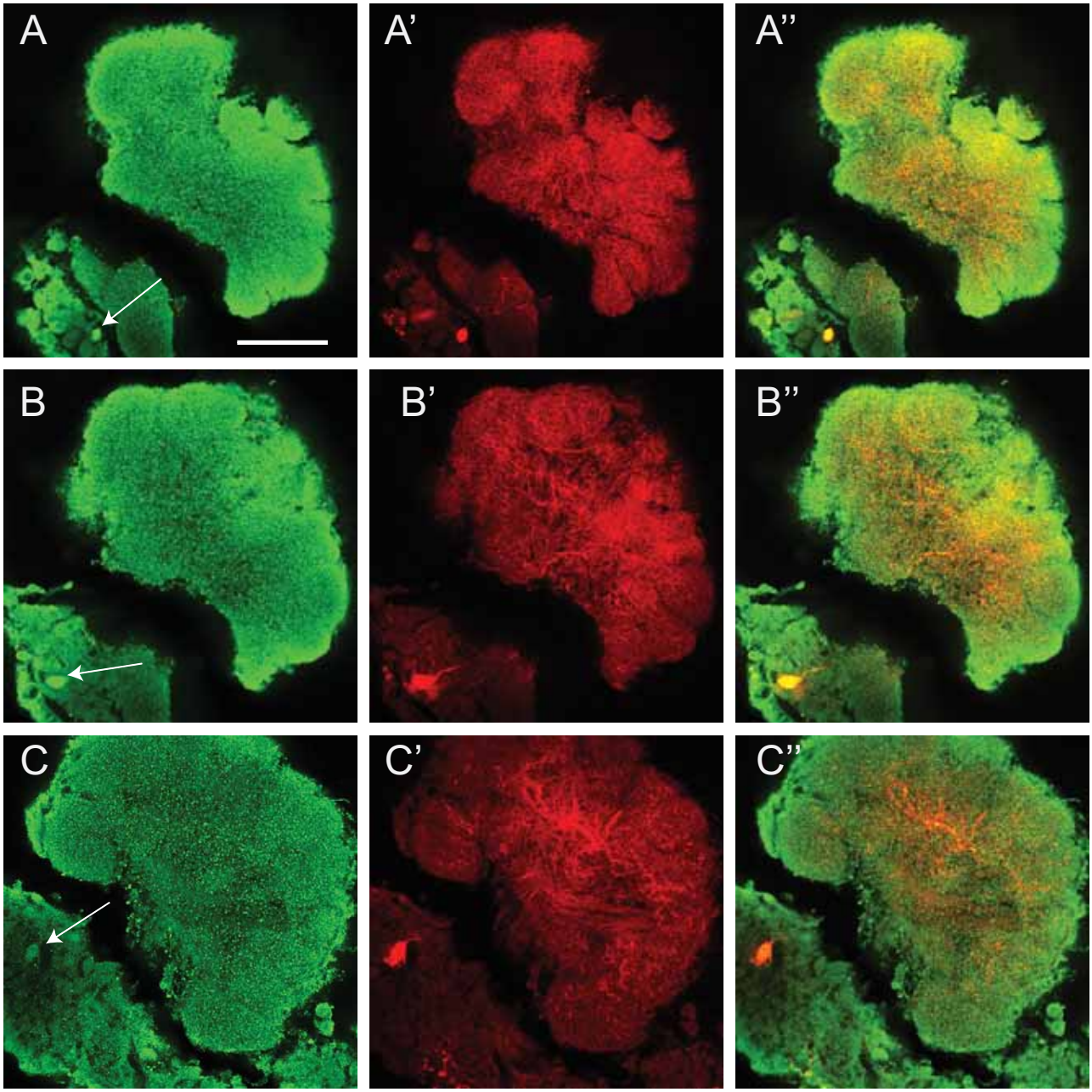


Figure 6: Confocal image sections from a frontally oriented pre-stained whole brain preparation. The preparation is double stained with anti GABA. The figure includes three rows of images **(A-C)** from three consecutive sections each showing the location of one stained local neuron somata. Left: GABA-staining (green). Middle: iotophoretic staining (red). Right: overlay of GABA-staining and iontophoretic staining. All three somata show GABA reactivity. The preparation is slightly damaged, hence the gap between the stained neurons in the lateral cellcluster and the rest of the antennal lobe. Scalebar: 100µm

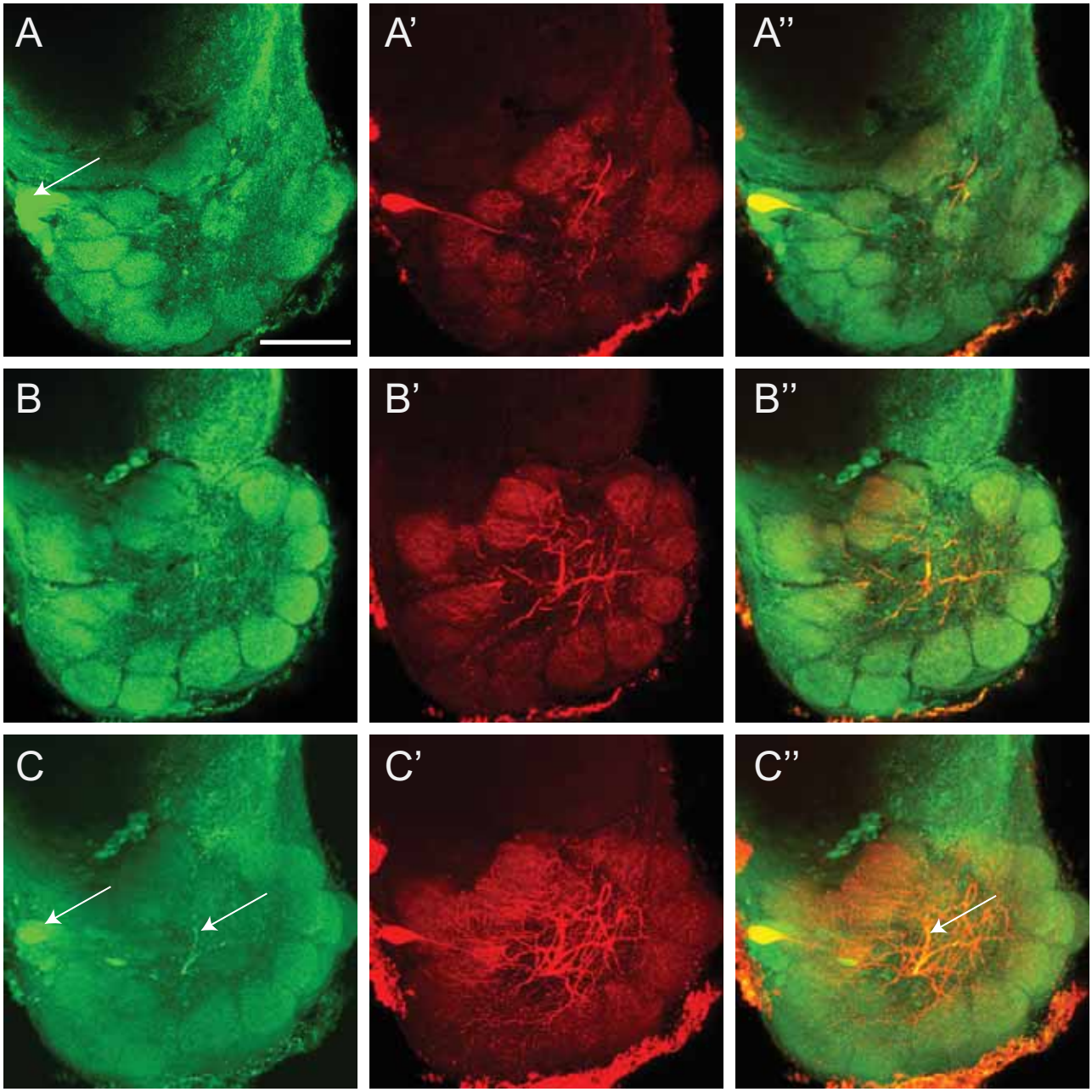


Figure 7: Confocal images from a dorsally oriented pre-stained whole brain preparation double stained with anti GABA. The antennal lobe is scanned with a 40x objective. The figure show two consecutive sections **(A-B)** and a 3D image **(C)**. Left: GABA-staining (green). Middle: iotophoretic staining (red). Right: overlay of GABA-staining and iontophoretic staining. **A**: A single large somata belonging to a stained neuron is marked by arrow in picture A-A' and the double labeling show it is GABAergic. **B**: The branches of the neuron throughout the antennal lobe. **C:** 3D picture showing the whole neuron. Some GABA staining can be seen on parts of the neuronal branches as indicated by the arrow in **C''**. Scalebar: 100µm.

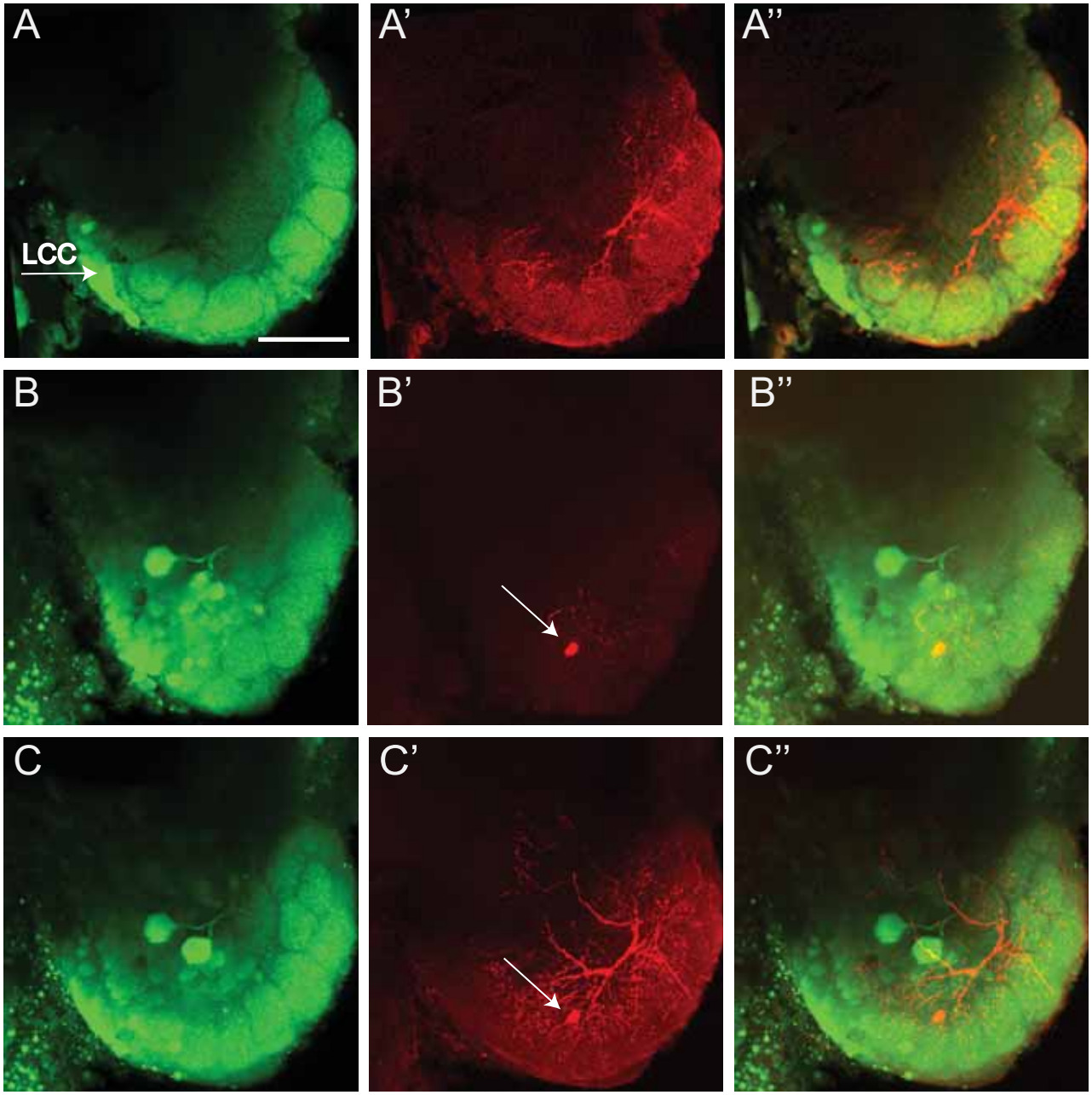


Figure 8: Confocal images from a dorsally oriented pre-stained whole brain preparation double stained with anti GABA. The antennal lobe is scanned with a 40x objective. The figure includes images from two consecutive sections (**A** and **B**) and one three-dimensional reconstruction (**C)**. Left: GABA-staining (green). Middle: iotophoretic staining (red). Right: overlay of GABA-staining and iontophoretic staining. **A:** The position of the lateral cellcluster (LCC) is indicated by an arrow. It is visible through several GABA stained somata. **B**: The position of the stained somata is marked with an arrow. It does not show GABA immunoreactivity. **C**: A 3D reconstruction of the whole neuron indicates it is not GABA immunoreactive. Scalebar: 100µm.

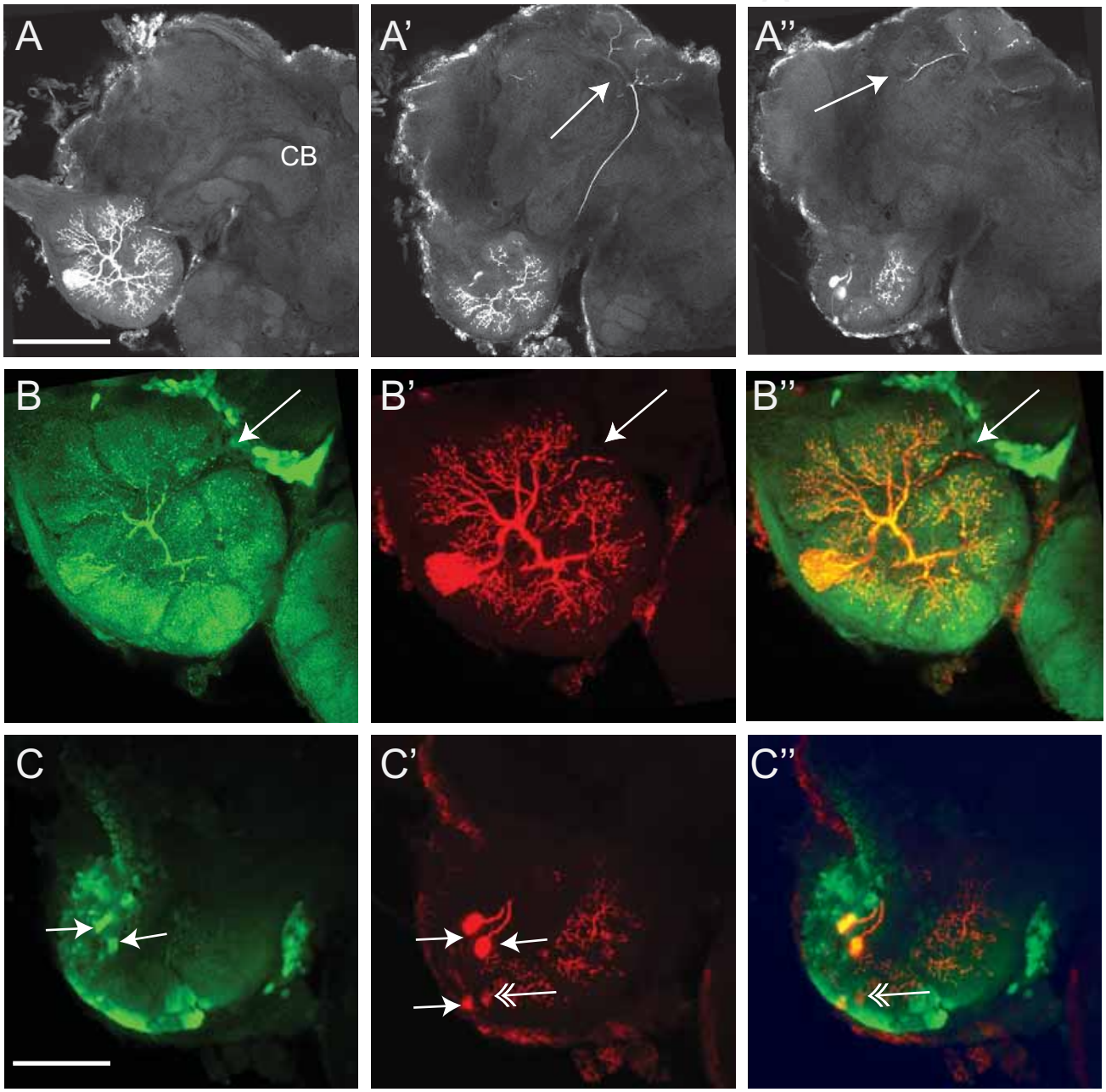


Figure 9: Confocal images from a dorsally oriented pre-stained whole brain preparation double stained with anti GABA. The brain is scanned with a 20x objective **(A)** and the antennal lobe is scanned with a 40x objective **(B-C)** A total of four neurons were iontophoretically stained. Left: GABA-staining (green). Middle: iotophoretic staining (red). Right: overlay of GABA-staining and iontophoretic staining. **A:** The neuronal branches of the stained neurons are visible across the antennal lobe. The central body (CB) is visualised. In picture **A'** the arrow indicates the projection neurons axional target area in the calyses. **A''**: Arrow points to the axonal projection target area in the lateral protocerebrum. Scalebar: 200 µm. **B:** Dendrites belonging to the iontophoretically stained neurons are also GABA imuno stained. Parts of the axon belonging to the projection neuron leaving the antennal lobe show GABA reactivity as indicated by the arrow in pictures **B-B''**. **C**: The arrows in picture **C** indicate two GABA immunoreactive cellbodies. In picture **C'** arrows point to a total of four stained neurons. **C''**: The overlay show two GABA ergic somata. One somata is not GABAergic (double arrow). The last soma is in the periphery of the preparation as indicated by the arrow and its reactivity is not vertified. Scalebar: 100µm.

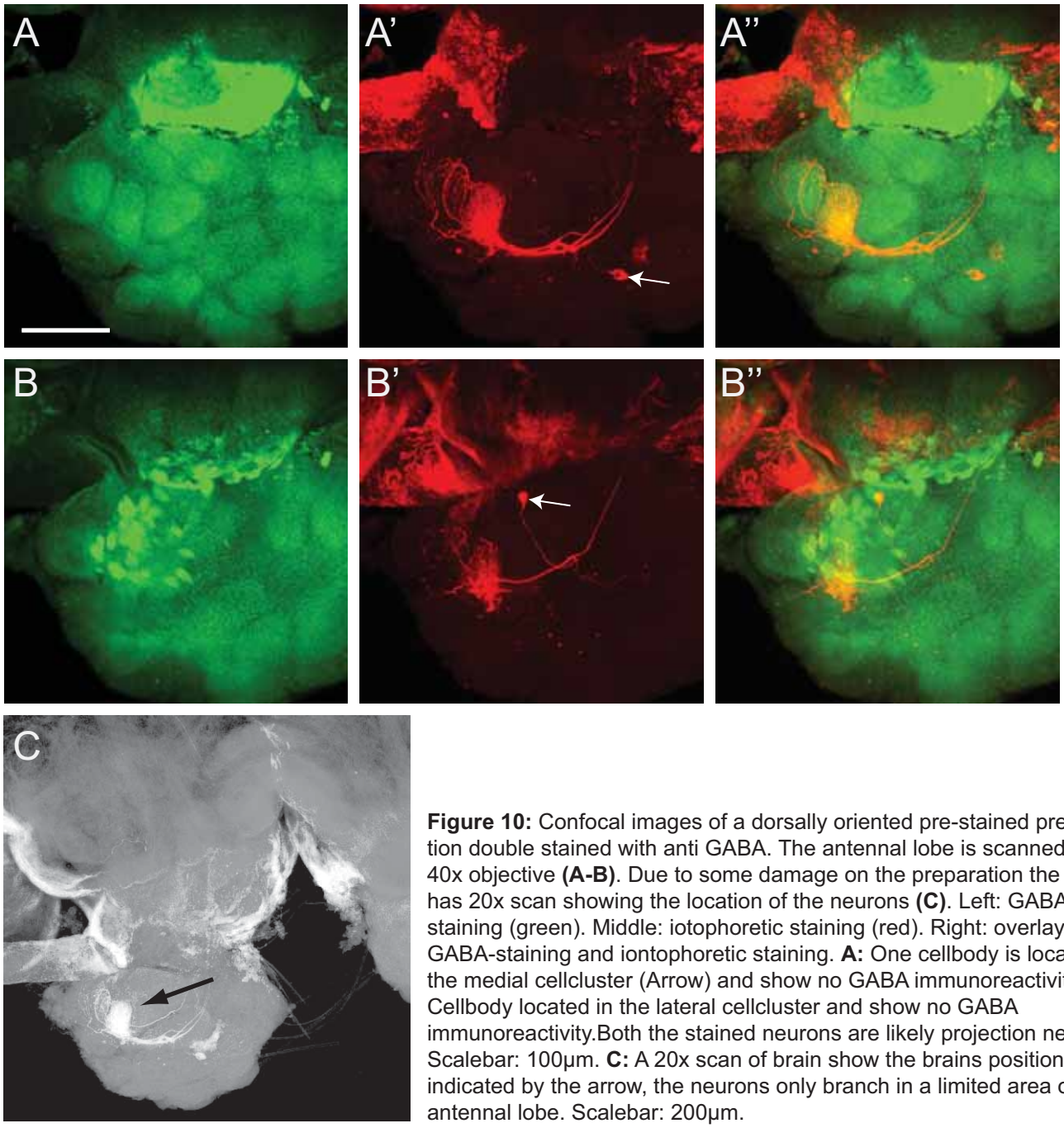


Figure 10: Confocal images of a dorsally oriented pre-stained preparation double stained with anti GABA. The antennal lobe is scanned with a 40x objective **(A-B)**. Due to some damage on the preparation the figure has 20x scan showing the location of the neurons **(C)**. Left: GABA-staining (green). Middle: iotophoretic staining (red). Right: overlay of GABA-staining and iontophoretic staining. **A:** One cellbody is located in the medial cellcluster (Arrow) and show no GABA immunoreactivity. **B:** Cellbody located in the lateral cellcluster and show no GABA immunoreactivity.Both the stained neurons are likely projection neurons. Scalebar: 100µm. **C:** A 20x scan of brain show the brains position. As indicated by the arrow, the neurons only branch in a limited area of the antennal lobe. Scalebar: 200µm.

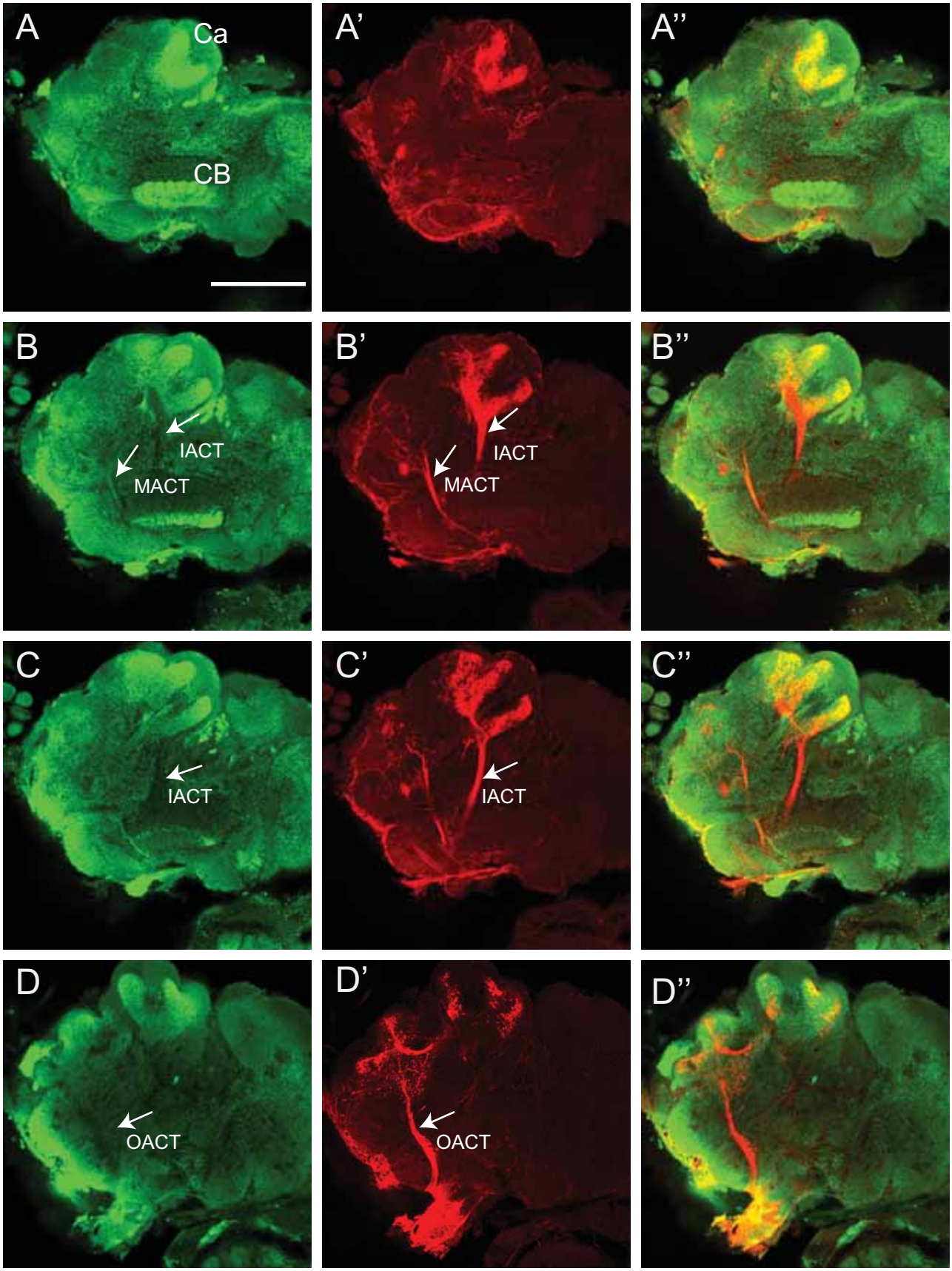


Figure 11: Confocal images from a dorsally oriented preparation scanned with a 20x objective. The preparation has tracts pre-stained through injection of dye in the antennal lobe. Left: GABA-staining (green). Middle: iotophoretic staining (red). Right: overlay of GABA-staining and iontophoretic staining. The figure show consecutive images dorsally through the image stack (**A-D**). **A:** The central body (CB) and the calyses of the mushroombodies (Ca) are distinctly stained with anti-GABA. **B:** GABA stained fibers are visualized passing through the middle antennocerebral tract (MACT). The inner atennocerebral tract (IACT) show no GABA reactivity **C-D:** IACT and the outer antennocerebral tract (OACT) show no GABA immunoreactivity. Scalebar 200 µm.

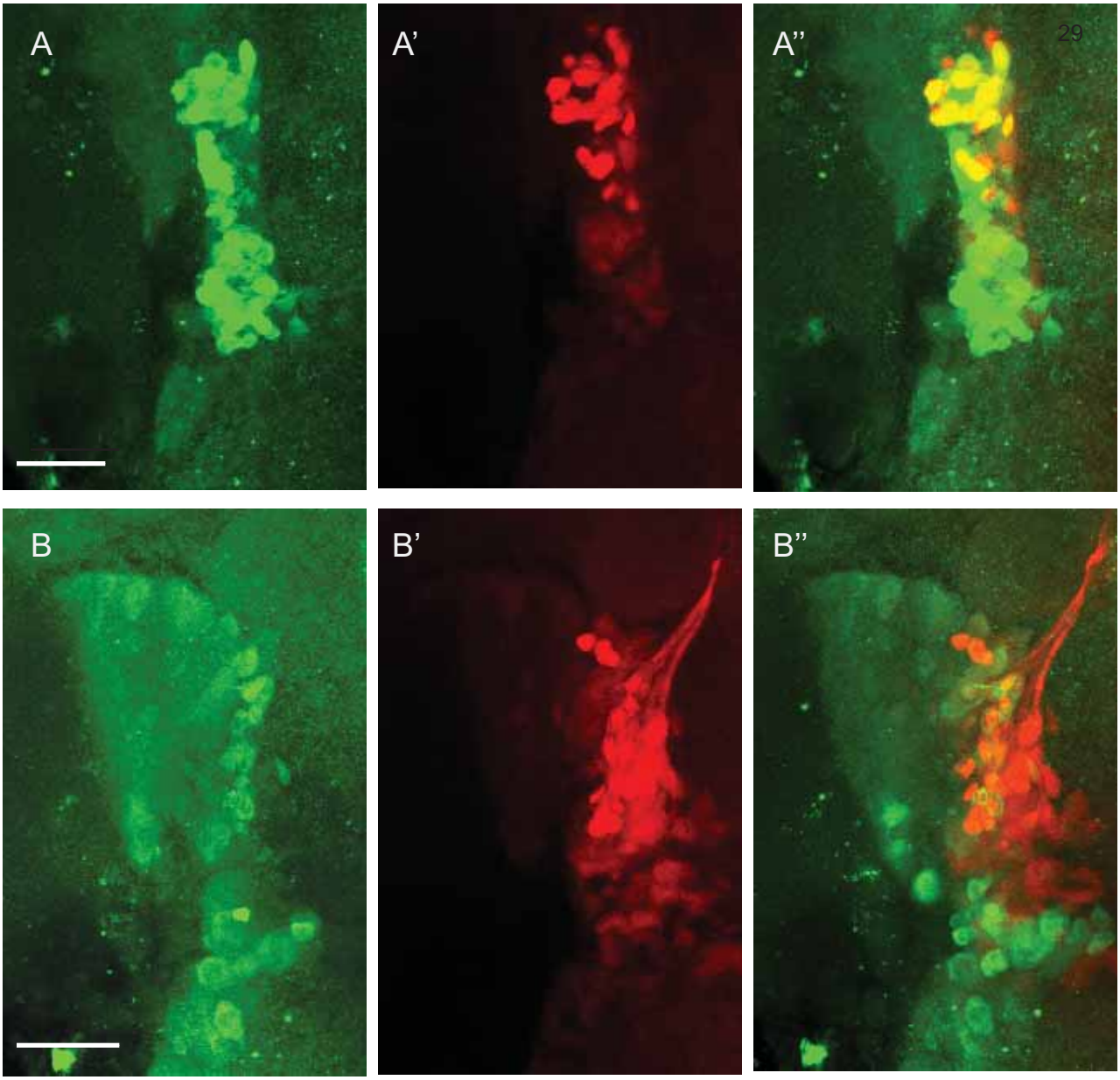


Figure 12: Confocal images from a preparation pre-stained with dye applied into the calyces through mass filling. The preparation is scanned with a 20x objective and show two consecutive images of the lateral cellcluster. Left: GABA-staining (green). Middle: iotophoretic staining (red). Right: overlay of GABA-staining and iontophoretic staining. **A**: Several projection neuron somata proximately grouped together show GABA immunoreactivety. **B**: A group of projectioneurons more ventrally located do not display GABA immunoreactivity. Scalebar: 100µm

4.0 Discussion:

4.1 The results of the study

4.1.2 A summary of the results

The present study revealed widespread GABA immunolabeling in sub-regions of the whole brain, including parts of the olfactory pathways. In particular, substantial staining was found in seemingly all glomeruli of the antennal lobe and in a large number of cell bodies located in the lateral cell cluster of the antennal lobe. Combined GABA-immunolabeling and mass staining of the antennocerebral tracts showed double-stained axons in the medial tract and a group of double-stained somata in the lateral cell cluster. Of the iontophoretically pre-stained antennal-lobe neurons, which included local interneurons and uni-glomerular projection neurons, all local neurons, except for one, were GABAergic while none of the the projection neurons were GABAergic.

4.1.3 General staining pattern of the olfactory pathway

The immunostaining of all antennal-lobe glomeruli, both ordinary and male specific, demonstrates the general importance of inhibitory input in processing of both pheromone and plant odour information. This glomerular staining pattern is in correspondence with previous reports dealing with moth (Berg et al., 2009; Seki & Kanzaki, 2009 & Hoskins et al., 1986) as well as other insect species (reviewed by Hansson & Anton, 2000).

The GABAergic somata observed in the antennal lobe were all located in the lateral cell cluster, none were observed in medial or anterior clusters. This is also in full agreement with former findings in several moth species as *H. virescens, M.sexta*, and *B. Mori*, all showing GABAergic somata located exclusively in the lateral cell cluster (Berg et al, 2009;Seki & Kanzaki, 2009 and Hoskins et al,1986). Concerning the species studied here, *H. virescens*, the number of GABAreactive antennal-lobe somata were counted in the previous study by Berg, Schachtner and Homberg (2009) and included a total of 327 ±12. As already mentioned, all local neurons of the antennal lobe have their cell bodies located in the lateral cell cluster (Hansson and Anton, 2000; Galizia & Rössler, 2010), and most of the labeled somata in the present study are therefore assumingly somata of this neuron category. However, like in previous studies, the present data also demonstrate the presence of some GABAergic projection neurons passing in the medial antennocerebral tract.

4.1.4 Pharmacological identification of iontophoretically pre-stained neurons according to GABA-immunoreactivity

The data from the present study include 8 successfully double-stained local interneurons. All but one of these displayed GABA-immunereactivity, a finding which confirms the generally accepted theory that most antennal-lobe local neurons provide inhibitory input by releasing GABA. Interestingly, one local interneuron was immunonegative. Recently, a sub-population of excitatory local neurons, using acethylcholin, was reported in the fruit fly (Shang et al. 2007). Also in *H.virescens* and *B.mori*, local neurons not containing GABA have been found (Berg et al. 2009; Iwano & Kanzaki, 2005). Whether these neurons use acethylcholin and act excitatory, like the sub-population reported in the fruit fly, is however not known. In *B.mori* it is estimated that the GABA negative neurons constitute approximately 20% of the local neurons (Iwano & Kanzaki, 2005) and it is reasonable to think that the number is transferrable to other moth species. The GABA-immunonegative neurons reported in moths could also be inhibitory trough the use of other transmitters like for instance histamine. In the honeybee *Apis mellifera*, a sub-population of inhibitory local neurons using histamin has namely been reported (Sachse, Peele, Silbering, Guhmann and Galizia, 2006). Generally, the functional significance of excitatory local neurons is subject to increasing interest (Shang et al, 2007).

As concerns the 3 single pre-stained projection neurons, none of them were GABAergic — a finding which verifies the assumption that uni-glomerular out-put neurons projecting in the inner antennocerebral tract, are GABA-immunonegative. Two of the neurons had their cell bodies located in the lateral cell cluster and one had its cell body located in the medial(?) cell cluster. This localization of projection-neuron somata has been described in earlier studies (Homberg et. al, 1989, Hansson and Anton, 2000).

In addition to the preparations containing the 3 iontophoretically stained projection neurons, GABA-immunostaining was performed on preparations containing projection neurons that were mass stained. In the retrogradely labeled preparations, projection-neuron somata were mapped in the lateral cell cluster and the projection-neuron axons were visualized through staining of the antennocerebral tracts. Interestingly, the sub-population of GABAreactive projection neurons seems to be gathered in a restricted area of the lateral cell cluster, located ventrally to the rest of the somata. And the double-labeled somata also seem to be relatively small in size. A similar organization has been reported in the *M.sexta* where a particular part of the lateral cell cluster is referred to as LC II (Homberg, Montague & Hildebrand, 1988).

This particular group is believed to contain somata of multiglomerular, GABAergic projection neurons passing in the medial tract (Galizia & Rössler, 2010). Due to the relatively poor penetration in deeper parts of the brain, the general distribution of GABA in the antennocerebral tracts could be analyzed only in a few preparations. However, as demonstrated in figure 11, the prominent inner tract is visible in shape of its GABA immunonegativity whereas the middle tract is weakly immunopositive. Also the outer tract can be identified based on a considerable amount of immunonegative fibers. The few GABAergic axons projecting in this particular tract, as previously reported in *H. virescens*, was not found in the present study.. In general, however, e the projection pattern of the tracts and their pharmacological properties fits with earlier findings (Berg, Schachtner & Homberg, 2009, Rø, Müller & Mustaparta, 2007; Homberg, Montague & Hildebrand, 1988).

4.2 The function of GABA in the olfactory system and implications of the results

4.2.1 Lateral inhibition

GABA-mediated lateral inhibition in the primary olfactory center of the moth brain was described more than 20 years ago (Waldorp et al, 1987). As mentioned above, GABA-mediated inhibition of neural circuits in this integration center is important for all olfactory signals, including those arising from plant odors, pheromones, and interspecifically acting substances. In the antennal lobe, inhibitory local neurons synapse both with receptor neurons, projection neurons, and other local neurons (reviewed by Anton and Homberg, 1999). The incoming signals that target one spesific glomerulus or sets of glomeruli are subject to what is called lateral inhibition. Lateral inhibition refers to the effect where an exited neuron reduces the activity of a neighbouring cell (Reviewed in Galizia & Szyszka, 2007, Galizia, 2008).

4.2.2 Spatial codes

Lateral inhibition is likely directly linked to what is called a spatial code. Because the principles of the olfactory system are so well preserved across different species, we have some ideas about its benefits. A major functional advantage can be found in the formation of similarly tuned cells into glomeruli. This kind of anatomical organization likely reduces the detection threshold for weak signals with a noisy background. Thus, these basic “hardwired”

connections from receptors to specific neuropil areas can afford fast processing. The olfactory stimuli are processed according to a principle of so-called odotopy which means that specific stimuli qualities are represented in distinct glomeruli. (Galizia & Szyszka, 2007; Sachse & Galizia, 2002; Lei, Christensen, Hildebrand, 2004). Recent experiments performed on honeybees and fruit flies including manipulation and restrictions of effects of lateral inhibition, demonstrated that the projection neurons became more broadly tuned and that odor discrimination thresholds were increased (Olsen & Wilson, 2008 and Mwilaria, Ghatak & Daly, 2008). This means that the underlying networks have a harder time distinguishing similar odors from each other. GABA is likely involved in odor processing by inhibiting the surrounding glomeruli of the glomerulus receiving a strong signal. In other words, the signal is beeing amplified by dampening the background noise (Wilson & Laurent, 2005). A good review of the corresponding vertebrate system is given by Wilson & Mainen, (2006).

4.2.3 Temporal codes

The inhibitory networks in the antennal lobe might also play a part in what is called temporal codes. This reefer to a possible synchronization in temporal spiking patterns of projection neurons so that network oscillations are formed (MacLeod and Laurent, 1996, Laurent 2002). The brain's ability to remember and discriminate odours might rely on a capability to interpret a combination of spatial and temporal codes. These codes might be processed in higher integration centres both in vertebrates and invertebrates. Many odour molecules are chemically and structurally similar. They therefore evoke similar spatial activation patterns. A temporal coding mechanism of timed action potential spiking might explain how fine tuned differentiations are obtained (Lledo, Gheusi and Vincent, 2005., Wilson and Mainen, 2006, Galizia, 2008).

Investigation into such codes in olfactory information processing can be traced back to E.D Adrian's research (1950). Most of the current explanation models are open to the possibility that temporal and spatial codes are intertwined and mutually dependent on each other. Overlapping spatial and temporal information expands the coding capacity as the number of glomeruli itself is limited (Sachse & Galizia, 2002, Lei, Christensen, Hildebrand, 2004. It is however not any conclusive evidence of precisely how these two coding principles might be intervened. What appears to be temporal codes in research data might actually be effects caused by administrating odor stimuli in certain ways during experiments. We also know too

little about the changes caused by experience and learning in sensory circuits (Christensen, 2005).

4.2.4 The effects of inhibitory projection neurons

This study, as well as recent publications dealing with moths, show that a collection of projection neurons are inhibitory (Berg et al., 2009, Seki et al, 2008; Hoskins et al. 1986). These neurons mainly pass through the middle antennocerebral tract. Further research is needed to understand the functions of the inhibitory signals. It is proposed however that the combined excitatory and inhibitory inputs from the tract to target brain areas, further enhances odor discrimination and sensitivity (Berg et al., 2009).

4.2.5 Local neurons not containing GABA

The local neuron that does not use GABA as a transmitter might use another inhibitory transmitter. Local neuron circuits can however also be excitatory, as mentioned above. A possible function of these circuits is to ensure transmission of excitatory signals from receptor neurons targeting one glomerulus to projection neurons innervating another glomerulus (Shang et al., 2007). They may also amplify the general response within the glomeruli (Galizia, 2008).

4.2.6 GABA mediated excitation

In general, GABA is recognized as the major inhibitory transmitter in the nervous system which means that its function is to dampen nervous activity. An increasing number of examples have however complicated the picture as GABA-mediated excitation has been demonstrated several times in mammals, predominantly in rats and mice. The effect was first observed in embryonic and early postnatal life only (Cherubini, Gaiarsa & Ben-Ari 1991, Ben-Ari, Khazipov, Leinekugel, Caillard & Gaiarsa, 1997). The role of excitatory GABA in early development is likely directly linked to modulatory functions as GABA induced excitations are shown to shape the early formation of networks into functional units and to establish oscillations (Ben-Ari, Gairsa, Tyzio & Khazipov, 2007) Although excitatory GABA-

synapses have not been reported in insects, these new insights raises the theoretical possibility that we might find similar arrangements in the future.

4.3 The validity of using insects as a model for humans

G. Galizia (2008) raises a question if the insect model is outdated as model organisms. The accessibility of the insect nervous system has historically ensured its relevance as a model, but technological and methodological developments have made vertebrate olfactory systems far more accessible. Maybe it is time to shift the focus more exclusively towards these models. Furthermore, new insights seem to indicate that there might be features of the insect olfactory system that are different from that of vertebrates, like the transduction mechanism in the olfactory receptor neurons (Nakagawa and Vosshall, 2009 and Pellegrino and Nakagawa 2009).

The main argument of using insects as a comparative model to understand humans is that olfactory processing principles are so well preserved across different species. Bipolar sensory neurons surrounded by supporting cells are found in the olfactory organ of both vertebrates and insects. Both systems also have olfactory receptor neurons with dendrites facing the external world through the surface of the olfactory organ. The inhibitory networks of the insect antennal lobe also find its counterpart in the olfactory bulb of vertebrates. The olfactory bulb includes a large number of local interneurons, many of which are GABAergic (Smith, 2008). The role of GABA generated oscillations is particularly well studied in rats and mice (Valley & Firestein, 2008).

One can raise the question of whether a system largely based on pheromone processing can give accurate descriptions of our own olfactory system. Smell as a chemical communicator between individuals is likely insignificant in humans as compared to the role pheromones play to most vertebrate and invertebrate species. Animals and insects use pheromones predominantly to find a suitable partner and reproduce, but in some species they also serve other behavioural functions. Examples are mammals marking territory or desert locust aggregating swarms (Ference and Seidelmann, 2003, Bear, Connors and Paradisio 2006). A widespread assumption is that in order to communicate with pheromones an *accessory olfactory system* is needed. The accessory system runs parallel to the primary olfactory systems and it is speculated if the *vomeronasal organ* in humans enters into such a system or

contains traces of an earlier, evolutionary significant system (Meridith, 2001 and Brennan & Zufall, 2006). Although more research is needed to clarify the functional specialization of olfactory systems in many species, the current literature does not justify a view of pheromone processing as qualitatively different from that of other odorants. The male specific macro glomerular complex seems to be affected by and integrated with ordinary glomeruli as part of the same networks, especially the local interneurons. It might not be fruitful to draw a clear cut functional distinction between primary and accessory olfactory systems (Christensen & Hildebrand, 2002). Both pheromone and non pheromone processing seem to rely on projection neurons with different temporal response properties and dynamic ranges (Galizia & Rössler, 2010).

At the level of functional neural networks similarities generally seem to outnumber the differences between species, even in evolutionary distant lineages. It seems as though nature has reinvented certain ways to deal with the challenge of detecting and perceiving chemical stimuli and that the olfactory sense modality is one of the most “well kept” structures throughout evolution (Hildebrand & Shepherd, 1997,Schactner, Schmidt & Homberg, 2005, Farris, 2004, 2008a).

Perhaps the most striking similarity across olfactory systems is the arrangement of neuropil into glomeruli. It seems as though glomeruli are formed when the intergration of neuronal activity reaches a critical level of synaptic complexity. Ouland & Tolbert, (1996) conclude that a critical number of axons is needed in order for glomerular structures to form. Manipulating the number of neurons can prevent formation of glomeruli. Examples of odour-driven insects without glomeruli like members of *hemipteria* can offer support to this explanation. These species share signs of low synaptic connectivity in the primary olfactory centre (Kristoffersen, Hansson, Anderbrant & Larsson, 2008).

Galizia (2008) underlines the fact that we are still far from understanding a single nervous system to its full extent. The comparative value of using various species in neuroscience is as valid as ever. The simpler organisms continue to play a key role in understanding all parts of the complexities of sensory systems. Even our understanding of the most fundamental principles is constantly being challenged by new insights.

4. 4 Methodological considerations

4.4.1 Establishing the protocol

The initial protocol was applied largely based on similar experiments, described in previous publications, in particular the recent GABA-staining of *B. mori* (Seki & Kanzaki, 2008) and *H. virescens* (Berg et al, 2009). The initial procedure produced sufficiently promising results from the start to encourage only minor changes in the procedure during the limited time period available for data collection. Different time intervals for incubation and fixation were tried out as well as different storing temperatures. It seems likely that conducting additional trials could help optimize the procedure systematically and reduce errors caused by insufficient laboratory skills.

One of the conclusions from establishing the protocol is that using whole brain preparations is preferable for several reasons. Whole brains generally provided the best quality of staining the antennal lobe and the most well stained preparations allowed good and illustrative 3D images and structures of interest could be visualized in one single confocal image stack.

4.4.2 Properties of the antibody

The antibody did provide good staining of antennal lobe structures like glomeruli and cellclusters. A major problem encountered in several preparations was a difficulty applying the antibody and the fluorescence in the centre of the preparations. The staining often seems to form a layer on the outer brain membrane and not penetrate it. Several causes for these results seem plausible.

The problems of deciding the time intervals under which administration to the different chemicals and conditions seems like a natural starting point and so are issues related to storage. It is plausible that the antibody needs more than three days to work optimally. Future trials could compare possible differences cased bye these variables, for example storage temperature. More time to perform consecutive trials could therefore help optimise the protocol. Another possible trick to make the absorption of staining easier might be to partially disrupt the sheet covering the brain. However, the risk of damaging the brain or removing parts of the anatomical areas of interest is a drawback. The strong labelling of the central body in several preparations indicate still that the staining reaches structures in the centre of the brain under certain conditions.

The use of vibratome sections rather than whole brains would probably be more ideal with an eye to characterizing immunostaining of the deeper brain structures as the olfactory tracts. Thin sections might be more thoroughly stained. However, due to the fact that whole brain preparations showed the most optimal immunostaining in the antennal lobe, we chose this method in the present study. In addition, handling vibratome sections requires accuracy and the ability of arranging consecutive slices from the same brain in the correct order, a skill that has to be developed during repeated trials.

A main objection against this particular GABA antibody concerns its specificty.. As reported in the results, there seems to occur a kind of cross reaction between the dye used for staining of single neurons, that means the rhodamin dextrane, and the GABA antibody. Thus, the antibody seems to strengthen the iotophoretic staining in particular neurons; more precisely, it seemed to cross react with previously stained GABAergic neurons so that they were intensified as compared to the GABAergic neurons that were not pre-stained. Pre-stained neurons classifying as GABA immunonegative, as for instance the uni-glomerular projection neurons, were, in general, not subject for such a cross reaction. In the present data, only one occasion was observed where the antibody attached itself to a small parts of a pre-stained neuron not displaying GABA-reactivity (figure 9). Actually, in most cases the effect of the cross reaction seems advantageous as it makes the pre-stained neuron somata and branches easier to spot in the data. In the present study, the results from the double-labeling experiments therefore demonstrated the presence versus absence of GABA in pre-stained neurons in a reliable way. However, the fact that the antibody binds to other components than just GABA, could be a threat against the validity of the results. Contact with the provider of the antibody has been established in order to inform about the weakness of the particular antibody as concerns specificity.

4.5 Future research

The current study indicates that the antibody and protocol used can generate valid data about the distribution of GABA in the olfactory pathway of the moth brain. The methodological data can likely be applied to further studies including general GABA immunostaining of particular brain compartments and immunolabeling of single pre-stained neurons. More time to conduct trials and optimise the protocol can hopefully improve the results. Combined with physiological data or staining with antisera against other transmitters, these data could enhance our understanding of the antennal lobe networks. One of the yet scarcely desribed parts of this network, is for example the physiological properties characterizing the sub-poulation of inhibitory antennal-lobe projection neuron

Future research has numerous ways to go to integrate previously fragmented knowledge about different parts of the olfactory system. Three dimensional atlases of antennal lobes and whole brains as well as standardized digital brain models are made for several species to better reveal general anatomical principles and integrate the information gained through immunocytochemistry, intracellular recordings combined with labelings, and other imaging techniques. Such an atlas exists for the antennal lobe of *H.virescens* and *H.assulta* (Berg et al 2002). Recently, standard brain models for the whole brain and the antennal lobe of *H.virescens* have been developed, with potential of integrating and generalizing anatomical, physiological, and pharmacological findings characterizing the neural networks (Kvello, Løfaldli, Rybak, Menzel and Mustaparta, 2009., Løvfaldli, Kvello and Mustaparta, 2010). A standard brain atlas was also recently published for the hawkmoth *M. sexta* (El Jundi, Huetteroth, Kurylas and Schachtner, 2009). The potentials of these imaging techniques are enormous provided the researchers find optimal ways to integrate the various forms of information. Furthermore, in *Drosophila*, the detailed mapping of the genome has provided new dimensions to the research as concerns functions of neuroactive substanses like GABA (Fei et al., 2010). To gain such an overview of the genome might also be possible for other species, as for instance the moth. The field of neuroscience is rapidly growing, and the possibilities for obtaining knowledge about previously unavailable areas seem ever increasing.

4.6 Conclusion

The main aim of the study — to establish a protocol for GABA immunostaining of the moth brain in order to map, by the double-fluorescence technique, the presence or absence of GABA in particular neurons of the central olfactory pathways — was in general fulfilled. In total, the results are scientifically interesting and also promising as concerns future projects intending to understand how inhibitory input influences chemosensory information processing.

1. Based on the protocol established in the present study, GABA immunoreactivity was demonstrated in particular regions of the olfactory pathways including the antennal lobe, the calyces, and the medial antennocerebral tract, a finding which is in correspondence with previous reports.

2. The double-labeling experiments succeeded in visualizing pharmacological properties of single antennal-lobe neurons. Of the local interneurons stained, all except for one were GABAergic whereas the uni-glomerular projection neurons were all immunonegative.

3. The anterogradely and retrogradely pre-stained brains revealed the presence of GABAergic fibers in the medial antennocerebral tract, whereas the inner and outer tract showed no GABA immunoreactivity. A group of double-labeled somata, assumingly belonging to the GABAergic fibers of the medial tract, were located in the ventral part of the lateral cell cluster.

4. The GABA antiserum used in the present study cross-reacted with the dextrane rhodamine dye in pre-stained GABAergic neurons. Due to this, using a different and more specific antiserum should be considered for this kind of double-labelings in future experiments.

5.0 REFERENCES

Adrian, E.D. (1950). The electrical activity of the mammalian olfactory bulb.*Electroencephalografi and Clinical Neurophysiology, 2*, 377-88.

Almaas, T.J. & Mustaparta, H. (1991). *Heliothis viriscence*: response characteristics of receptor neurons in sensilla trichodea type 1 and 2. *Journal of chemical ecology, 17,* 953-972.

Almaas, T.J. & Mustaparta, H. (1990). Pheromone reception in tobacco budworm moth, *Heliothis viriscence. Journal of chemical ecology, 16,* 1331-1347.

Anton, S. & Homberg, U. (1999) Antennal lobe structure. In: B.S. Hansson (ed.), Insect olfaction. (pp.97-124). Berlin: Springer-verlag.

Bear, M. F., Connors, B.W. & Paradiso, M. A. (2007) *Neuroscience exploring the brain* (3rd ed.). Baltimore : Lippincott Williams & Wilkins.

Ben-Ari, Y., Gaiarsa,J., Tyzio,.R & Khazipov, R. (2007). GABA: A pioneer transmitter that excites immature neurons and generates primitive oscillations. *Physiological reviews, 87*,1215-1284

Ben-Ari, Y., Khazipov, R., Leinkugel, X., Caillard, O., Gaiarsa, J.-L. (1997). $GABA_A$, NMDA and AMPA receptors: a developmentallyregulated 'menage a trois'. *Trends in Neurosci, 20*, 523–529.

Berg, B.G., Almaas, T.J., Bjaalie, J.G. & Mustaparta, H. (1998) The macroglomerular complex of the antennal lobe in tobacco budworm moth *Heliothis virescens*: specified subdivision in four compartments according to information about biologically significant compounds. *The Journal of Comparative Physiology A, 183*, 669-682.

Berg, B.G., Galizia, G., Brandt, R. & Mustaparta, H. (2002). Digital atlases of the antennal lobe in two species of tobacco budworm moths, the oriental *Helicoverpa assulta* (male) and the American *Heliothis virescens* (male and female). *The journal of comparative neurology, 446,*123-134.

Berg, B.G., Schachtner, J. & Homberg, U.(2009). Gamma-aminobutyric acid immunostaining in the antennal lobe of the moth Heliothis virescens and its colocalization with neuropeptides. *Cell and Tissue Research, 353* (3):593:603

Brennan, P.A. & Zufall, F. (2006). Pheromonal communication in vertebrates. *Nature, 444,* 308-315.

Brodal, P. (2007). *Sentralnervesystemet* (4th ed.). Oslo: Universitetsforlaget.

Breer, H. (1994). Odor recognition and second messenger signaling in olfactory receptor neurons. *Cell biology, 5*, 25-32.

Buck, L. & Axel, R. (1991). A novel multigene family may encode odorant receptors: A molecular basis for odorant recognition. *Cell, 65*, 175-187.

Butenandt, A., Beckmann, R., Stamm, D. & Hecker, E. (1959) Über den sexuallockstoff den Seidenspinners *Bombyx mori.* Reindarstellung und konstitution. *Zeitschrift für Naturforchung, 14,* 283-284.

Caveney , S & Donly, B.C. (2002). Neurotransmitter transporters in the insect nervous system. *Advances in Insect Physiology. 29, 55-159.*

Cherubini E, Gaiarsa, J.L, Ben-Ari, Y. (1991). GABA: an excitatory transmitter in early postnatal life. *Trends in Neuroscencei.*(12):515-9.

Cho, S., Mitchell, A., Mitter, C., Regier, J., Matthews, M. & Robertson, R. (2008). Molecular phylogenetics of heliothine moths (Lepidoptera: Noctudinea: Heliothinae), with comments on the evolution of host range and pest status. *Systematic Etomology, 33*, 581-594.

Christensen, T.A. (2005). Making scents out of spatial and temporal codes in specialist and generalist olfactory networks. *Chemical Senses.* 30, 283-284.

Christensen, T.A. & Hildebrand, J.G. (2002). Pheromonal and host-odor processing in the insect antennal lobe: how different? *Current opinion in neurobiology, 12*, 393-399.

Carlsson, M.A. & Hansson, B.S. (2003). Plasticity and coding mechanisms in the insect antennal lobe. In: G. Blomquist & R. Vogt (Eds.), Insect pheromone detection biochemistry and molecular biology. Heidelberg: Elesevier academic press.

Fahrbach, S.E. (2006). Structure of the insect mushroom bodies of the insect brain. *Annual Review of Entomology, 51*, 209-32.

Farris, S.M. (2004). Evolution of insect mushroom bodies: old clues, new insights. *Arthropod structure & development, 34*, 211-234.

Fahrbach, S.E. (2006). Structure of the insect mushroom bodies of the insect brain. *Annual Review of Entomology, 51*, 209-32.

Farris, S.M. (2004). Evolution of insect mushroom bodies: old clues, new insights. *Arthropod structure & development, 34*, 211-234.

Farris, S.M. (2008a). Structural, functional and developmental convergence of the insect mushroom bodies with higher brain centers of vertebrates. *Brain Behav Evol, 72*, 1-15.

Farris, S.M. (2008b). Tritocerebral tract input to the insect mushroom bodies. *Arthropod structure & development, 37,* 492-503.

Ferenz, H. & Seidelmann, K. (2003). Pheromones in relation to aggregation and reproduction in desert locust. *Physological etomology, 28,* 11-18.

Galizia, C.G. (2008). Insect olfaction. In: Firestein, F. & G.K. Beauchamp (Eds.) *The senses: A comprehensive reference. Vol 4 olfaction and taste*. (pp.725-770). San Diego: Academic.

Galizia, C.G. & Rössler, W. (2010). Parallel olfactory systems in insects: Anatomy and function. *Annual Review of Entomology, 55,* 399-420.

Galizia, C.G. & Szyszka, P. (2008). Olfactory coding in the insect brain:molecular receptive ranges, spatial and temporal coding. *Entomologia Experimentalis et Applicata* 128 (1) 81-92

Guerenstein, P., Christensen, T.A. & Hildebrand, J.G. (2004). Sensory processing of ambient-CO2 information in the brain of the moth *Manduca sexta*. *Journal of Comparative Physiology, 190*, 707–725.

Hammer, M. (1997). The neural basis of associative reward learning in honeybees. *Trends in Neuroscience 20*, 245-252

Hansson, B.S. & Anton, S. (2000). Function and morphology of the antennal lobe: new developments. *Annual Review of Entomology, 45,* 203-231.

Hildebrand, J.G & Shepherd, G.M. (1997). Mechanisms of olfactory discrimination: Converging evidence for common principles across phyla. *Annual Review of Neuroscience*, *20,* 595-631.

Homberg, U., Christensen, T.A. & Hildebrand, J.G. (1989). Structure and function of the deutocerebrum in insects. *Annual Review of Entomology, 34*, 477-501.

Homberg, U., Montague, R.A. & Hildebrand, J.G. (1988). Anatomy of antenna-cerebral pathways in the brain of sphinx moth *Manduca Sexta. Cell Tissue Research, 254,* 255-281.

Homberg, U. & Müller, U. (1999). Neuroreactive substances in the antennal lobe. In: B.S. Hansson (ed.). *Insect olfaction* (pp. 97-124). Berlin: Springer-verlag.

Hoskins, S.G., Homberg, U., Kingan, T.G., Christensen, T.A. & Hildebrand, J.G. (1986). Immunocytochemistry of GABA in the antennal lobes of the sphinx moth *Manduca sexta*. *Cell and tissue research, 244*, 243-252.

Iwano, M & Kanzaki, R. (2005). Immunocytochemical Identification of Neuroactive Substances in the Antennal Lobe of the Male Silkworm Moth *Bombyx mori. Zoological Science Zoological Science* 22(2):199-211.

Jorgensen, E.M. (2005). GABA. In The *C. elegans* Research Community (Ed). WormBook, doi/10.1895/wormbook.1.7.1, http://www.wormbook.org

Karlson, P. & Lüscher, M. (1959). Pheromones: a New Term for a Class of Biologically Active Substances. *Nature, 183*, 55-56.

Kent, K.S., Harrow, I.D., Quatararo, P. & Hildebrand, J.G. (1986). An accessory olfactory pathway in Lepidoptera: the labial pit organ and its central projections in *Manduca sexta* and certain other sphinx moths and silk moths. *Cell and Tissue Research*, *245,* 237-245.

Kerkut, GA., Pitman, R.M. & Walker, R.J. (1969). Iontophoretic application of acetylcholine and GABA onto insect central neurons. *Comparative Biochemistry and Physiology, 31,* 611-633.

King, JR., Christensen, T.A. & Hildebrand, J.G. (2000). Response characteristics of an indentified, sexually dismorphic olfactory glomerulus. *The journal of neuroscience, 20*, 2391-2399.

Kristoffersen, L., Hansson, B.S., Anderbrant, O. & Larsson, M.C (2008). Glomerular hemipteran antennal lobes- basic neuroanatomy of a small nose. *Chemical senses, 33*, 771-778.

Kvello, P., Løfaldli, B.B., Rybak, J., Metzel. R. & Mustaparta, H. (2009). Digital, three-dimensional average shaped atlas of the *Heliothis virescens* brain with integrated gustatory and olfactory neurons. *Frontiers in systems neuroscience, 3* (14), 1-14.

Laurent, G. (2002). Olfactory network dynamics and the coding of multidimensional signals. *Nature Reviews Neuroscience, 3*, 884-95.

Lei, H., Christensen, A. & Hildebrand, J.G. (2004) Spatial and temporal organization of ensemble representations for different odor classes in the moth antennal lobe. *The journal of neuroscience, 24*, 11108-11119.

Lledo, P., Gheusi, G. & Vincent, J. (2005). Informantionprocessing in the mammalian olfactory system. *Physol Rev, 85,* 281-317.

Løfaldli, B.B., Kvello, P. & Mustaparta, H. (2010). Integration of the antennal lobe glomeruli and three projection neurons in the standard brain atlas of the moth *Heliothis virescens. Frontiers in systems neuroscience, 4* (5)*,* 1-12.

MacLeod, K. & Laurent, G. (1996). Distinct mechanisms for synchronization and temporal patterning of odor encoding neural assemblies. *Science, 274*, 976-979.

Masante-Roca, I., Gadenne, C. & Anton, S. (2005). Three dimensional antennal lobe atlas of male and female moths, *Lobesia botrana* (Lepidoptera: Tortricidae) and glomerular representations of plant volatiles in females. *The journal of experimental biology, 208*, 1147-1159.

Meredith, M. (2001) Human vomeronasal organ function: a critical review of best and worst cases. *Chem senses, 26*, 433-445.

Mombaerts, P. (1999). Seven-transmembrane proteins as odorant and chemosensory receptors. *Science, 268*, 707-711.

Mwilaria, E.K., Ghatak, C. & Daly, K.C. (2008). Disruption of $GABA_A$ in the insect antennal lobe generally increases odor detection and discrimination thresholds. *Chemical Senses, 33,* 267-281.

Nakagawa, T. & Vosshall, L.B. (2009). Controversy and consensus: noncanonical signaling mechanisms in the insect olfactory system. *Current opinion in neurobiology, 19*, 284-292.

Oland, L.A. & Tolbert, L.P. (1996). Multiple factors shape development of olfactory glomeruli: Insights from an insect model system. *Journal of neurobiology,* 30, 92-109.

Olsen, S. & Wilson, R.I. (2008). Lateral presynaptic inhibition mediates gain control in an olfactory circuit. *Nature, 425*, 956-960.

Osborne, R.H. (1996). Insect neurotransmission: neurotransmission and their receptors. *Pharmacology & Therapeutics*, *69*, 117-142.

Otsuka, M., Iversen, L.L., Hall, Z.W. & Kravitz, E.A. (1966). Release of gamma-aminobutyric acid from inhibitory nerves of lobster. *Procedings of the National Academy of Science, 56,*1110-1115.

Pellegrino, M. & Nakagawa, T. (2009). Commentary. Smelling thedifference: controversial ideas in insect olfaction. *The journal of experimental biology, 212*, 1973-1979.

Roberts, E. (2000). Adventures with GABA: fifty years on. In: D.L. Martin & Olsen, R.W. (Eds.). *GABA in the nervous system: The view of fifty years* (pp.1-24). New York : Lippincott Williams & Wilkins.

Royet, J.P., Distel, H., Hudson, R. & Gervais, R. (1998). A re-estimation of the number of glomeruli and mitral cells in the olfactory bulb in rabbit. *Brain research, 788*, 35-42.

Rø, H., Müller, V. & Mustaparta, H. (2007). Anatomical organization of antennal lobe projection neurons in the moth *Heliothis viriscence*. *The journal of comparative neurology, 500,* 658-675.

Sachse, S. & Galizia, G. (2002). Role of inhibition for temporal and spatial odor representation in olfactory output neurons: A calcium imaging study. *J Neurophysol, 87*, 1106-1117.

Sachse, S., Peele, P., Silbering, AF., Gühmann, M & Galizia, C.G. (2006). Role of histamine as a putative inhibitory transmitter in the honeybee antennal lobe. *Frontiers of Zoology, 3*-22.

Sato, K., Pellegrino, M., Nakagawa, T., Nakagawa, T., Vosshall, L.B. & Touhara, K. (2008). Insect olfactory receptors are heteromic ligand-gated ion channels. *Nature, 452*, 1002-1007.

Seki, Y. & Kanzaki, R. (2008). Comprehensive morphological identification and GABA immunocytochemistry of antennal lobe local interneurons in *Bombyx mori*. *The journal of comparative neurology, 506*, 93-107.

Schachtner, J., Smidt, M. & Homberg, U. (2005). Organization and evolutionary trends of primary olfactory brain centers in Tetraconata (Crustacea + Hexapoda). *Arthopod structure & development, 34*, 257-299.

Shang, Y., Claridge-Chang, A., Sjulson, L., Pypaert, M & Miesenböck G. (2007). Excitatory local circuits and their implications for olfactory processing in the fly antennal lobe. *Cell.* 128(3):601-12.

Shepherd, G.M. (1972). Synaptic organization of the mammalian olfactory bulb. *Physiological Reviews*, *52*, 864-917.

Shepherd, G.M. & Greer, C.A. (1990). Olfactory bulb. In: G.M Shepherd (Ed), *The synaptic organization of the brain* (pp.159-203). (3rd ed.).Oxford: Oxford university press.

Shepherd, G.M. (2006). Smell images and the flavor system in the human brain. *Nature,* 444, 316-321.

Smith, C.U.M. (2008). *Biology of sensory systems* (2nd ed.). Oxford : Wiley-Blackwell.

Stranden, M., Liblikas, I., Köning, W.A., Almaas, A., Borg-Karlson, K. & Mustaparta, H. (2003). (-) – Germacrane D receptor neurons in three species of heliothine moths: structure activity relationships. *The Journal of Comparative Physiology A, 189,* 563-577.

Todd, E.L. (1978). Maculation characters for separation of the species of the *Heliothis virescens* complex. *The Florida Entomologist, 61*, 197-199.

Valley, M.T. & Firestein, S. (2008). A lateral look at olfactory bulb lateral inhibition. *Neuron, 59*, 682-684.

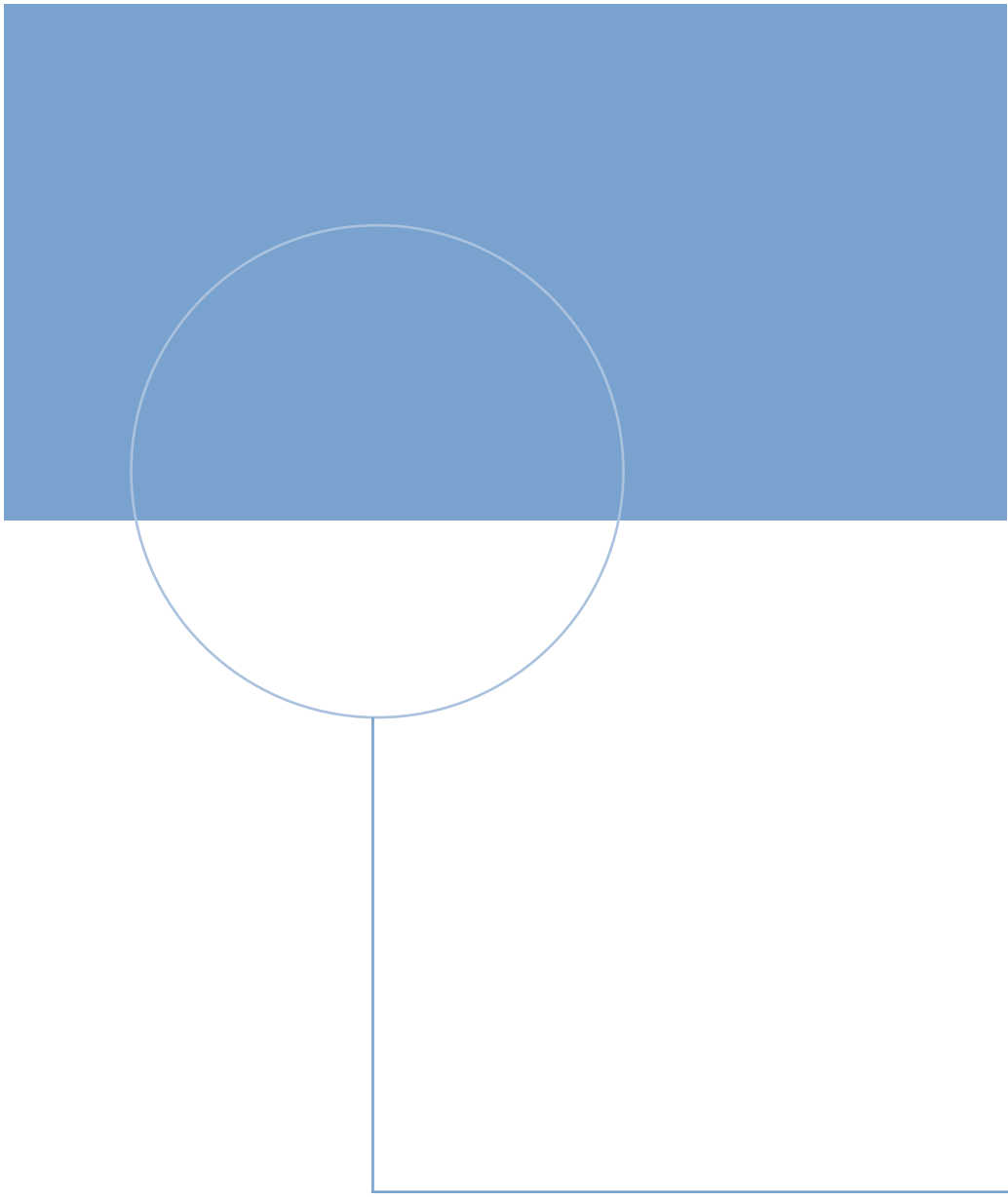
Waldrop, B., Christensen, T.A. & Hildebrand, J.G. (1987) GABA-mediated synaptic inhibition of projection neurons in the antennal lobes of the sphinx moth, *Manduca sexta*. *J Comp Physiol A, 161*, 23-32.

Wicher, D., Shäfer, R., Bauernfeind., Stensmyr, M.C., Heller, R., Heinemann, S.H. & Hansson, B.S. (2008). *Drosophila* odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. *Nature, 452*, 1007-1012.

Wilson, R.I. & Laurent, G. (2005). Role of GABAergic inhibition in shaping odor-evoked spatiotemporal patterns in the *Drosophila* antennal lobe. *The journal of neuroscience, 25*, 9069-9079.

Wilson, R.I. & Mainen, Z.F. (2006). Early events in olfactory processing. *Annual Review of Neuroscience,* 29, 163-201.

Zhao, X.C & Berg, B.G. (2009). Morphological and Physiological Characteristics of the Serotonin-Immunoreactive Neuron in the Antennal Lobe of the Male Oriental Tobacco Budworm, *Helicoverpa assulta.Chemical Senses, 34,* 363-372.



NTNU – Trondheim
Norwegian University of
Science and Technology