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GABA Immunostaining of the Olfactory Pathway in the Heliothine Moth Brain

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Bente Jacobsen

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Sammendrag

Ettersom GABA (γ-aminosmørsyre) er til stede i alle kjente nervesystem, hvor den fyller rollen som den mest utbredte inhibitoriske nevrotransmitteren, er den utbredt både i luktebananene og andre deler av invertebrat hjernen. For å kartlegge inhibitoriske nettverk i luktekesystemet, ble det foretatt GABA-immunofarging av hjernen hos nattsvermeren *Heliothis virescens*. Denne typen immunohistokjemisk eksperiment innebærer benyttelse av et antistoff mot GABA for å visualisere GABAerge strukturer, og nevroner. Fargingen viste sterk immunoreaktivitet i antennelobene, det laterale protocerebrum og i mushroom bodies; alle disse strukturene spiller en viktig rolle i duftprosessering hos insekter. Immunofargingen i det laterale protocerebrum avslørte blant annet en trakt som projiserer fra den mest laterale delen i protocerebrum, nær øyets lobula, til mushroom bodies. Denne trakten er med all sannsynlighet ekvivalent med den såkalte protocerebral calycal tract som også er identifisert hos andre arter. Av de tre hovedbanene fra antenneloben til høyere hjernesentre, de antenno-protocerebrale traktene, viste fibere i den medio-laterale trakten sterk GABA immunoreaktivitet. Dobbelfarging av to intracellulært forhåndsfargede nevroner fra denne trakten viste derimot at de to nevronene ikke var GABAerge og dermed at den medio-lateral trakten ikke er utelukkende inhibitorisk. På bakgrunn av projeksjonsmønstrene til de to immunonegative nevronene, samt tidligere funn som omfatter to strukturelt ulike typer av projeksjonsnevroner i den medio-laterale trakten, kan det synes som om de to nevronene undersøkt her tilhører en egen morfologisk klasse som skiller seg fra de GABAerge nevronene.

Noen metodiske problemer ble også avdekt i denne studien; det viktigste var at identiteten til det sekundære antistoffet - så vel som det primære - har betydning for kvaliteten på resultatene ved immunofarging.

Abstract

γ-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in all known nervous systems, and it is widely distributed in the olfactory circuit as well as other parts of the invertebrate brain. In order to further understand the GABAergic olfactory circuitry, the distribution of GABA in the brain of a nuctoid moth, *Heliothis virescens,* was investigated. Immunohistochemical experiments with an antibody against GABA were performed in order to visualize GABAergic structures and neurons. The stainings revealed strong immunoreactivity in the antennal lobes, the lateral protocerebrum, and the mushroom bodies, all of which are areas involved in olfactory information processing. The dense immunostaining in the lateral protocerebrum included a brightly immunoreactive cell cluster linked to processes innervating the mushroom bodies; this likely corresponds to the protocerebral calycal tract described in other species. Of the main antenno-protocerebral tracts, fibers in the medio-lateral showed strong immunoreactivity. However, double labeling of two intracellularly pre-stained projection neurons passing in this tract showed that not all fibers therein are GABAergic. Looking at the projection pattern of these neurons, it seems likely that they belong to a different morphological class of projection neurons than the GABAergic ones.

Some methodologically important issues were also discovered in the study, the most significant being that the identity of not only the primary but also of the secondary antibody is of great importance to the quality of the results.

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Introduction

The sense of smell is considered the most ancient sense of all, and mechanisms for detection of chemicals are found in all living organisms. Animals depend on odor cues in order to carry out vital activities such as finding food, avoiding toxins, locating a suitable partner, and finding an appropriate place to lay eggs. Olfaction is also closely connected to memory formation and emotions in many species, and associations made with scents are often robust and long lasting. The olfactory system of many invertebrates is well developed; it is often easily accessible as well. This renders them excellent organisms for experimental research, both with regard to species-specific functions, but also with respect to global knowledge about olfaction.

The Olfactory Pathway

The odorant receptor neurons (ORNs), which are capable of detecting volatile molecules in the air, are located in olfactory sensilla on the antennae of the insect, shown in figure 1. Inside the sensilla, the dendritic part of the receptor neuron is surrounded by sensillum lymph that holds a high concentration of odor binding proteins. These carrier proteins ensure transportation of the lipophilic odorant molecules to the odorant receptors that are located on the dendrite (Vogt et al. 1991; Steinbrecht 1998). In contrast to mammals, the molecular mechanism of signal transduction is not fully known in insects. However, two studies indicate that the receptors are an ionotrophic heterodimers acting as ligand-gated ion channels (Sato et al. 2008; Wicher et al. 2008). The transformed signals, i.e. the action potentials, are mediated via the ORN axons projecting directly to the antennal lobe, the primary olfactory center of the insect brain. Here, the ORNs terminate in spherical structures called glomeruli. The glomeruli are highly organized structures; one glomerulus only receives input from one particular type of ORN, and each ORN holds only one kind of odorant receptor. When an animal is exposed to a mixture of odorants, a subset of glomeruli will be stimulated, causing a specific pattern of activation for that particular stimulus. The number of glomeruli in an antennal lobe varies between species (Flanagan and Mercer 1989; Rospars and Hildebrand 1992; Berg et al. 2002; Skiri et al. 2005). Minor differences in the number of glomeruli present in the antennal lobe may also be found between sexes in some species (Rospars 1983).

Two main types of antennal-lobe neurons - local interneurons and projection neurons - have been described in insects, moths included. The local interneurons, which are restricted within the antennal lobe, run between and within the glomeruli, and thereby mediate information laterally. Most local interneurons contain GABA and thus provide inhibition within the antennal lobe. It is thought that these lateral interactions between glomeruli are especially important for odor processing and that they may help to enhance information about important components and reduce the impact of less important components in a blend (Hansson and Christensen 1999).

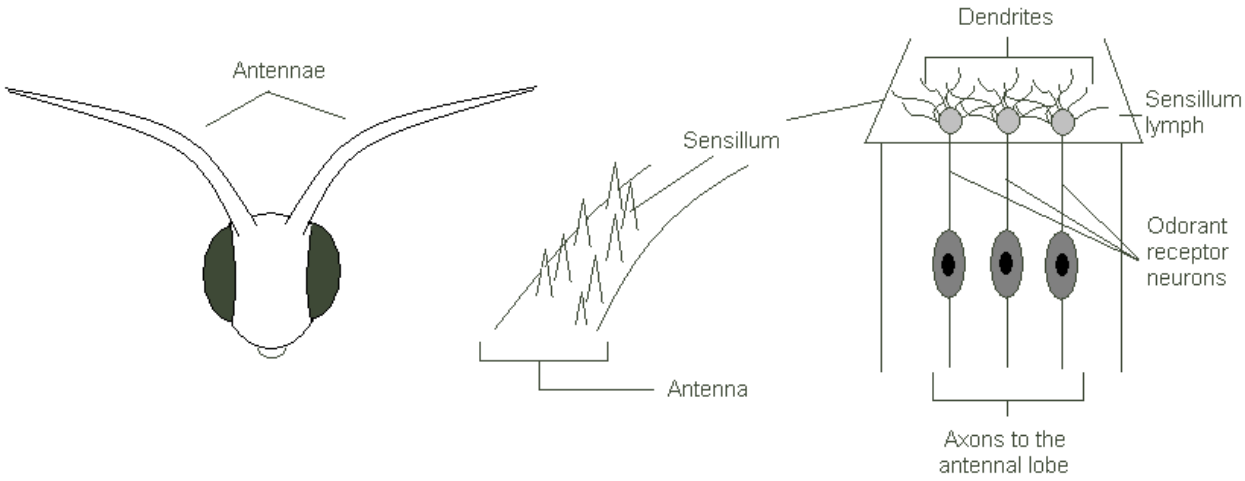

Figure 1: The basic structures in the moth olfactory system.

The Antenno-protocerebral Tracts

From the antennal lobe, projection neurons carry information to higher brain centers for further processing. There are several morphological subtypes of projection neurons; one rough division is made between uni- and multi-glomerularar ones. This distinction refers to their arborizations in the antennal lobe and the number of glomeruli they send collaterals to. In *H. virescens* and in other species of moths the antennal lobe projection neurons are organized in five tracts projecting to two main areas in the protocerebrum: the mushroom body calyces and the lateral protocerebrum (Homberg et al. 1988). These tracts were previously termed the antenno-cerebral tracts, however in order to comply with the nomenclature in honeybees and the fruit fly they will now be referred to as antenno-protocerebral tracts (APT); (Galizia and Rossler 2010). There is a medial (m-APT), a medio-lateral (ml-APT), a lateral (l-APT), a dorsal (d-APT) and a dorso-medial tract (dm-APT).

Their names indicate their positions relative to each other in the brain of the insect, and do not imply functional homology between species. Figure 2 shows an overview of the three main tracts.

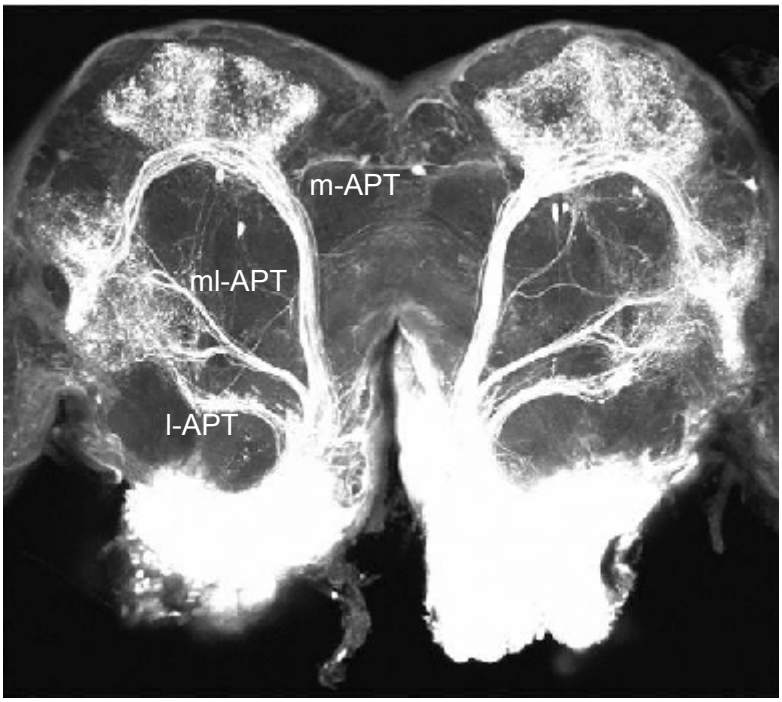

Figure 2: The three main antenno-protocerebral tracts in the moth brain, (shown with permission from Xin-Cheng Zhao).

The m-APT is by far the most prominent tract. It contains mainly uni-glomerular projection neurons that target the calyces of the mushroom bodies before innervating the lateral protocerebrum (Homberg et al. 1988; Rø et al. 2007). Sexual dimorphisms are found in the m-APT, due to output from the male-specific glomeruli of the macroglomerular complex (Homberg et al. 1988). The ml-APT exits the antennal lobe in the postero-medial part, between the roots of the m-APT. The ml-APT then bends off laterally and targets the lateral protocerebrum (Homberg et al. 1988; Rø et al. 2007). All projection neurons in this tract are assumed to be multi-glomerular (Homberg et al. 1988; Rø et al. 2007) and, as will be discussed in further detail later, the ml-APT is known to contain many GABAergic fibers (Homberg et al. 1987; Berg et al. 2009). The l-APT is located more ventrally than any of the other tracts. From the antennal lobe it projects in the lateral direction where it has several diffuse arborizations in the lateral protocerebrum. A small portion of the l-APT also innervates the ipsilateral mushroom body calyces (Homberg et al. 1988). Both uni- and multi-glomerular projection neurons run in the l-APT (Homberg et al. 1988; Rø et

al. 2007), and there are some sexual dimorphisms in this tract as well (Homberg et al. 1988).

The d-APT and dm-APT are far less prominent than the aforementioned tracts and their morphology and physiology is poorly described. The dm-APT is the most dorsal of all tracts, and its exact point of termination is unknown. The tract seems to innervate only a few glomeruli and holds very few, fairly thick fibers (Homberg et al. 1988). The d-APT is also a small tract with widespread target regions (Homberg et al. 1988); at least some of the fibers running in this tract show GABA immunoreactivity (Berg et al. 2009).

Integration Centers in the Brain

The mushroom bodies are bilateral symmetrical structures in the insect brain. They are recognized as the primary centers of multi-sensory integration, learning and memory, and also play an important role in motor control (Hammer and Menzel 1995; 1998; Heisenberg 1998; Menzel 2001; Muller et al. 2002; Heisenberg 2003). Structurally the mushroom bodies consist of three main parts: the calyces (median and lateral calyx), the pedunculus, and the lobes (α-, β- and γ-lobe). The calyces appear as two satellite dish shaped objects in the posterior-dorsal part of the brain. They are made up of mushroom body intrinsic neurons; the Kenyon cells, as well as a number of so-called mushroom body extrinsic neurons with various functions. The somata of Kenyon cells are located just outside the calyces. The pedunculus is a connective structure made from the axons of Kenyon cells, and the lobes are putative output structures that hold the axon terminals of the Kenyon cells.

The lateral protocerebrum is a putative premotoric area of the invertebrate brain (reviewed by DeBelle and Kanzaki 1999). Many of the neurons in the lateral protocerebrum respond in a fashion that suggests that integration has occurred; mixed olfactory stimulation evokes a response more often than single odorant substances in these neurons, and single neurons have also shown responses to several sensory modalities.

Neurotransmitters

The main mechanism for transduction of signals between neurons is through neurotransmitters. Several different transmitter substances have been found in the insect brain. However, the full physiological functions of many neurotransmitters have yet to be determined.

Acetylcholine is a small amine transmitter that is abundant in the nervous systems of both vertebrates and invertebrates. It is thought to be the main excitatory neurotransmitter in the central nervous system (CNS) of insects (Homberg and Müller 1999). The ORNs of many insect species have shown immunoreactivity to acetylcholine or to molecules associated with acetylcholine function and synthesis (Homberg et al. 1995; Homberg and Müller 1999). Several members of a group of modulatory neurotransmitters called biogenic amines are found in the invertebrate brain. These are dopamine, serotonin, octopamine, and histamine. Many of the neurons that hold these transmitters are centrifugal neurons, having dendrites in various regions of the CNS and axonal projections in the antennal lobe. One such neuron is the octopaminergic VUMmx1 neuron in honeybees (Hammer 1993). Centrifugal neurons are assumed to carry out multiple functions in the brain.

Neuropeptides, which are so-called non-classical transmitters, are found in great numbers in the invertebrate brain (reviewed by Salio et al. 2006); however their functions have yet to be characterized. They can either appear as the only transmitters of a particular neuron or be co-localized with other transmitters (Berg et al. 2009).

γ-aminobutyric acid

The most important inhibitory neurotransmitter in the invertebrate (as well as the vertebrate) brain is GABA. Glycine and histamine may also carry out inhibitory functions, but to a far lesser extent. GABA was first identified in neural tissue by Roberts and Frankel (1950). However, it took several years for its inhibitory properties to be discovered (Bazemore et al. 1957; Otsuka et al. 1966). Today it is recognized as the main fast inhibitory neurotransmitter in the CNS in both vertebrates and invertebrates. GABA is synthesized from L-glutamate by the enzyme glutamic

acid decarboxylase (Roberts and Frankel 1950), and it is widespread in all nervous systems. In mammals, imbalances in inhibition are the cause of many disorders, one of which is epilepsy. Thus, many drugs have been developed specifically to target the GABAergic system.

GABA receptors

GABA can induce both short and long term effects by binding to one of two receptor types; the $GABA_A$ and the $GABA_B$ receptor, respectively. These are well described in mammals, and structural and functional analogues have been found in the invertebrate brain as well (Henderson et al. 1993; Mezler et al. 2001).

The $GABA_A$ receptor is a ligand-gated ionotrophic receptor. It functions as an ion channel that is selectively permeable to chloride ions. Upon binding of GABA, there is a transient hyperpolarization of the postsynaptic membrane – a so called inhibitory postsynaptic potential (IPSP) that renders the postsynaptic cell harder to excite for a couple of milliseconds (Hammond 2001).

The $GABA_B$ receptor is a metabotrophic receptor, i.e. a G-protein coupled receptor that has multiple and longer lasting effects upon the binding of GABA. $GABA_B$ receptors are localized on both pre- and post synaptic membranes. The two main effects of GABA binding to this receptor type are opening of potassium channels, causing a hyperpolarization, and an inhibition of the voltage-dependent calcium channels. Inhibiting calcium channels has an important effect in the pre-synapse as calcium regulates the release of neurotransmitters. Thus, GABA has a self regulating mechanism (Mott 2001).

GABA in the Invertebrate Brain

Several studies have demonstrated a plethora of GABA in the invertebrate brain (Hoskins et al. 1986; Schäfer and Bicker 1986; Homberg et al. 1987; Distler 1989). All parts of the antennal lobe are densely innervated by GABA immunoreactive neurons; these are numerous local interneurons, plus a small population of multi-glomerularar projection neurons (Hoskins et al. 1986; Homberg et al. 1987; Berg et al. 2009). The local interneurons of the antennal lobe run both between and within

glomeruli (Hoskins et al. 1986; Homberg et al. 1987; Berg et al. 2009). The cell bodies of the local interneurons are located in the lateral cell cluster, which is the only cluster displaying GABA immunoreactivity. In addition to the numerous GABAergic local neurons, the immunoreactive somata belong to the population of GABAergic projection neurons (Hoskins et al. 1986; Berg et al. 2009). Of the five previously described tracts, only the ml-APT and the d-APT show substantial immunoreactivity to GABA (Homberg et al. 1987; Berg et al. 2009). The strong immunoreactivity to GABA in the mushroom bodies is mainly attributed to extrinsic neurons from the protocerebral-calycal tract (PCT), a GABAergic pathway that has been identified in many species (Mobbs 1982; Bicker et al. 1985; Homberg et al. 1987; Leitch and Laurent 1996; Grünewald 1999a).

Inhibitory Circuits

As further research into the inhibitory circuits of the brain has been carried out, several classes of inhibitory networks have been distinguished, highlighting the complexity of the inhibitory system – a system that was long considered to be simple and straight forward. We generally classify three different types of inhibition: feedforward inhibition, feedback inhibition, and lateral inhibition, as indicated in figure 3. All three kinds may display conventional axon-dendrite synaptic connections or dendrodendritic connections. The dendrodendritic connections are important in forming inhibitory microcircuits, which are attributed increasingly important roles in neural networks, particularly with regards to sensory processing (Shepherd 2004).

Feedforward inhibition

Shepherd (2004) describes feedforward inhibition as “an inhibitory shaping of excitatory events.” Feedforward inhibition is a process in which an excitatory neuron gives output onto both an excitatory neuron as well as an inhibitory interneuron. This inhibitory neuron, in turn, provides inhibitory output onto the excitatory neuron or some neuron downstream in its path (Fig. 3A). Dendrodendritic microcircuits of feedforward inhibition are thought to be particularly important in sensory systems (Shepherd 2004).

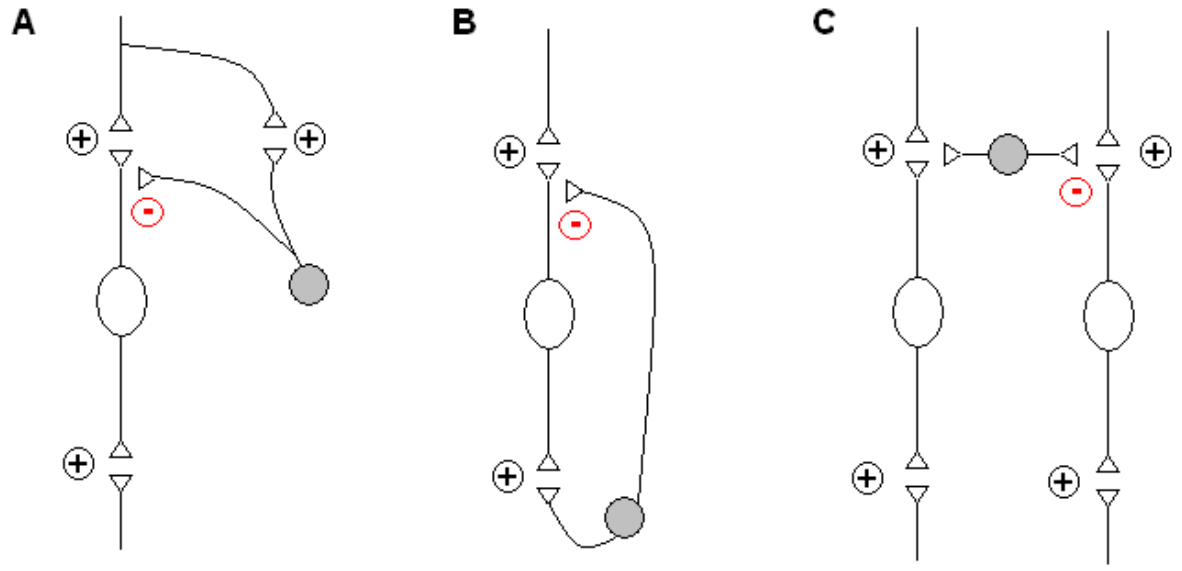

Figure 3: Inhibitory circuits. (**A**) Feedforward inhibition, the top neuron provides excitatory input to both the principal neuron and an inhibitory interneuron that inhibits the principal neuron. (**B**) Feedback inhibition; the top neurons excites the principal neuron, which in turn excites the inhibitory neuron. The inhibitory neuron provides inhibition back onto the principal neuron. (**C**) Lateral inhibition, the top neuron in the left pathway excites an inhibitory neuron that provides inhibition of the neighboring pathway.

Feedback inhibition

Recurrent or reciprocal inhibitory loops are also abundant in the CNS. Feedback inhibition is a process by which excitation of a neuron leads to activity in an inhibitory neuron that in turn inhibits that initial excitatory neuron; forming a loop where excitation drives inhibition (Fig. 3B). The function of these loops is thought to be particularly important for regulating hyper-excitability of neural networks, and for synchronizing and coordinating neural activity; i.e. creating network oscillations (Shepherd 2004).

Lateral inhibition

Networks for feedforward and feedback inhibition may also be a part of arrangements performing lateral inhibition. Lateral inhibition serves to attenuate the response in neighboring processing streams in order to promote the activity in one particular stream of information (Fig. 3C). One well-known example is the amacrine cells in the retina that carry out contrast enhancement via the principle of lateral inhibition (Shepherd 2004).

The Aim of the Thesis

To analyze general GABA immunoreactivity of neuronal populations in the olfactory pathway of the moth brain and to determine the neurotransmitter of single olfactory neurons that are ionotophoretically pre-stained.

i.) To establish a protocol for immunolabeling of brain preparations including general GABA immunostaining and double-labeling of pre-stained preparations.

ii.) To test two different primary GABA antibodies.

iii.) To describe the prominent inhibitory tract, the PCT, targeting the mushroom bodies.

iv.) To characterize the GABA immunoreactivity of intracellularly pre-stained projection neurons of the ml-APT and an interneuron in the lateral protocerebrum.

Materials and Methods

Insects

The work in the lab is carried out in accordance with Norwegian law regarding animal rights (lov om dyrevern §2), under which most invertebrates are not regulated. However, the lab does its best to avoid putting the animals under unnecessary strain and stress. Pupae of the tobacco budworm, *Heliothis Virescens* (Lepidoptera: Noctuidae), were kindly provided from a laboratory culture at Syngenta, Basel, Switzerland. Male and female pupae were sorted into different hatching boxes and kept in a climate chamber (Refritherm 6E, Struers) at 26-28˚C, with a high relative humidity and a light:dark photoperiod of 14:10h. After ecdysis the moths were moved to separate acrylic tubes where they were given the opportunity to feed on a sucrose solution (50g/L water). Experiments were performed 1-4 days after hatching on both male and female individuals; double staining was performed exclusively on female animals.

Antisera

Primary antibodies against GABA

Two different antibodies were tested for the GABA immunostaining, the first a non-commercial polyclonal antibody kindly delivered by Dr. Heinrich Dircksen, Dept. of Zoology, Stockholm University, Sweden (see Kreissl et al. 2010). Several stainings were carried out using this antibody without positive results, thus it was considered necessary to change the primary antibody.

The antibody utilized for the successful GABA immunostaining (anti-γ-aminobutyric acid, affinity isolated antigen specific antibody) is produced by Sigma (product no. A2052). It is a GABA antibody developed in rabbit with GABA-bovine serum albumin (BSA) as the immunogen. The antibody is isolated through an immuno-specific purification method from rabbit antiserum; the accuracy of the purification method is very good such that it removes all rabbit serum proteins that do not specifically bind to GABA, including immunoglobulins. The final product shows positive binding to GABA and GABA-keyhole limpet hemocyanin but negative binding to BSA in a blot plot assay, thus ensuring its high specificity to GABA in the preparations (Wolansky

et al. 2007). The minimum working dilution, the lowest concentration at which one can expect sufficient staining, is 1:10 000. However for our purposes higher concentrations (1:1000/1:2000) were necessary for optimal results.

Secondary antibodies

Three different secondary antibodies were used: CY5, CY2 and Alexa Fluor® 488 (Jackson Immunoresearch Inc., Pennsylvania). All secondary antibodies are purified from antisera of rabbits and react with whole molecule rabbit IgG, and to some extent also with other rabbit immunoglobulins. Cross reactions with immunoglobulins from other species may occur. CY5 has an excitation max at 650nm and an emission max at 670nm, and was excited by the 633 line of the Helium-Neon laser. CY2 has an excitation max at 492nm and emission max at 510nm, and Alexa Fluor® 488 an excitation max at 493nm and an emission max at 519nm. Both CY2 and Alexa Fluor® 488 were scanned with an argon laser, 488 nm.

Immunohistochemistry

GABA immunostaining was performed on both whole mount brain preparations and free floating vibratome sections. Animals were collected from the climate chamber and fixed in small plastic tubes with dental wax (Kerr Corporations, Romulus, MI, USA), leaving only the head and antennae exposed. Hairs and scales were removed from the surface of their heads exposing the cuticle. After this the proboscis and other mouth parts were removed before the animals were decapitated. For stability, the head was mounted in a small glass dish with some dental wax and embedded in a cold (4˚C) Ringer's solution (in Mm: 150NaCl, 3CaCl2, 3KCl, 25 sucrose, 10 TES buffer, pH 6.9) in which the dissection was carried out. All muscular and non-neural tissue was removed from the head capsule such that the brain was completely exposed. Before releasing it completely from the cuticle the esophagus was removed leaving a clean brain ready for staining. The freshly dissected brain was immediately placed in a fixing solution; 4% paraformaldehyde in a phosphate-buffered saline 0.1M (PBS: 684mM NaCl, 13mM KCl, 50.7mM Na_2HPO_4, 5mM KH_2PO_4, pH7.4) or Roti Histofix© at 4˚C over night.

Whole brains

The day following the dissection, the brains were washed for 4x15 min in PBS 0.1 M pH 7.4. The preparations were then preincubated in PBS containing 0.5% Triton X-100 (PBSX) and 5% Natural goat serum (NGS; Sigma, St. Louis, MO) for 3 hours at room temperature. Finally they were incubated with the primary antibody; anti-GABA in PBSX containing 5% NGS. The antibody was tested at different concentrations; several test runs proved 1:1000 as being the most suitable concentration for these experiments. The preparations were incubated with the primary antibody for 5 days at 4˚C. On the fifth day the brains were washed at room temperature in PBS 6x15 min, before incubation with the secondary antibody CY5/CY2/AlexaFluor® 488 (1:300) in PBSX, for 3 days at 4˚C. After the three days of incubation with the secondary antibody the preparations were washed 4x15 min in PBS and dehydrated in alcohol series 50%, 70%, 90%, 96%, 100%, 100% each for 10 min before clearing in methyl salicylate. The brains were kept in methyl salicylate until mounting on double sided aluminum plates for scanning in the confocal microscope.

Free-floating vibratome sections

After being fixed overnight the brains were washed for 4x15 min in PBS. Next they were embedded in a heated gelatine albumine gel (max 60˚C). Each brain was placed in the preferred position within the gel, before it was left to dry for 10 min, and after this fixed for 20 minutes (Roti Histofix©) before the gelatine/albumine mount was removed from the mold and further fixed (Roti Histofix©) for 16-18 hours at 4˚C. To stop the fixing process, the mount was moved to a NaPi solution. Then, the gelatin/albumine preparations were dried off and cropped to small pyramids before being mounted with superglue on a metal plate for the vibratome. Free floating vibratome sections were made in a NaPi solution, on ice. The slice thickness was 40µm. The staining process for the vibratome sections was the same as that for whole brains, described above. Before dehydration, the free floating sections were mounted on gelatin coated (1g gelatin, 0.1g Chromium(III) potassium sulfate/100mL) microscope slips, in such a fashion that the most ventral sections appeared first and the dorsal-most last. Sections were dried before being washed 4x15 min in PBS and after this dehydrated according to the protocol above. Finally the sections were cleared in xylol before a coverslip was mounted with Permount ™.

Double-Labeling

Double-labeling was performed both on previously iontophoretically stained preparations as well as on preparations stained by mass-filling from the antennal lobe. Both kinds of staining were done with micro ruby as the dye. The preparations were rehydrated in an alcohol series 100%, 100%, 96%, 90%, 70%, 50% each for 10 min, before the protocol could commence. The same protocol as described above was followed.

Images

After staining, the preparations (whole mounts and sections that were mounted on microscope slides) were scanned with a confocal microscope (LSM 510, META Zeiss /Leica TCS SP5). Scans were made with a Helium Neon laser at 633 nm for CY5 and an Argon laser at 488 nm for CY2 and AlexaFluor® 488. The pixel resolution was 1024 x 1024, and 10x and 20x objectives were used (Plan-Neofluar 10x/0.3, Plan-Neofluar 20x/0.5 I). Stacks of optical sections were made, and image processing software (Amira, GIMP, LSM Image Browser) was utilized to create pictures of selected parts of the brain.

Results

Immunolabeling the moth brain for GABA gave rise to a characteristic staining pattern. Figure 4 shows a confocal scan of an immunostained vibratome section, in which several prominent GABA reactive structures are visible. These include the antennal lobes (Fig. 4B), the lower division of the central body (Fig. 4C), and the calyces of the mushroom bodies (Fig. 4D). The eye lobes (not shown) are also amongst the most reactive structures of the brain; however, they have not been a focus of this study.

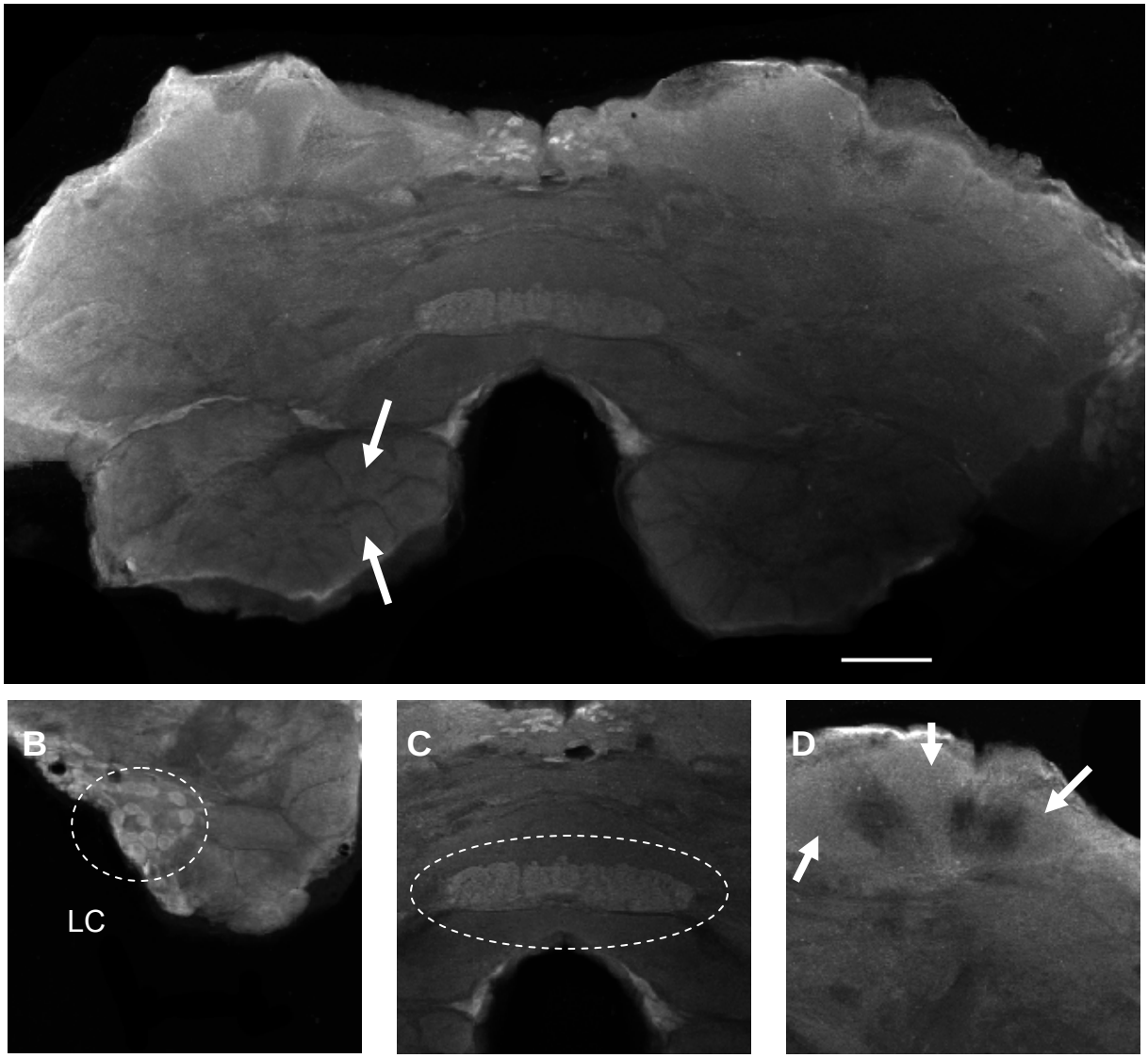

Figure 4: GABA immunostained vibratome section. **(A)** Dorsal vibratome section with some of the most prominent GABA immunoreactive structures. The arrows indicate glomeruli of the antennal lobe. **(B)** The antennal lobe with numerous stained somata in the lateral cell cluster (LC, circled). **(C)** The lower division of the central body (circled). **(D)** The calyces of the mushroom body (arrows). Confocal scan, 10x objective, scale bar 100µm.

Immunolabeling in the Antennal Lobes

There is a large population of GABAergic neurons in the antennal lobes; shown by numerous labeled somata that are localized in the lateral cell cluster (Fig. 4B). Also the glomeruli show strong immunoreactivity and thus appear as distinctive structures (arrows, Fig. 4A). The medial and the anterior cell clusters (not shown) are on the other hand completely devoid of immunoreactive cell bodies.

Immunolabeling in the Antenno-Protocerebral Tracts

Of the three most prominent antenno-protocerebral tracts (APTs) visualized by anterograde staining from the antennal lobe (using micro ruby; figure 5), the medio-lateral tract displayed GABA immunoreactivity. As shown in the vibratome section in figure 6, this tract is clearly visible as it leaves the antennal lobe and projects laterally. Due to the sectioning, the whole tract is not visible in the current image. In whole mount preparations the GABA-immunoreactivity of the ml-APT was hard to visualize due to insufficient penetration of the antibody. The medial and the lateral antenno-protocerbral tract (m-APT and l-APT, respectively) did not show any immunoreactivity (data not shown).

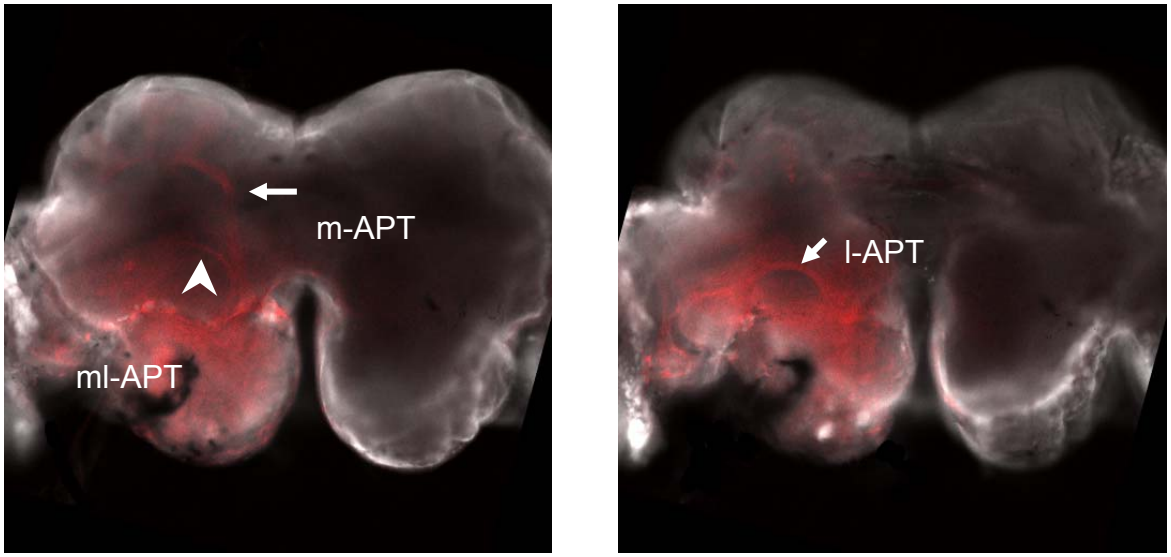

Figure 5: The three main antenno-protocerebral tracts (APT) visualized by anterograde staining, in a whole mount preparation. **(A)** The medial APT (arrow) and medio-lateral APT (arrowhead). **(B)** The lateral APT. Confocal scan, 10x objective, scale bar 100µm.

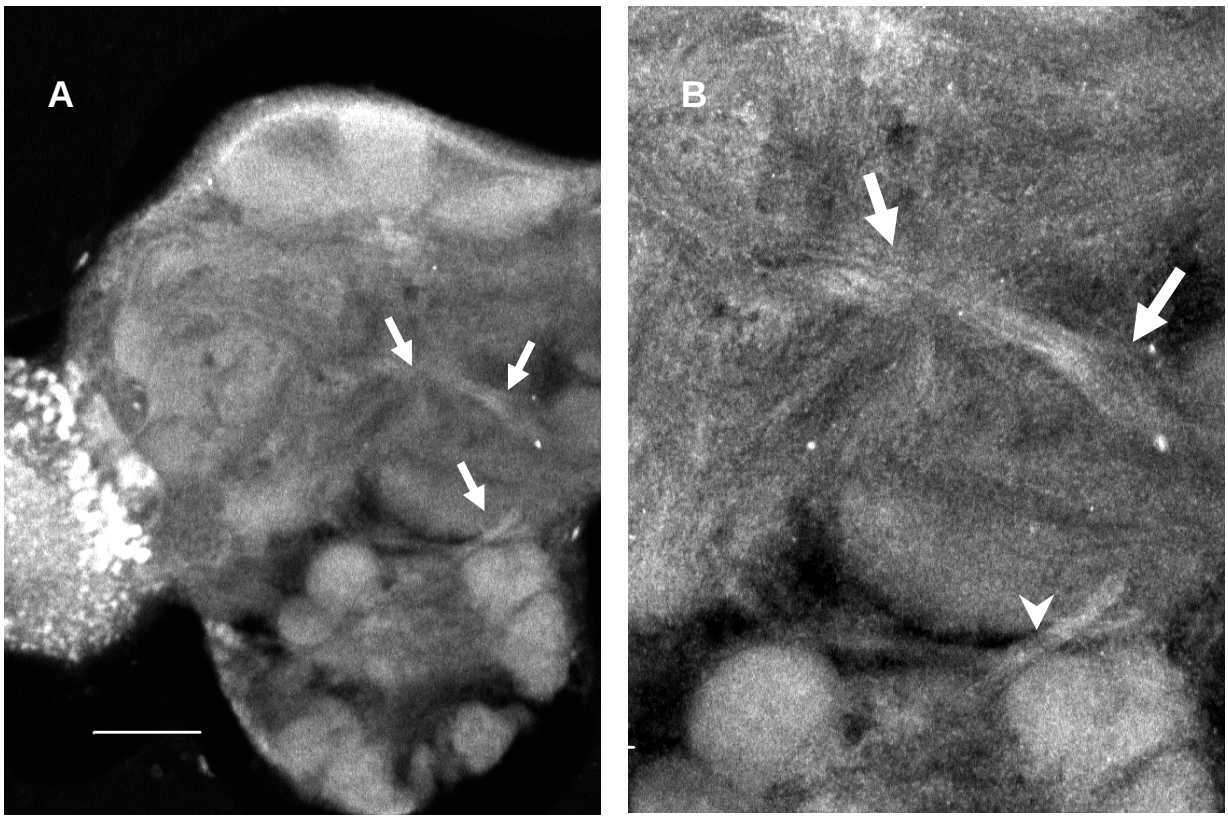

Figure 6: The medio-lateral antenno-protocerebral tract (ml-APT) visualized by GABA-immunostaining in a vibratome section. **(A)** An overview of one brain hemisphere. The ml-APT is indicated by the arrows, confocal scan, 10x objective. **(B)** Magnified image showing the ml-APT as it exits the antennal lobe (arrowhead) and bends off laterally toward the lateral protocerebrum (arrows); confocal scan, 20x objective. Scale bar 100 µm.

Immunolabeling in the Protocerebrum

In the lateral protocerebrum, GABAergic neurons were dispersed throughout the area as a fine meshwork of neurites. In addition, several cell clusters were immunostained, one of which is the origin of the prominent tract innervating the mushroom bodies, the so-called protocerebral calycal tract (PCT). As shown in figure 7, the current soma cluster is located in the lateral-most part of the lateral protocerebrum, near the lobula of the optic lobe. From here a bundle of fibers project medially thorough the protocerebrum to the pedunculus of the mushroom body, figures 7 and 8.

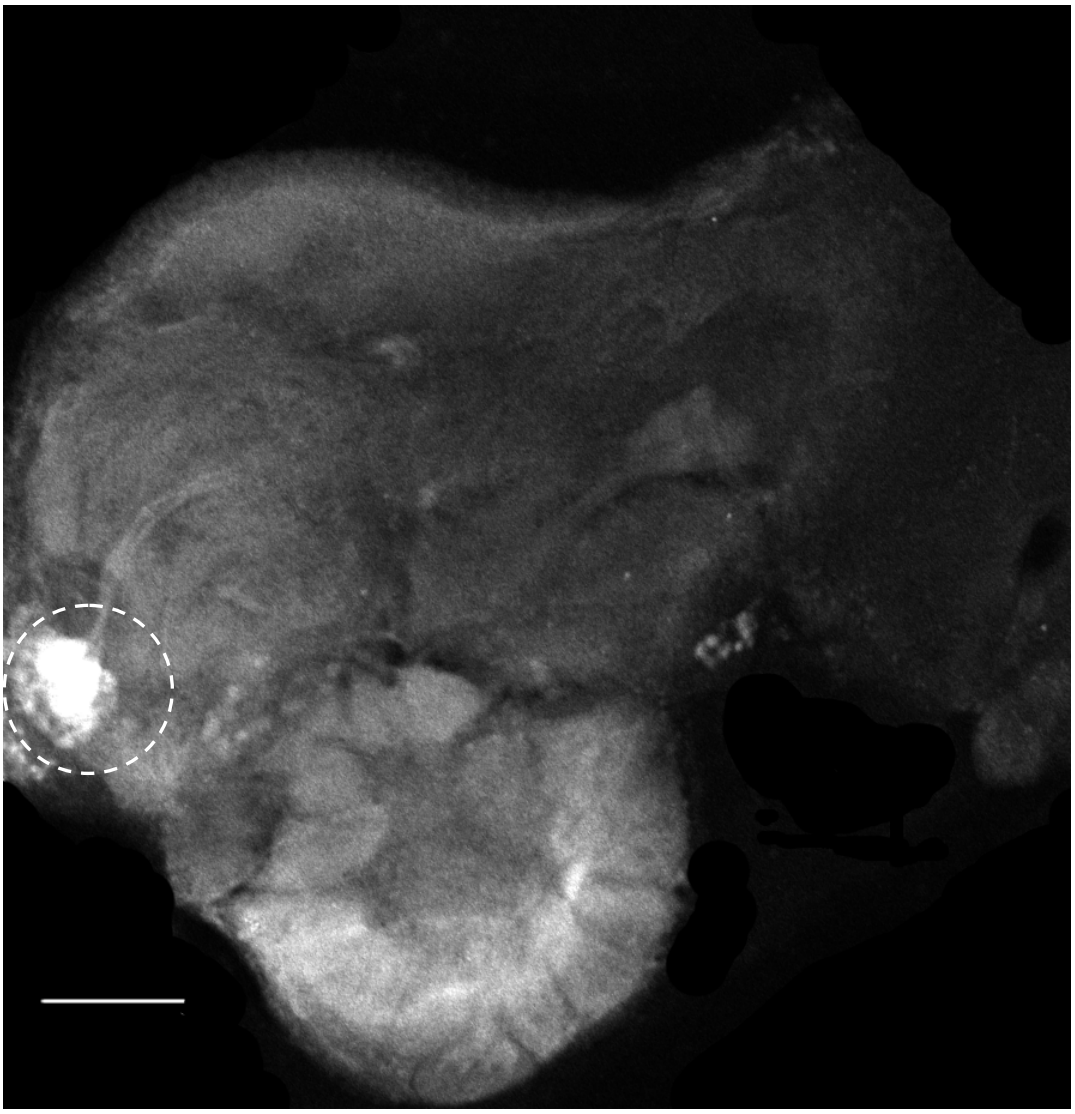

Figure 7: The origin of the protocerebral calycal tract, shown by the strongly labeled cell cluster located near the lobula of the optic lobe. GABA immunostained preparation sectioned on the vibratome. Confocal scan, 10 x objective, scale bar 100µm.

All parts of the mushroom bodies including the medial and the lateral calyces, the pedunculus, and the lobes, show GABA immunoreactivity (Fig. 8). The tract shown in the aforementioned figure gives rise to most of, if not all the GABA reactive fibers in the mushroom bodies. As can be seen in figure 8 (A-D) the tract projects through the lateral protocerebrum before entering the mushroom body through the pedunculus. Here it bifurcates, sending branches both to the lobes and the calyces. The calyces are very dense structures and there is an abundance of fine GABAergic neurites in this area of the brain. The arborizations in the mushroom body lobes are also easy to see (Fig. 8D), however the defined structures of the lobes are not visible in these preparations.

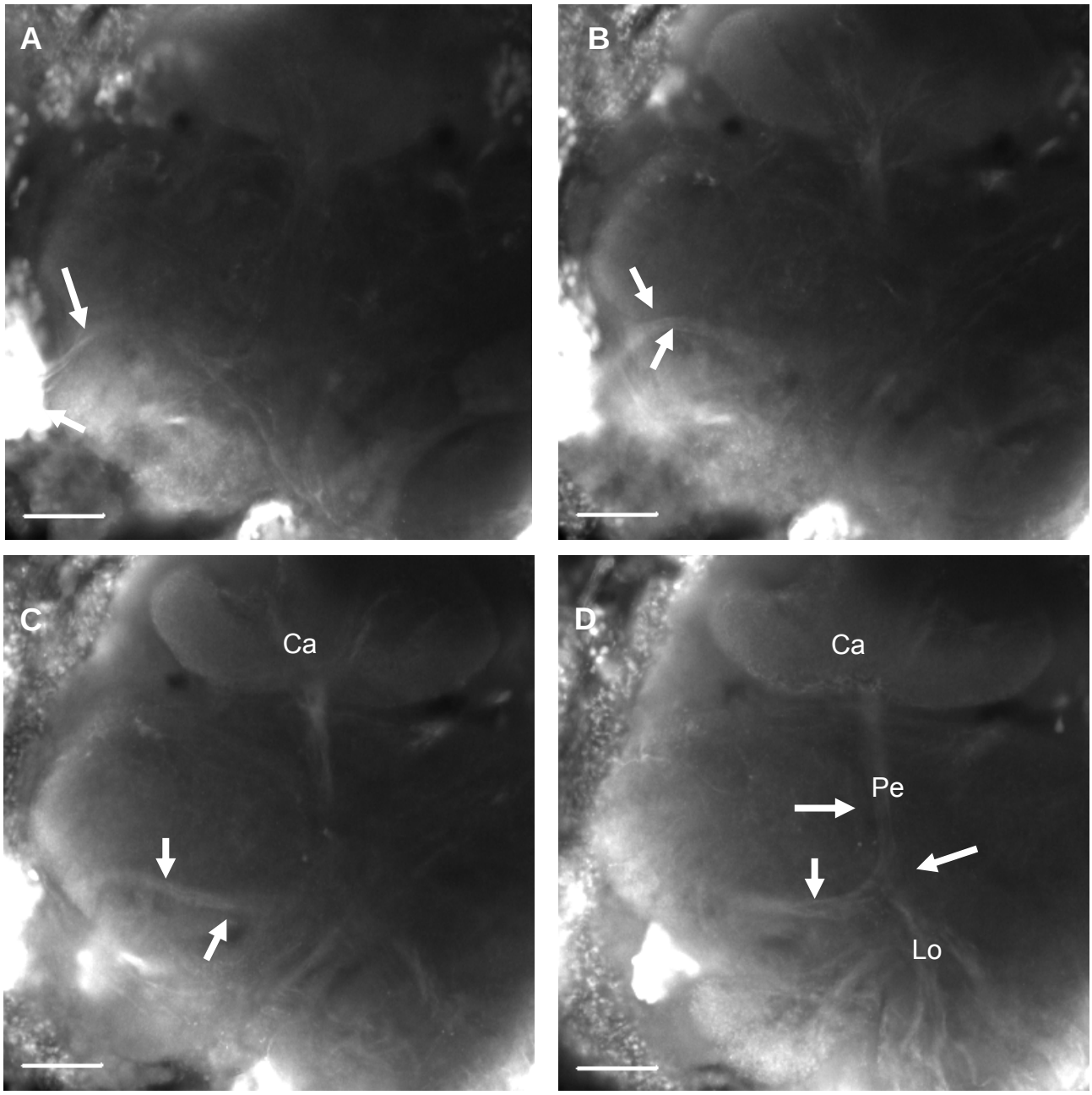

Figure 8: Optical sections from ventral to dorsal position showing the protocerebral calycal tract (PCT) in a GABA immunostained whole mount preparation. **(A)** Ventral-most section showing the tract (arrow) emerging from the soma cluster located in the lateral part of the lateral protocerebrum, near the optic lobe (arrowhead). **(B)** and **(C)** More dorsal sections showing the tract projecting through the protocerebrum, bound for the pedunculus (Pe) of the mushroom body. **(D)** Dorsal-most section showing the PCT entering the ventral part of the pedunculus where it bifurcates; one branch sends processes to the lobes (Lo) and the other to the calyces (Ca). Arrows indicate the PCT. Scale bar 100 µm, confocal scan, 20x objective.

Double Staining Experiments

Among the neurons previously stained by the iontophoretic technique, none turned out to be GABAergic.

Double-labeling of preparations containing pre-stained antennal-lobe projection neurons passing in the medio-lateral antenno-protocerebral tract

As the ml-APT is one of the tracts that shows strong GABA immunoreactivity (Fig. 3), double-labeling of iontophoretically pre-stained neurons passing in this pathway was performed. Figures 9, 10, 11, and 12 clearly demonstrate that the intracellularly stained neuron(s) are not GABAergic. In figures 9 and 10, the GABA immunoreactive part of the ml-APT is visible; the intracellularly stained neurons on the other hand seem to run dorsal to this portion of the tract, and their exit point from the antennal lobe seems to deviate slightly from that seen for the GABAergic portion of the ml-APT, as indicated by arrows in figure 10. Figures 11 and 12 show the cell body of one of the intracellularly stained neurons; it is quite clear that the cell body in question is located dorsal to the part of the cluster containing GABA, which verifies the observation from the protocerebrum that the neuron(s) in question are not GABAergic.

GABA immunostaining of the preparation shown in figures 13 and 14 also included an immuno-negative projection neuron passing in the ml-APT. The GABA immunostaining in the preparation in question is not as good as that seen in other preparations, yet it is fully possible to conclude that the neuron in question is not immunoreactive.

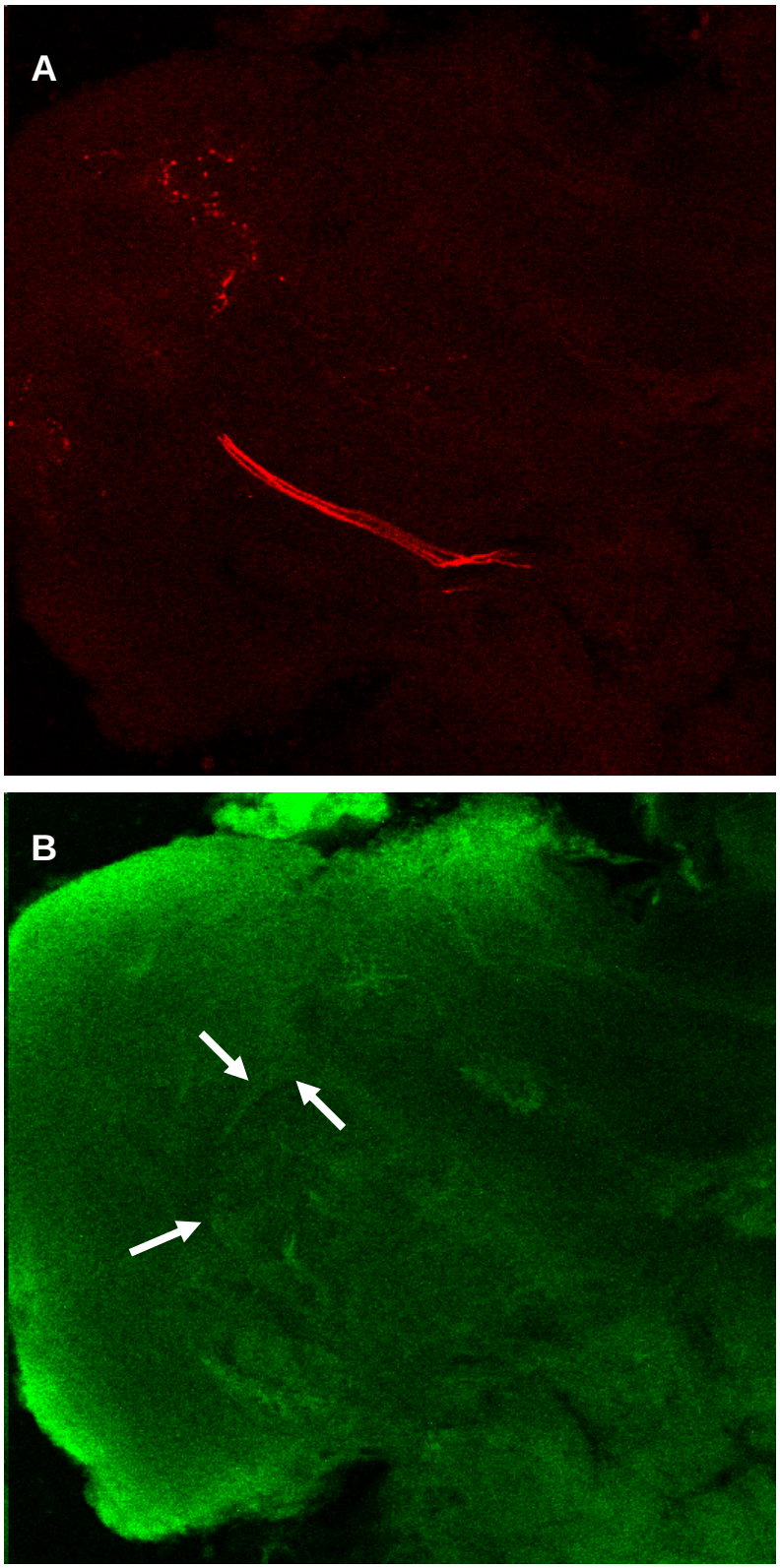

Figure 9: GABA immunolabeling of two iontophoretically pre-stained projection neurons passing in the medio-lateral protocerebral tract (ml-APT), vibratome section. (**A)** The iontophoretically stained neurons (red). **(B)** The GABA immunoreactivity of the brain preparation (green). The arrows indicate the ml-APT. Confocal scan, 20x objective.

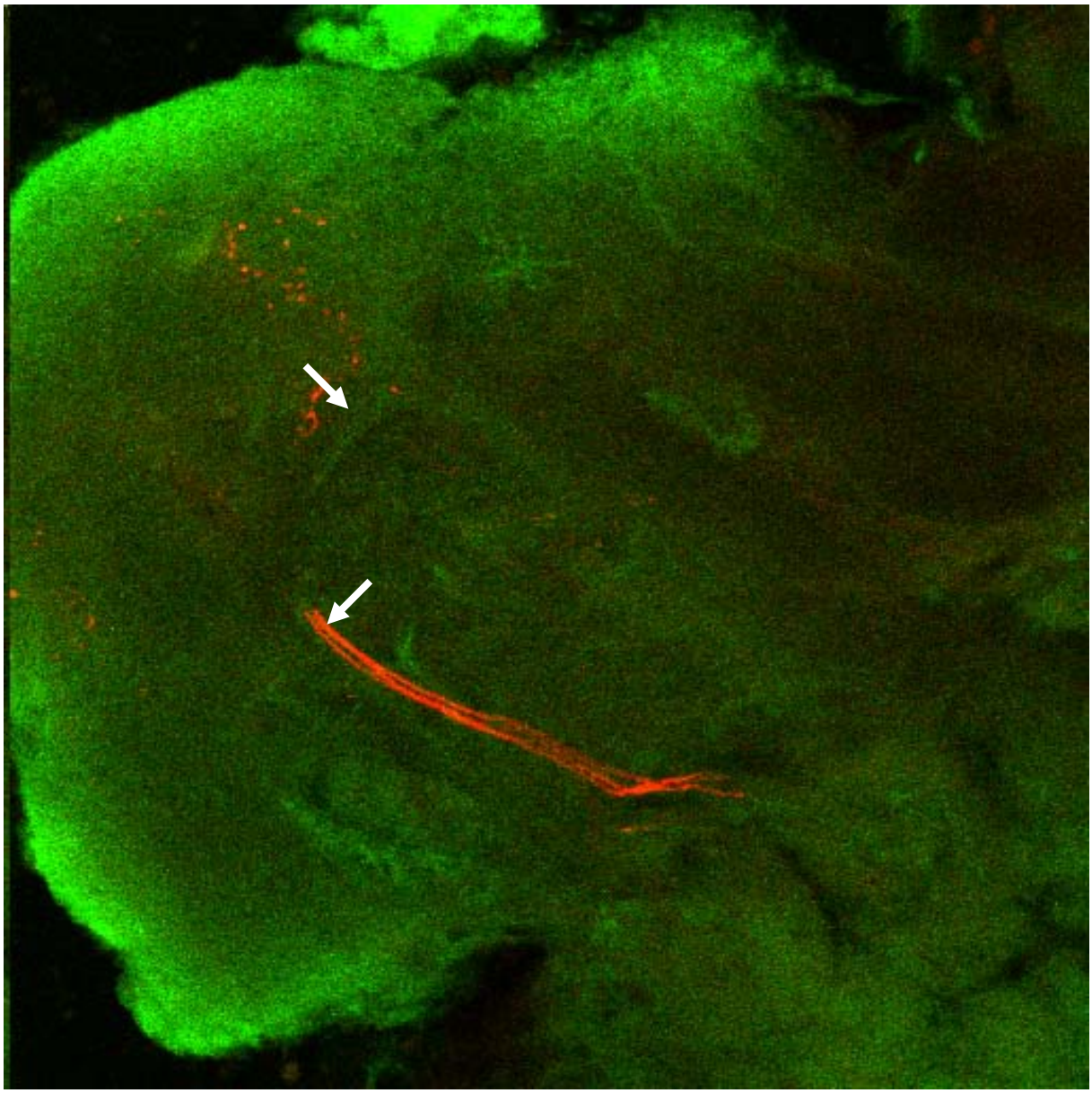

Figure 10: Overlay of the GABA stain and the iontophoretical stain shown in Figures 9A and B. Arrows indicate the GABAergic portion of the ml-APT. The neurons in question do not appear to display GABA immunoreactivity. Confocal scan, 20x objective.

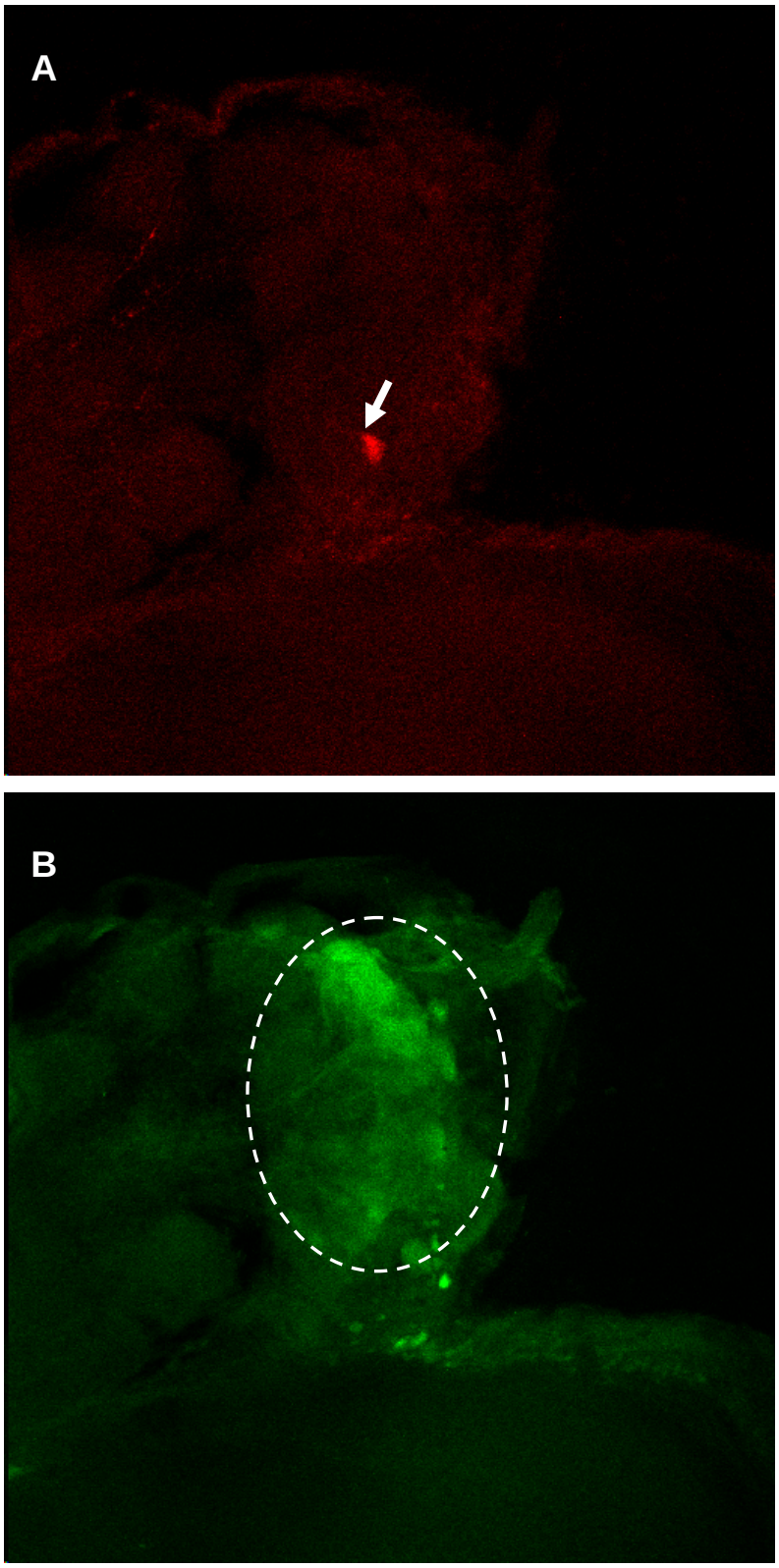

Figure 11: Double-labeling of the lateral cell cluster, vibratome section from the same preparation shown in figures 9 and 10. **(A)** The intracellularly stained cell body (red). **(B)** The GABA immunoreactivity of the lateral cell cluster (green). Confocal scan, 20x objective.

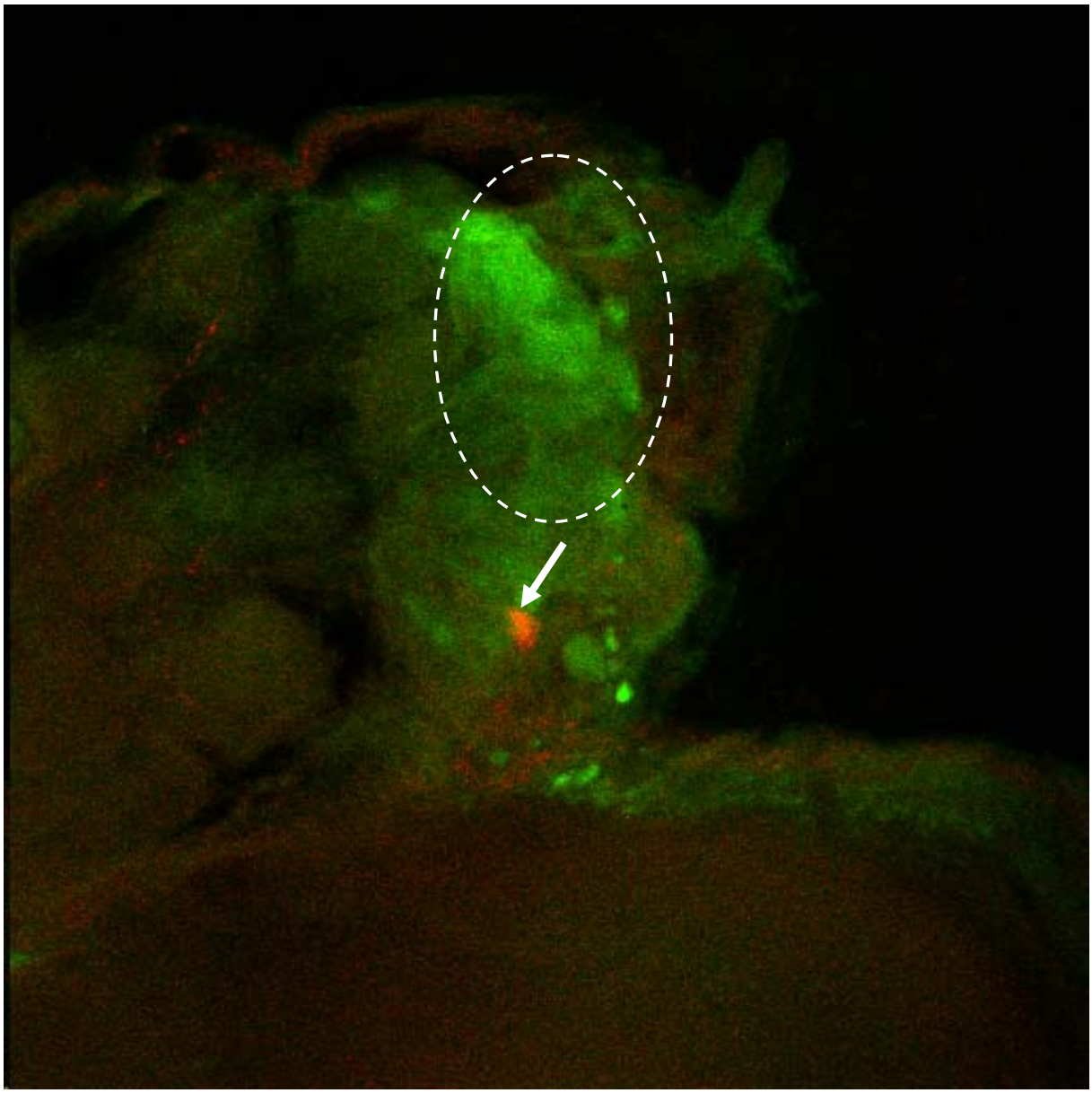

Figure 12: Overlay of the intracellular stain and the GABA stain from figures 11A and B. The GABAergic cell bodies appear to be located in a different portion of the lateral cell cluster than does the cell body in question. The cell body does not display GABA immunoreactivity. Confocal scan, 20x objective.

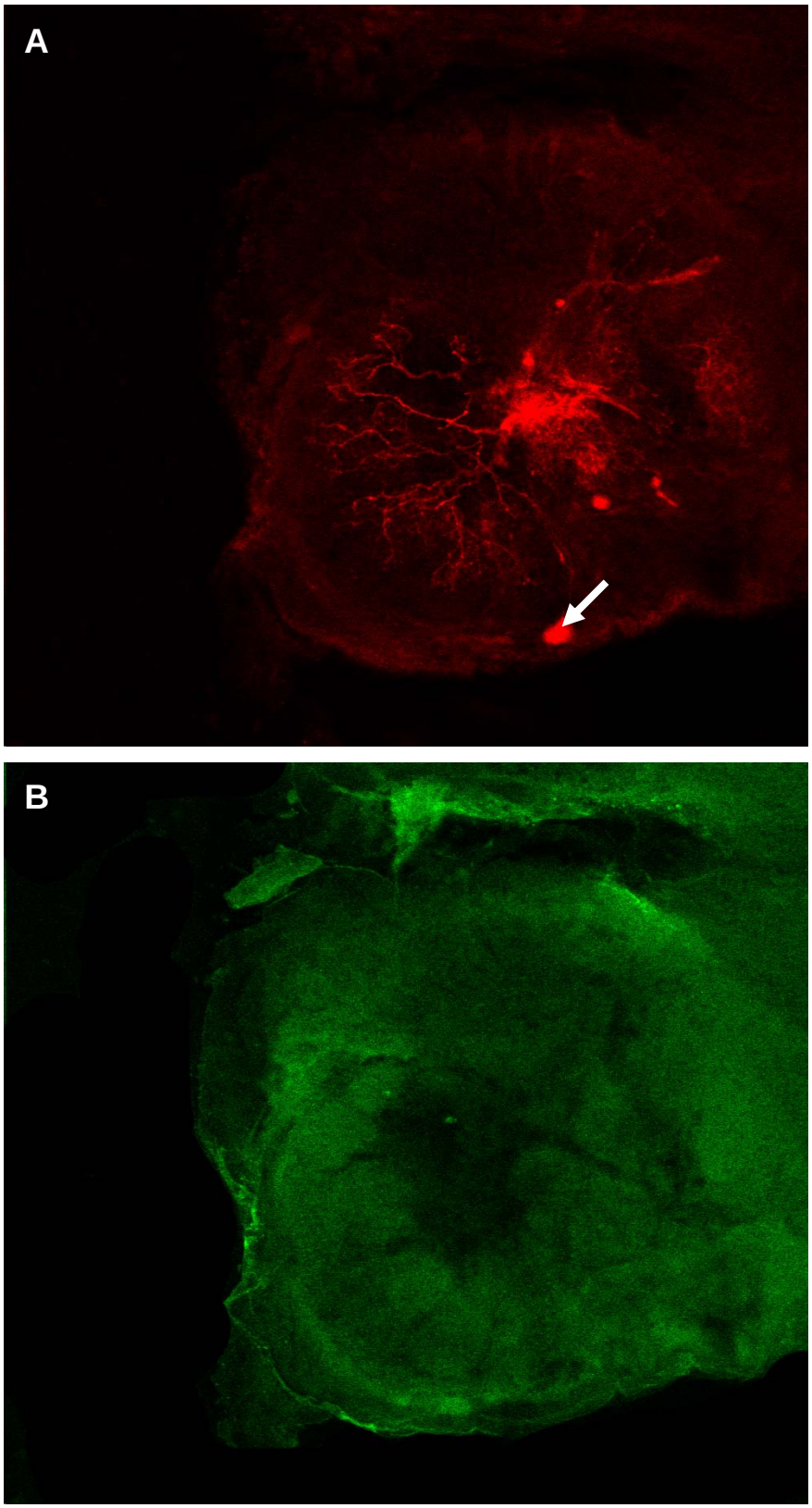

Figure 13: GABA immunoreactivity of the antennal lobe, and an iontophoretically stained neuron, vibratome section. **(A)** The intracellularly pre-stained neuron with dendrites in the antennal lobe (red). **(B)** GABA immunoreactivity of the antennal lobe. Confocal scan, 20x.

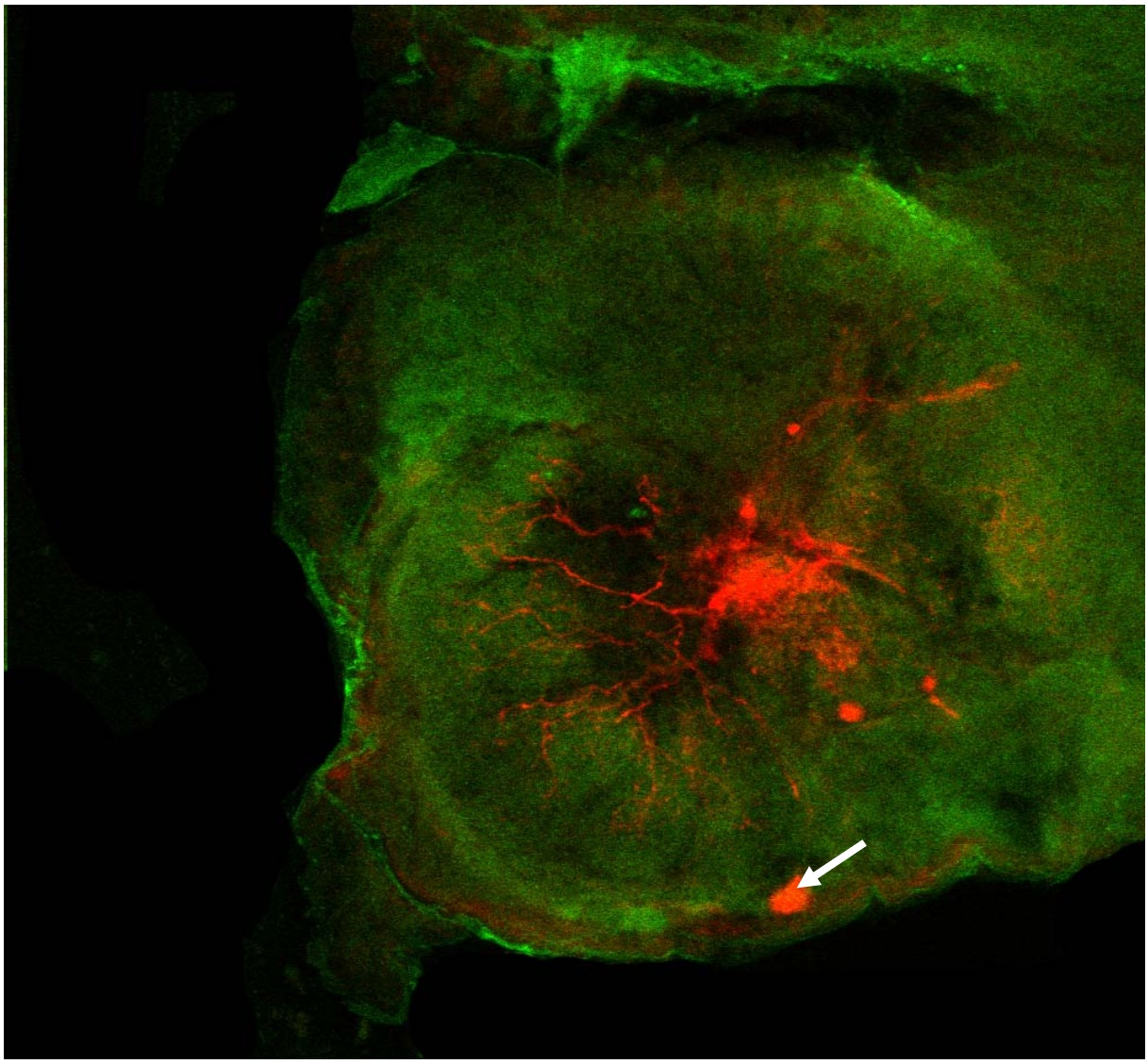

Figure 14: Overlay of the GABA stain and the iontophoretical stain shown in figure 13. The arrow indicates the cell body of the intracellularly stained neuron.

Double-labeling of a preparation containing a higher order neuron

A preparation with a pre-stained neuron (Fig. 15A) that runs from the lateral to the superior protocerebrum was immunostained for GABA. The result shows that there are many GABA immunoreactive neurons in the area in question (Fig. 15B). In spite of the relatively weak iontophoretic stain, it is obvious that the neuron at hand does not display GABA immunoreactivity, as shown by the overlay (Fig. 15C). The fine meshwork of GABAergic neurons in the lateral protocerebrum, as described above, can easily be seen in this preparation.

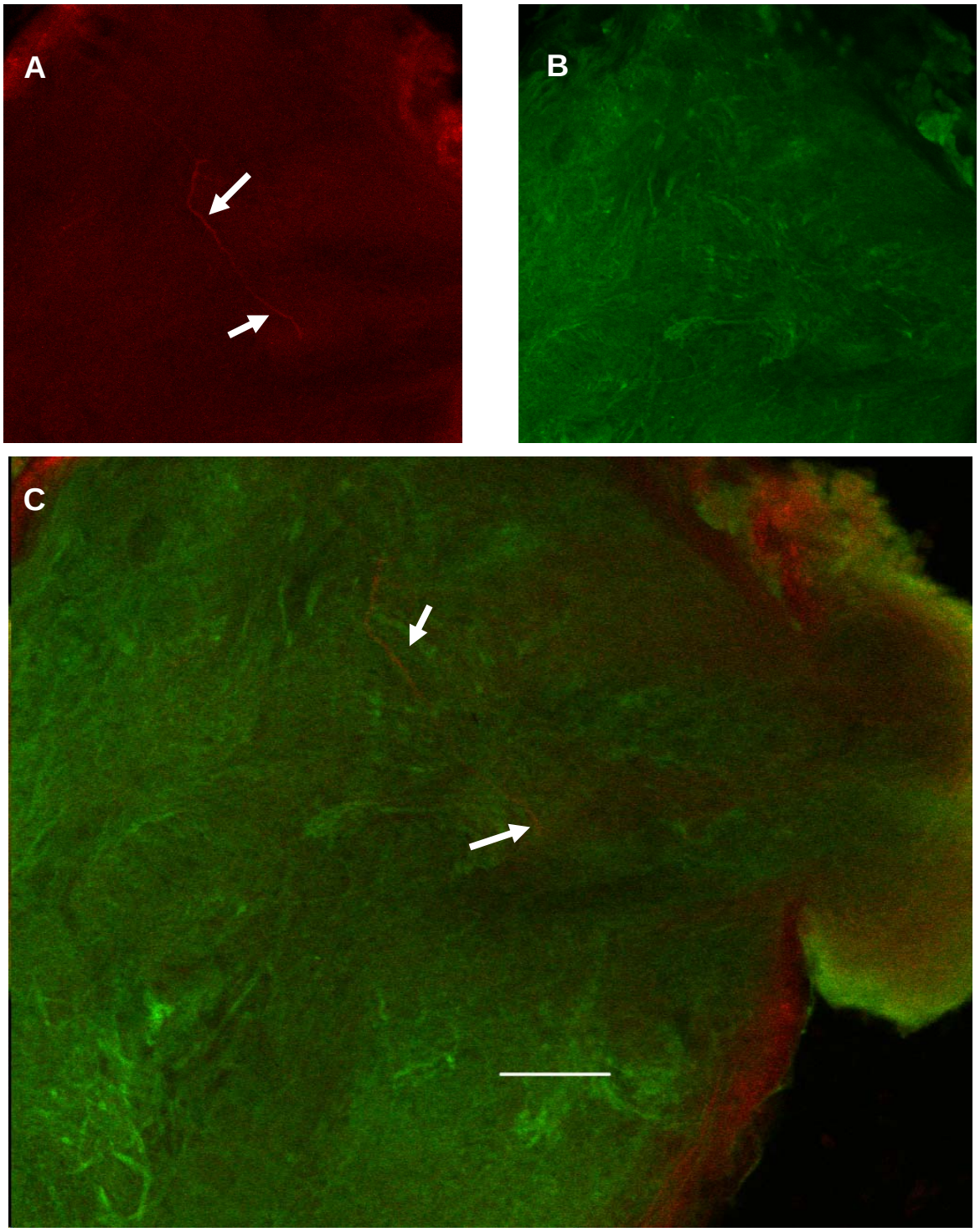

Figure 15: Higher order neuron in the lateral protocerebrum, vibratome section. **(A)** Optical section showing a higher order neuron (arrows), visualized via intracellular staining (red). **(B)** GABA immunostaining of the same section (green). **(C)** Overlay of the two images demonstrating that the neuron in question does not display GABA immunoreactivity. Confocal scan, 20x objective. Scale bar 100µm.

Discussion

Result Summary

This immunohistological study uncovered strong GABA-labeling in various regions of the moth brain. Areas of particular interest with regards to the olfactory pathway are the antennal lobes, the lateral protocerebrum, and the mushroom bodies; all of which displayed robust immunoreactivity. The two most important foci of the study were the prominent GABA-labeling of the mI-APT, and the GABAergic input to the mushroom bodies by virtue of the protocerebral calycal tract (PCT). The latter structure has been described in several species, and its physiology is just beginning to be elucidated. This study confirms its existence in *H. virescens*.

The Antennal Lobes and the Antenno-protocerebral Tracts

The antennal lobe

The results presented here, including numerous immunoreactive somata in the largest cell cluster of the antennal lobe (Fig. 4B) and consistent GABA-staining of seemingly all glomeruli (Fig. 4A), indicate the importance of GABA for odor information processing. The present data are in complete accordance with previous findings (Hoskins et al. 1986; Berg et al. 2009). This includes the observation concerning the occurrence of GABAergic somata in the lateral cell cluster exclusively, which indicates that a considerable amount of the immunoreactive neurons are local interneurons, as all their cell bodies are located within this cell group (Hoskins et al. 1986; Homberg et al. 1988). These inhibitory connections have been thoroughly studied, and much is known about their morphology and physiology (Matsumoto and Hildebrand 1981; Christensen et al. 1993). Thus, putative functions of the GABAergic antennal-lobe neurons include lateral inhibition as well as synchronization of neural activity for establishing oscillations (Matsumoto and Hildebrand 1981; Christensen et al. 1993; Laurent and Davidowitz 1994; Leitch and Laurent 1996; MacLeod and Laurent 1996; Stopfer et al. 1999). Such oscillations have been detected via measurement of local field potentials in the antennal lobe. The role of oscillations in the nervous system is still in the process of being completely elucidated.

GABA immunoreactivity in the antenno-protocerebral tracts

GABA immunostaining of vibratome sections revealed the ml-APT as a highly GABA immunoreactive pathway (Fig. 6). This is in accordance with previous immunohistological studies of the olfactory system of moths and other insects (Hoskins et al. 1986; Schäfer and Bicker 1986; Homberg et al. 1987; Berg et al. 2009). In some preparations, a void in the immunostain could be observed in the position of the m-APT (results not shown); whereas the l-APT was hard to make out. Previous studies have found that the m-APT is immuno-negative whereas the l-APT holds some GABAergic fibers (Homberg et al. 1987; Berg et al. 2009) – this study was unable to verify the latter finding.

Double staining experiments of two (or three) iontophoretically pre-stained neurons passing in the ml-APT were performed in order to explore their immunoreactivity against GABA (Fig. 9-14). The physiology of these neurons has been presented by Løfaldli et al. (2012). Surprisingly, the intracellularly stained multi-glomerularar projection neurons of the ml-APT turned out to be GABA immuno-negative. As shown in figures 9-11, presenting images of one preparation, there was no overlap between the iontophoretic stain and the GABA-stain either for the neural processes or the soma. The second preparation, which contained another pre-stained projection neuron passing in the ml-APT, also revealed a total lack of overlap between the iontophoretic labeling and the GABA-labeling. Based on the fact that the ml-APT in moths is assumed to hold approximately 120 neurons (Homberg et al. 1988); and that only 40-70 of these are estimated to display immunoreactivity to GABA (Berg et al. 2009), the current data seem sensible. Interestingly, two previous morphological studies have indicated that the ml-APT contains at least two subtypes of projection neurons; one that only innervates the lateral protocerebrum and another type that has vast arborizations in both the lateral and superior protocerebrum (Homberg et al. 1988; Rø et al. 2007). As can be seen from the previous results of Berg et al. (2009) dealing with GABAergic projection neurons passing in the ml-APT, there is abundant staining in the lateral protocerebrum and almost no staining in the superior protocerebrum. Actually, the GABAergic part of the ml-APT does not seem to innervate the superior area of the protocerebrum at all. In the present study, this general staining pattern is not evident, partly due to the sectioning of the preparations. However, the two iontophoretically stained neurons investigated here

seem to belong to the vastly arborizing subtype. As shown in figure 16, the projections of the two ml-APT neurons have extensive terminations in the area where GABAergic neurons are less abundant, i.e. the superior protocerebrum. Based on the findings in this master's project and the observations of the previous data from Berg et al. (2009), it is tempting to hypothesize that the two morphological subtypes of neurons in the ml-APT may also differ with regard to their neurotransmitter. This in turn means that a particular kind of neuron probably provides excitatory input to a large area in the protocerebrum, including a lateral and superior region, whereas the other kind of neuron provides inhibitory input only to the lateral protocerebrum.

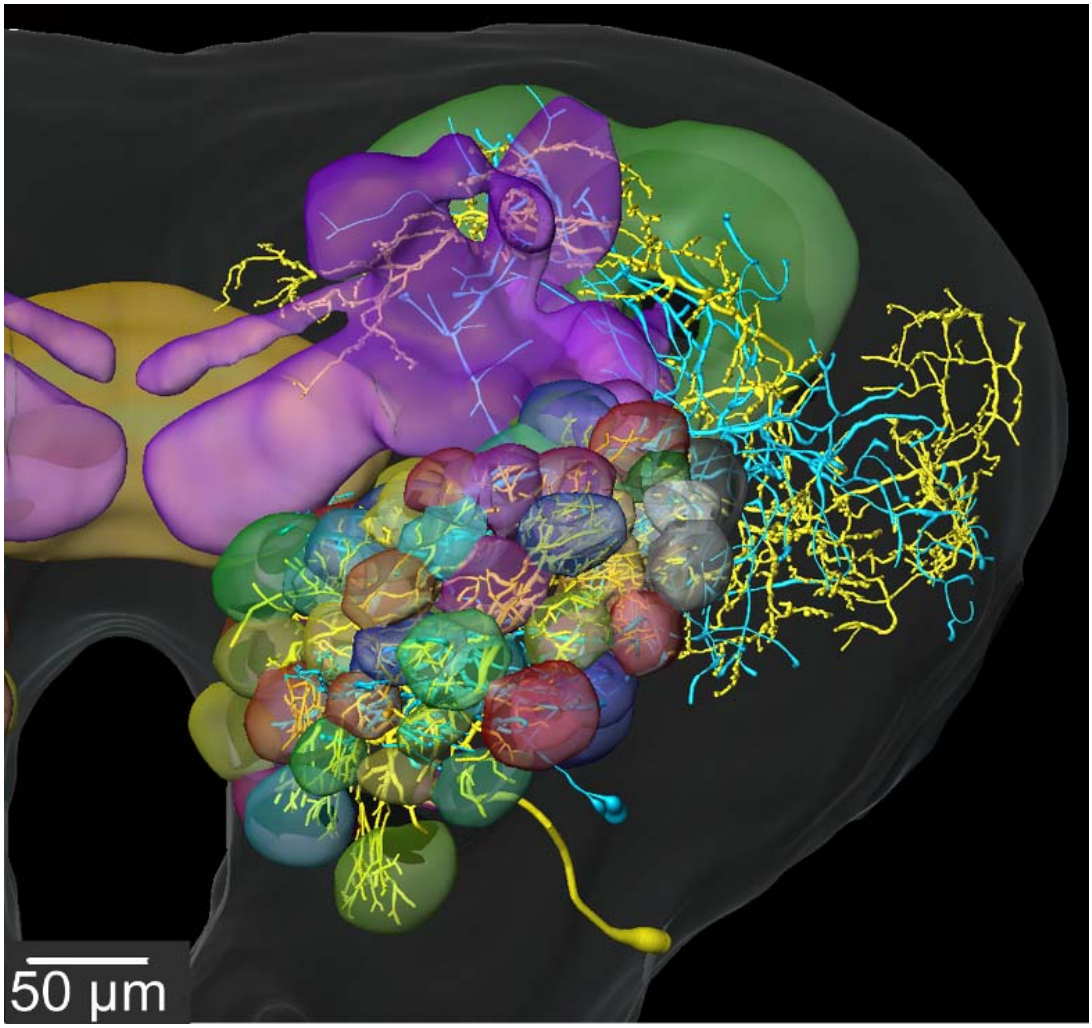

Figure 16: Reconstruction of the two iontophoretically stained medio-lateral tract antennal-lobe projection neurons shown in figures 9-14. Both neurons have multi-glomerularar arborizations in the antennal lobe and target widespread regions of the lateral and superior protocerebrum. The frontally oriented model is made by the visualization software AMIRA (Løfaldli et al. 2012).

Identification of the other neurotransmitter(s) of the ml-APT was not a topic of this study, but they are likely not inhibitory. Unfortunately the main neurotransmitter of the antennal-lobe projection neurons of moths has yet to be conclusively established. However, cholinergic projection neurons have been reported in flies, honeybees, and in the sphinx moth, *M.sexta (Buchner et al. 1986; Kreissl and Bicker 1989; Homberg et al. 1995)*, thus acetylcholine is a likely candidate as the main excitatory neurotransmitter in the projection neurons.

GABA and the medio-lateral antenno-protocerebral tract in parallel processing streams

When looking at the three most prominent APTs, the m-APT, the ml-APT, and the l-APT, as in this study, the ml-APT is the only one that shows significant GABA immunoreactivity, and is thus likely the only APT-pathway capable of mediating any significant level of inhibition[*]. The ml-APT surpasses the mushroom bodies completely, which indicates that the information it carries is likely not subject to associative plasticity, but rather has a role that is more directly connected to output from the brain. We can only speculate as to what the functional properties of the ml-APT are. As the information in the prominent m-APT goes through the mushroom bodies, this pathway is probably subject to a higher level of integrative processing. Concerning the inhibitory portion of the ml-APT, it might serve in attenuating and modulating its own output due to the limited processing the information goes through. (It also seems that an inherent property of nervous systems is ridding of redundant loops; thus it is feasible that some information streams omit the mushroom bodies simply because they do not require any of the modifications these structures can offer). The ml-APT projection neurons are to our knowledge all multi-glomerular (Homberg et al. 1988; Rø et al. 2007); a logical conclusion to draw from this observation is that the tract may carry information about odor blends rather than single odorants. Indeed, the electrophysiological recordings performed from the neurons that were double-stained in the current study show that they respond to odor mixtures and not to single substances (Løfaldli et al. 2012). It is not at all surprising that activation from more than one glomerulus would be required in order to activate

[*] The GABA immunoreactivity of the dorsal APT is not taken into consideration here.

a neuron having such a wide-spread net of dendrites. The sheer morphology of the ml-APT projection neurons seems to exclude the possibility that the tract could carry information about odors of one particular biological relevancy to the animal. This is in contrast to the olfactory processing in fish, which has been shown to process information about food odorants, sex pheromones, and other social cues along separate tracts (Hamdani and Døving 2007).

Separation of sensory information into several processing streams is called parallel processing, and is a debated subject in sensory neurophysiology. In all of the sensory subsystems, anatomical segregation into different information streams is evident (somatosensory: Gasser and Erlanger 1929; vision Bishop 1933; olfaction: Kirschner et al. 2006). However, the function of these information streams remains to be completely deciphered. It has been suggested that parallel pathways aid the process of compressing sensory information such as to accommodate the limited anatomical coding space of the brain (Nassi and Callaway 2009). Rössler and Galizia (2010) introduced two terms in order to describe two basic types of parallel processing. The first, segregated parallel pathways, refers to systems in which different parallel processing streams handle different types of information. Dual parallel processing pathways, on the other hand, refers to different processing streams where different pieces of information about a particular stimulus are handled separately. There is evidence that both types of information processing exist; segregated parallel pathways are found in the olfactory system of fish (Hamdani and Døving 2007), whereas the visual system provides an example of dual parallel pathways. Here, the mango-, parvo- and koniocellular pathways carry different pieces of information about the same visual percept from the retina through the lateral geniculate nucleus to area V1 in the visual cortex (review: Nassi and Callaway 2009).

If parallel processing streams serve to compress information, it is not hard to imagine that they could be useful in odorant processing, due to the complexity of the olfactory world. In the olfactory system of insects, multiple processing pathways have indeed been revealed in several species (Homberg et al. 1988; Kirschner et al. 2006; Galizia and Rossler 2010). The concept of segregated parallel pathways is instantly appealing, especially in light of the findings in fish (Hamdani and Døving 2007).

However, Homberg et al. (1988) showed that projection neurons from sex pheromone specific glomeruli run in all three APTs; thus, the evident anatomical segregation does not seem to carry information about odorants with different biological relevancies to the animal. Due to the diversity of the tracts it seems more likely that the different information streams from the antennal lobe to the higher brain centers function as parallel pathways that process different parts of the same information, i.e. dual parallel pathways. This is supported by what we know about the ml-APT in moths.

Inhibitory Circuits in the Protocerebrum

The lateral protocerebrum

As described in the result, the inhibitory connections of the lateral protocerebrum appear as a meshwork of fine neurites. These neurons are likely interneurons that provide many different kinds of inhibition in order to prepare the neural signal for propagation in descending neurons to the ganglia that carry out behaviors. Very little is known about the physiology of inhibitory neurons in the lateral protocerebrum, thus we can only speculate what their functions might be. It is conceivable that they play an important role in filtering the information that is passed on to descending pathways, and that they can act as a mechanism by which “less important” information is eliminated from reaching descending pathways if for example a danger is to occur, or if some more urgent task suddenly needs to be carried out. An iontophoretically pre-stained protocerebral inter-neuron with known electrophysiology was stained for GABA in the current study (Fig. 15), but proved not to be GABAergic; thus we are no closer to unraveling the physiology of single GABAergic neurons in the lateral protocerebrum.

The protocerebral calycal tract

Immunolabeling the moth brain for GABA revealed a prominent reactive structure that projects from a cell cluster in the lateral part of the lateral protocerebrum and enters the pedunculus of the mushroom bodies. Figure 7 gives a clear view of the cell cluster from which the tract originates, and figure 8 follows the projection of the tract from the lateral protocerebrum to the mushroom body pedunculus. The tract enters the pedunculus through its ventral side, bifurcates, and sends collaterals to

both the lobes and calyces. It is evident from the present immunostained preparations that the calyces, in particular, are brightly immunoreactive. No Kenyon cell bodies show immunoreactivity in this or other studies; thus it is assumed that Kenyon cells do not hold GABA as their transmitter. The tract seen in the present study closely matches other findings from *M. sexta (Homberg et al. 1987)* and honeybees (Mobbs 1982; Grünewald 1999a; b). The tract has been given several names, however PCT is now the most commonly used. The neurons in the PCT were shown to have branches in the lobes displaying dendrite like specializations whereas the branches in the calyces had axonal specializations (Mobbs 1982; Rybak and Menzel 1993; Grünewald 1999a). On the basis of these observations as well as electrophysiological recordings (Grünewald 1999b) the connectivity of the tract was originally believed to be that of a straight forward inhibitory feedback loop, figure 17; which means that the Kenyon cells give excitation to the PCT neurons in the mushroom body lobes, and receive inhibition from the PCT neurons in the calyx.

However, two electron microscopy studies have shown that the connectivity of the PCT neurons is more complex (Leitch and Laurent 1996; Ganeshina and Menzel 2001). This is in accordance with findings in flies where a subunit of a GABA receptor has been found not only in the lobes but also in the calyces (Harrison et al. 1996). The electron microscopy studies do indeed confirm that there are GABAergic axon terminals in the calyces of the mushroom bodies, and that these provide output onto Kenyon cell dendrites. Yet, they also show tight connections between what is assumed to be the axon terminals of antennal lobe projection neurons and PCT neurons. Also, the connections between the GABAergic neurons and projection neurons are assumed to be bidirectional (Ganeshina and Menzel 2001). Thus, in addition to forming a ‘structurally global’ inhibitory feedback loop (Fig. 17), the PCT neurons are also involved in several microcircuits within the mushroom body calyx. Figure 18 shows a schematic overview of the putative inhibitory microcircuits in the mushroom body calyx. These microcircuits are thought to include feedforward inhibition from the projection neurons to Kenyon cells through PCT-neurons, and feedback loops from PCT-neurons to projection neurons (Leitch and Laurent 1996; Ganeshina and Menzel 2001). The only neurons present in the mushroom body calyx that do not seem to display considerable connectivity to PCT-neurons are dense core axonal terminals; these are assumed to be axonal collaterals from modulatory

neurons that hold octopamine, serotonin etc. Thus the modulatory input to the mushroom body calyx does not seem to be influenced by inhibition within the mushroom bodies (Ganeshina and Menzel 2001).

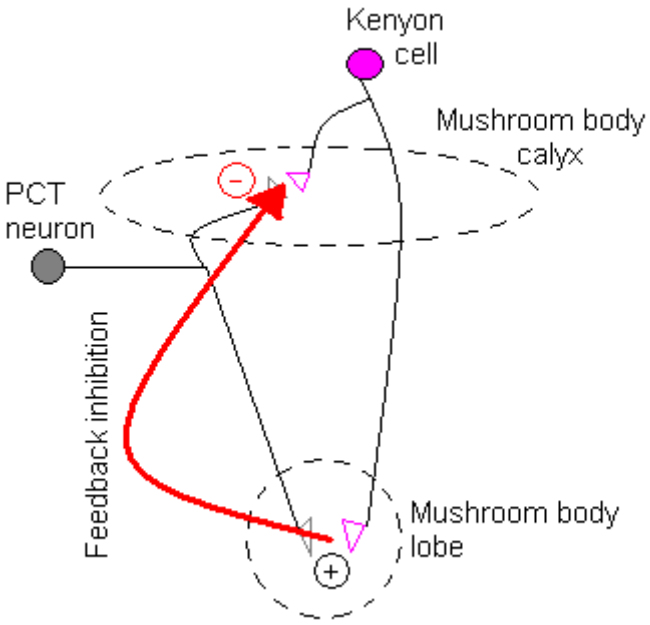

Figure 17: The inhibitory feedback loop between the Kenyon cells and PCT neurons.

As can be seen in figures 8C and D, the calyces of the mushroom bodies and the mushroom body lobes both display strong immunoreactivity. The multitude of connections involving inhibitory neurons found within the mushroom body indicates that these neurons serve several functions. The recurrent feedback loop from the lobes to the calyces might perform an attenuation of the excitatory signals in the Kenyon cells that prevents hyper-excitability. The feedback loop might also be involved in generating the oscillations observed in the local field potential in the current region (Laurent and Naraghi 1994). Finally, the fibers of the tract are reported to display experience dependent plasticity (Grünewald 1999b; Haehnel and Menzel 2010).

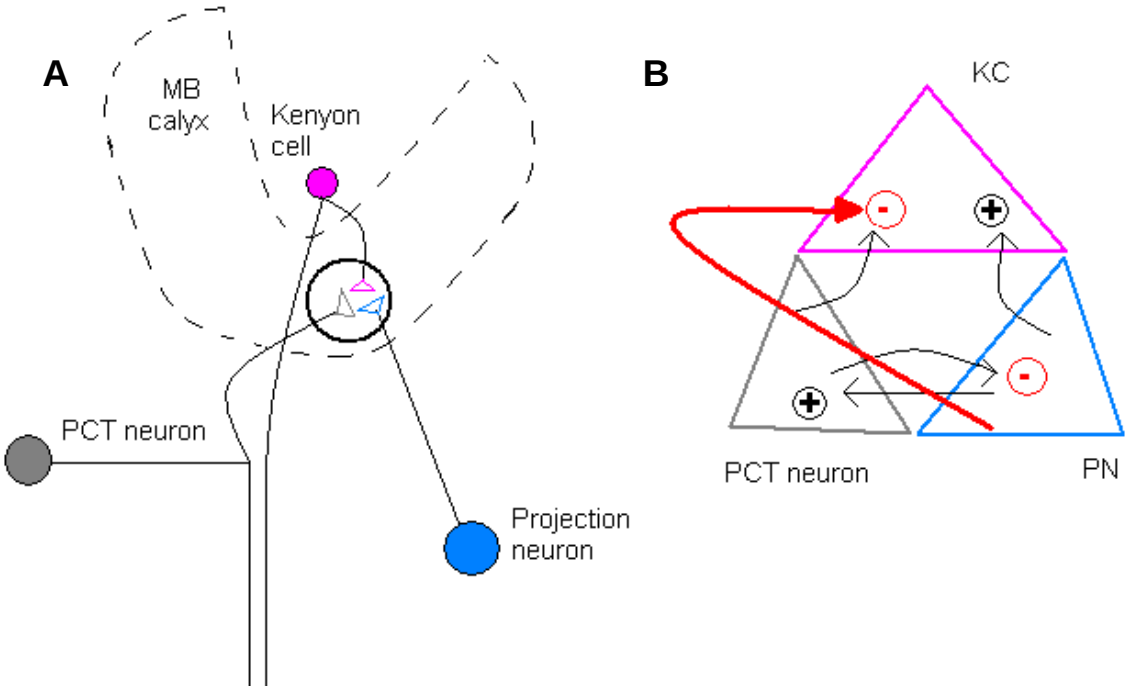

Figure 18: Microcircuits in the mushroom body calyx. (**A**) Schematic drawing of the mushroom body calyx and the neurons involved in the circuit. (**B**) Close up of the synapse showing the synaptic connectivities between the neurons. The projection neuron excites the PCT neuron which in turn inhibits the Kenyon cell creating a feedforward inhibitory loop (red arrow). There is also a local feedback loop between the PCT neuron and the projection neuron, in addition to the expected excitatory connection between the projection neuron and the Kenyon cell. Kenyon cell (KC), Projection neuron (PN), Protocerebral calycal tract neuron (PCT neuron).

Feedforward inhibition in the mushroom bodies

The local microcircuits seem to provide a mechanism for stabilizing the excitation of Kenyon cells (Ganeshina and Menzel 2001). Figure 18 shows a schematic figure of the mushroom body calyx and some of the neurons that synapse within it. Figure 18B explains the putative local inhibitory feedforward loop from projection neurons to Kenyon cells in the mushroom body calyx. Kenyon cells are known for sparse firing, and they have next to no spontaneous activity in spite of a constant drive of spontaneous firing by the projection neurons (Stopfer et al. 2003). This is partly attributed to a high threshold for activation, but may also be facilitated by strong feedforward inhibition by the PCT-neurons (Perez-Orive et al. 2002). Feedforward inhibition is known to be an effective regulator according to temporal integration properties of neurons (Shepherd 2004). A computational study by Assisi et al. (2007), suggests that feedforward inhibition from the projection neurons through PCT-neurons to Kenyon cells in the calyx is a main contributor to the sparseness of the

Kenyon cell firing over a wide variety of stimulus conditions, and that changes in projection neuron activity modulates the phase of the feedforward inhibition. When the coherence of the input from the projection neurons increase, the firing in the PCT neurons follow, thus creating even stronger inhibition onto the Kenyon cells (Assisi et al. 2007). This is a property that has proved to be incredibly resilient, and which is maintained even when odors are presented at overwhelming concentrations – suggesting that it is an important mechanism (Stopfer et al. 2003).

Oscillations and feedback inhibition in the mushroom bodies

One kind of oscillation in neural assemblies is observed via measurement of so-called local field potentials. A local field potential is a weighted average of the fluctuations in the local electrical potential around the electrode during an extracellular electrophysiological recording (Laurent 2002). These fluctuations are caused by synaptic currents in the areas surrounding the electrode. The invertebrate nervous system in which oscillatory activity has been most intensively studied is that of the locust (Laurent and Naraghi 1994; MacLeod and Laurent 1996; Laurent 2002; Perez-Orive et al. 2002). The locust olfactory system is in many respects different from those of higher order insects such as honeybees and moths. However, there are many similarities and the mushroom bodies seem closely related structurally in many species of insects. I will discuss the PCT's putative role in generating oscillations in the Kenyon cells of the mushroom body, and possible functional implications of this oscillatory synchrony, keeping in mind that the nervous systems of the moth and locust are likely to have differences. Still, it is conceivable that the main mechanisms behind, and the functions of, oscillatory synchronization are similar across a wide variety of species. Inhibitory loops, such as the one in the mushroom bodies revealed in this study, are thought to be of importance in generating oscillatory activity (Laurent and Naraghi 1994; MacLeod and Laurent 1996; Perez-Orive et al. 2002). The following paragraph contains a short summary of how oscillations may affect neural processing in the primary learning center of insects.

In 1994, Laurent and Naraghi reported synchronous oscillations in the local field potential in the mushroom bodies of locusts upon odorant stimulation. The membrane potentials of the Kenyon cells fluctuated in phase with the local field potential. Such oscillations may be a result of intrinsic or synaptic events related to

the Kenyon cells. Most likely, they result from a combination of these (Laurent and Naraghi 1994). The oscillations were not present in the absence of odorants, and were not elicited by puffs of clean odorless air. The frequency at which the local field potential oscillated was ~20Hz; this was invariant of the quality of the odor presented. Action potentials always occurred in phase with the oscillations, maximally one action potential per cycle. Spontaneous oscillations did sometimes occur, suggesting that the Kenyon cells have an intrinsic tendency to form fluctuations. However, membrane properties of the Kenyon cells alone would not be able to initiate oscillations in a neuronal assembly, but might rather facilitate them (Laurent and Naraghi 1994). MacLeod and Laurent (1996) later demonstrated that oscillations in the antennal lobes help generate the oscillations in the mushroom body calyces, but also that the mushroom body calyces are fully capable of generating such oscillations when oscillatory activity in the antennal lobe is abolished. Fast inhibition – inhibition that is mediated by $GABA_A$-like receptors – is important for generating oscillations in the antennal lobe (Review:MacLeod and Laurent 1996; Stopfer et al. 1999). The local interneurons of the antennal lobe are probably responsible for these oscillations (Laurent and Davidowitz 1994; Leitch and Laurent 1996; MacLeod and Laurent 1996; Stopfer et al. 1999; Laurent 2002).

In the mushroom body, the neurons from the PCT are thought to play a similar role as the interneurons in the antennal lobe. This because the Kenyon cell oscillatory response does seem to have an inhibitory component (Laurent and Naraghi 1994). Kenyon cells are both the source and target of PCT inhibition, thus they provide a mechanism for indirectly inhibiting themselves (Leitch and Laurent 1996; Grünewald 1999b; a; Ganeshina and Menzel 2001). The inhibitory neuron provides an inhibitory post synaptic potential onto the Kenyon cells, creating a window of time where the Kenyon cells in question can not depolarize even if an excitatory synaptic input was to occur. When the inhibition passes, the Kenyon cell may once again be susceptible for excitatory input, which may lead to a spike. These fluctuations in the Kenyon cells' ability to spike could help generate the oscillations recorded in the local field potential of the mushroom body. The PCT is estimated to contain approximately 50 cell bodies (Homberg et al. 1987; Grünewald 1999a); (such a count could not be carried out on the basis of the results in this study), whereas the Kenyon cells can count up to 300 000 in some species (DeBelle and Kanzaki 1999). When looking at the

distribution of GABA reactivity in the mushroom body calyces (Fig. 8C and D), it is evident that the entire calyx contains GABAergic processes – which means that each PCT neuron makes an exceptional number of synaptic contacts with Kenyon cells. The higher the number of cells the inhibitory neurons connect, the larger an area of the neuronal assembly they can coordinate. Based on a sheer numerical observation it is likely that the PCT neurons coordinate large parts, if not the entire mushroom body calyx.

The role of oscillations in coding of olfactory information remains to be fully elucidated; however, there are several suggestions as to what may be the function(s). One such function is odor discrimination. By manipulating GABA receptors such that oscillations are ablated, it has been shown that odor discrimination is impaired (Stopfer et al. 1997). The most popular theories, however, are those that relate to learning and memory. Oscillations may form the basis for Hebbian learning ("neurons that fire together wire together") – by synchronizing the neuronal assembly, afferent input is received in such a way that stronger synaptic association occurs, e.g. long-term potentiation (Laurent and Naraghi 1994; Laurent 2002). Oscillations may also aid the feedforward mechanism (described earlier) in providing a small window for which Kenyon cells are susceptible to projection neuron input, thus adding another level of control to ensure sparse Kenyon cell firing. Maintaining sparseness of firing in Kenyon cells is thought to be an important property with regards to learning and memory tasks. Sparse representations offer many advantages; overlapping between individual representations of sensory stimuli is less likely in sparse coding, because only very few of a huge assembly of neurons are active at any given time. At the same time, given that the sparseness is not extreme, the coding space offered by sparse representations is very large. (Perez-Orive et al. 2002). There are also significant energy savings to sparse neural activity (Olshausen and Field 2004).

Methodological Considerations

The main aim of this master's project was to successfully perform GABA immunostaining in particular parts of the moth brain. The following are some methodological considerations regarding this immunostaining method.

First a new non-commercial primary antibody for GABA, which was kindly provided by Dr. Dirckson, was tested. We used a protocol with a long incubation period for both the primary and secondary antibody as well as several different concentrations of the primary antibody (from 1:500 to 1:10000). None of the stainings were successful, and no specific staining could be seen in any of the preparations. This was likely due to difficulties concerning the fixation method used for the brains. In our lab we primarily fixate brains in paraformaldehyde or Roti Histofix© (both of which are formalin based fixation mediums), whereas the recommended method of fixation for the primary antibody was glutaraldehyde. The fix likely affects the ability of the antibody to penetrate and/or bind in the neural tissue rendering the brains devoid of specific staining. Due to the fact that the iontophoretically stained preparations were already fixed in paraformaldehyde, changing the fix was not an option, and further attempts to make successful stainings with this primary antibody were abandoned.

We then moved on to a commercially synthesized primary antibody from Sigma. This was the same antibody previously utilized by Jens M. Halvorsen in his master's project (Halvosen 2011); however he had some caveats about its use. First, he experienced that the antibody did not always achieve satisfactory staining. By extending the incubation period from 24 hours to 5 days, thus ensuring ample time for the antibodies to penetrate and bind to any GABA molecules present in the brain, this problem was eliminated in the current study. Another problem he had was that of penetration; he experienced that structures that lie deep within the tissue appear as less prominent than the ones located more peripherally. This was still the case, even after the extended incubation period of the new protocol, and is likely due to impaired penetration of the antibodies. By sectioning the preparations on the vibratome, as done in the present study, the centrally positioned brain structures become more robustly labeled. One last problem in the previous study was that of a cross-reaction between the iontophoretical stain in GABAergic neurons and the immunostain. It seemed that neurons that showed GABA immunoreactivity and were intracellularly stained had stronger coloration by the immunostain than general GABAergic neurons that had not been intracellularly stained. This could also be due to cross-reactions between immunoglobulins of different species and the secondary antibody. This was never a problem in this study as none of the neurons in question turned out to display immunoreactivity to GABA.

The Sigma antibody was tested at several different concentrations; 1:1000 proved to give the best stain with the least amount of unspecific staining.

With regards to the secondary antibody, most of the preliminary studies were performed with CY5. It was assumed that the identity of the secondary antibody would not make much of a difference. For the double staining experiments, changing the secondary antibody proved to be necessary because the preparations had been stained with synapsin and CY5 in the earlier experiment. CY2 was chosen as the new secondary antibody, under the assumption that it would achieve as good results as CY5 – however this turned out not to be the case. The CY2 staining was weaker and had little or no specific staining to GABA; indicating that the different secondary antibodies have different penetrating and/or binding abilities. A new secondary antibody; AlexaFluor® 488, with approximately the same absorption and emission maxima as CY2 but with better binding and penetration properties, was tested out in the end and gave good results.

Another methodological consideration concerns sectioning the preparations vs. keeping the whole mount preparation. As can be seen from the results, both methods were used (e.g. Fig. 6 and 8). Whole mount preparations are easy to handle, they do not break and all parts (usually) remain intact throughout the staining process. Large immunoreactive structures are easily visible in the preparations, and there is relatively little unspecific staining. The disadvantage of whole mount preparations is that smaller structures are hard to see, and that structures embedded deep in the neural tissue appear as less reactive than more superficial ones. This is probably due to the fact that the antibodies have more trouble penetrating deep within the tissue, combined with limitations of the microscope in penetrating the tissue with the laser. Vibratome sections expose individual neurons to a greater extent, and all GABAergic structures appear very prominently. A disadvantage of the procedure however is that structures break easily in the vibratome, and are often spread out over several sections, thus rendering them harder to recognize. Because penetration is improved there is also a problem of unspecific staining; however to the trained eye distinguishing GABAergic structures from surrounding unspecific staining is usually not a significant problem.

An attempt was also made to visualize the APTs through mass-staining from the antennal lobe, before immunostaining the preparations such that any differences between the mass-stained tracts and the parts visible by the immunostaining could be revealed. Mass-staining does however cause significant damage to the neurons that are being stained, and because of this carrying out immunostaining for GABA on these preparations proved difficult at best. It seems that stabbing the neurons with the electrode caused them to “leak” neurotransmitter, thus immunostaining did not give fruitful results. The procedure was attempted several times on both my own and older anterogradely stained preparations; all immunostainings performed on such preparations were unsuccessful.

Conclusion

Upon immunostaining the *H. virescens*' brain, the importance of GABA in neural processing of odor signals is immediately evident. Primary relays in the central olfactory pathway of the insect, such as the antennal lobes, the lateral protocerebrum, and the mushroom bodies, show GABA immunoreactivity.

Of the three main antenno-protocerebral tracts, the medio-lateral (ml-APT) does display substantial immunoreactivity. However, GABA staining of two iontophoretically pre-stained projection neurons passing in the ml-APT showed that they were immuno-negative – confirming the assumption that GABA reactivity does not apply to all projection neurons in the tract. The protocerebral regions targeted by the immuno-negative neurons indicate that they constitute a particular category innervating not only the lateral protocerebrum but also the superior protocerebrum.

The strong GABA-labeling of the protocerebral calycal tract (PCT) proves its presence in Heliothine moths. The current tract is made up by a population of neurons which have their somata clustered in the lateral protocerebrum and project a thick bundle of neurites into the pedunculus of the mushroom body, innervating the lobes and the calyces.

As concerns the methods, the Sigma primary antibody proved superior to the non-commercial, probably due to restrictions with regards to the fixation method in the current project. Extending the incubation period for the primary antibody proved fruitful with regards to obtaining consistent staining on every trial, a known problem when using the Sigma antibody. In order to minimize the problem of weak staining in structures embedded deep in the tissue, vibratome sectioning turned out to be appropriate. The study also shows that the identity of the secondary antibody is of great importance to the quality of the result, and that similar results cannot be expected on the basis of the choice of primary antibody alone – this is something to keep in mind for future experiments.

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