MAPPING OF CONTRALATERAL PROJECTIONS IN THE CENTRAL OLFACTORY PATHWAY IN THE MOTH SPECIES *HELICOVERPA ARMIGERA* AND *HELIOTHOSIS VIRESCENS*

Ola Auran

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Supervisor: Bente G. Berg

Norwegian University of Science and Technology

Faculty of Social Sciences and Technology Management

Department of Psychology

Acknowledgments

Sammendrag

Fylogenetisk sett er luktesansen den mest primitive sansen. Den er også en sans som vekker mange følelser i oss mennesker, fra avsky og vemmelse, til å vekke gode minner fra barndommen. Luktesansen er hovedsakelig et ipsilateralt organisert system i alle arter, i motsetning til andre sansesystemer som er hovedsakelig kontralateralt organiserte. Det er funnet bevis for at det er noen kontralaterale projeksjoner som går på tvers av midtlinjen hos noen insektarter. I denne studien undersøkes slike kontralaterale projeksjoner hos møllartene *Helicoverpa armigera* og *Heliothosis virescens*. Bevis for slike kontralaterale projeksjoner er blitt funnet og blir beskrevet i denne oppgaven. Hvor projeksjonene går og hvilke prosesser de kan være involvert i blir diskutert. Likevel understreker bevisene at luktesystemet er hovedsakelig ipsilateralt organisert, noe som leder til det spennende spørsmålet om hvorfor dette systemet skiller seg fra andre sensoriske systemer. Konklusjonsmessig blir det derfor diskutert hvorvidt dette kan ha en sammenheng med dets utvikling før andre sensoriske systemer, at det er en forløper og en nødvendighet for utviklingen av et sentralnervesystem, og at dette er grunnen for dets unike organisering.

Abstract

Olfaction is phylogenetically the most primitive sense. It is also a sense that evokes a lot of feelings in us humans, from disgust to nostalgic childhood memories. Olfaction is primarily an ipsilaterally organized system in most species, contrary to other sensory system which are primarily contralaterally organized. There are however some evidence of contralateral projections in insect olfactory systems. In this study such contralateral projections are investigated in the moth species *Helicoverpa armigera* and *Heliothosis virescens*. Evidence for some contralateral projectional axons are obtained and described. However, they are few in numbers. Their anatomy and what processes these neurons may be implemented in is discussed. However, the pictures obtained underline the ipsilateral organization of the olfactory system. This leads to the intriguing question of why this sensory system is so differently arranged compared to other sensory systems. Conclusion wise it is discussed whether this is a result of the early phylogenetically nature of this sensory system, that it evolved before other sensory systems, and therefore is a precursor and a necessity for the development of a centralized nervous system.

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Introduction

Olfaction is phylogenetically the oldest of all senses. In some way, many would argue that the sense of olfaction has lost its importance among humans at the expense of vision and audition. However, the mere fact that perfume is a multibillion-dollar industry proves the central role of olfaction in the human experience. We are also all aware of the uncomfortable experience of the gagging reflex which can occur if a very bad smell is present, or the salient hunger feeling that arises with the smell of freshly baked cinnamon buns. Adding to this, we have all been reminded of olfaction's importance regarding the sensation of taste when we catch the common cold. Some studies have also shed light on probable subconscious processes linked to olfaction and pheromones, i.e. menstrual synchrony among women (McClintock, 1971) and how the length of women's menstrual cycle is affected by the pheromones of other women (Stern & McClintock, 1998). In the following sections information regarding the olfactory system in both insects and mammals will be provided for the reader, but first a short introduction to why insects are used as model organisms in research on olfaction.

Comparative studies including insects as model organisms
Genetic heritage for olfaction in mammals is found to be 200 million years old, starting with seaborne creatures. Insects, on the other hand developed about 480 million years ago. Early investigations presumed that the olfactory systems of insects and other terrestrial beings like reptiles and mammals did not share a common origin, but rather developed independently (Benton, Sachse, Michnick, & Vosshall, 2006). However, new research into this matter has revealed similarities beyond mere chance, arguing that a common ancestor to both protostomes and deuterostomes (and thus ancestors to both mammals and insects) already had developed chemosensory integrating brain centers (Tomer, Denes, Tessmar-Raible, & Arendt, 2010) (Strausfeld & Hirth, 2013). The ability to process chemosensory information seems to be very advantageous for terrestrial life, offering life forms the most basic way of interacting with other molecules in their environment. Irrespective of details concerning the evolutionary development, however, it is an undisputed fact that the olfactory system is particularly well conserved across various organisms. The many similarities in anatomical and physiological principles typifying the olfactory system offer an opportunity to study favorable model organisms, such as insects, for exploring chemosensory mechanisms in general. Compared to

mammals, insects have a more accessible nervous system, are easier to keep, and have shorter life cycles. In addition, the insect brain is small enough to be placed under a confocal microscope without having to slice it.

In the following section contemporary research and knowledge about general insect neural anatomy and specifically neural areas related to olfaction will be provided. Primarily the antennal lobes, the mushroom bodies, the lateral horns and the antennal lobe tracts (ALTs) will be discussed. With the insect olfactory system in mind, the mammal olfactory system will be discussed in context to this.

The insect olfactory system

The supraesophageal ganglion and the subesophageal ganglia
The supraesophageal ganglion is located dorsally to the esophagus of the insect and include three different brain regions, the protocerebrum, the deutocerebrum and the tritocerebrum. The protocerebrum, which comprises the optic lobes and the midbrain, is involved in higher order processing. The deutocerebrum includes the antennal lobe (AL) and the dorsal lobe. The AL processes olfactory information from the antenna while the dorsal lobe is involved in processing mechanosensory information. The dorsal lobe is also implemented in the motor control of the antenna. The tritocerebrum integrates information from the protocerebrum and the deutocerebrum and connects these parts with the subesophageal ganglion (SEG), which is located ventrally. The SEG is the primary brain center for gustatory information.

The antenna
In insects, odorants are recognized by dendrites of *olfactory sensory neurons* (OSNs) located in small hair like structures called *sensilla* covering the antennal surface. The OSNs dendrites express g-protein coupled chemosensory receptors called *olfactory receptors* (ORs), and they respond in an excitatory fashion when the receptors are coupled with a relevant stimulus, which may be a pheromone or another odorant (Vosshall & Stocker, 2007) (Galizia & Sachse, 2010)**.** Some OSNs respond to a single molecule, while others may respond to classes of molecules, such as alcohols or aldehydes. Usually however, it is difficult to distinguish between molecules and that is the task of the complex neural olfactory system (Hansson B., 2010).

A single sensillum in itself may contain a different number of OSNs. The sensilla also differ in their anatomical shape, which is often implicated in a structure-function relationship

(Galizia & Řössler, 2010). The tract rearrange in to a dense network of new tracts in the AL in an area called the *sorting zone*. In moths, each AL receives input from the one ipsilaterally located antenna by means of the antenna tract (Galizia & Sachse, 2010).

The antennal lobe

The AL is located rostrally in the moth brain, and is analogous to the olfactory bulb in vertebrates (Carlsson, Galizia, & Hansson, 2002). The AL consists of two main neuron types: the local neurons (LNs) which are confined to the AL and the projection neurons (PN) that connect the AL to higher processing areas of the insect brain. The LNs have connections to PNs, OSNs and other LNs. Together with the sensory terminals the AL neurons form globular formations called glomeruli. The cell bodies of LNs and PNs are located in distinct clusters in the periphery of the AL, outside of the glomeruli. Moths are reported to have approximately 65 such glomeruli (Berg, Galizia, Brandt, & Mustaparta, 2002). Within the glomeruli the first processing of odorants is conducted and research has shown that these glomeruli are functional units representing different odors (Hansson & Stensmyr, 2011). PNs have been found to be close to firing threshold, which offers the insect an increased sensitivity to miniscule concentration changes of odor molecules (Sachse, Peele, Silbering, Gühmann, & Galizia, 2006). LNs are primarily GABAergic meaning they form inhibitory connections to the PNs, thus keeping the PNs below firing threshold. One possible reason for this is ensuring that PNs are not affected by spontaneous background activity. Another functional property of this setup may be to evoke lateral inhibition between glomeruli, which could increase the signal to noise ratio within the system. Studies have found correlations between inhibition of LNs and excitation of PNs (Christensen, Waldrop, Harrow, & Hildebran, 1993).

Research has found that glomeruli can have inhibitory connections to one another and the strongest inhibitory links are found among glomeruli that have the same odor-response profiles (Linster, Sachse, & Galizia, 2005). Furthermore, these connections can be a-symmetrical. This reduces response overlap in the PNs for similar odors. The asymmetry reflects the fact that an odor X might be more similar to odor Y, then the other way around (Guerrieri, Schubert, Sandoz, & Giurfa, 2005). In many OSNs an increase in concentration of the odorant molecule in the surrounding environment of the insect mediates an increase in the impulse frequency of the neuron. The neurons in the AL also show concentration dependent firing rates, but they have been found to have a lower threshold than the OSNs. This allows the neurons in the AL to summate input from spatially segregated OSNs – which allows them to increase the sensitivity of the olfactory system substantially (Hansson B., 2010). In

honeybees as well as moths and rats, molecule chain length has been found to be the best predictor for glomeruli activation, and also the other way around. One can predict to a certain extent the properties of an odorant stimulus by observing glomeruli activation (Guerrieri, Schubert, Sandoz, & Giurfa, 2005). One can conclude that olfactory glomeruli in the AL seem to represent an address for a specific odorant feature, which then allows the brain to use information across these glomeruli to identify the correct odor blend (Hansson B., 2010).
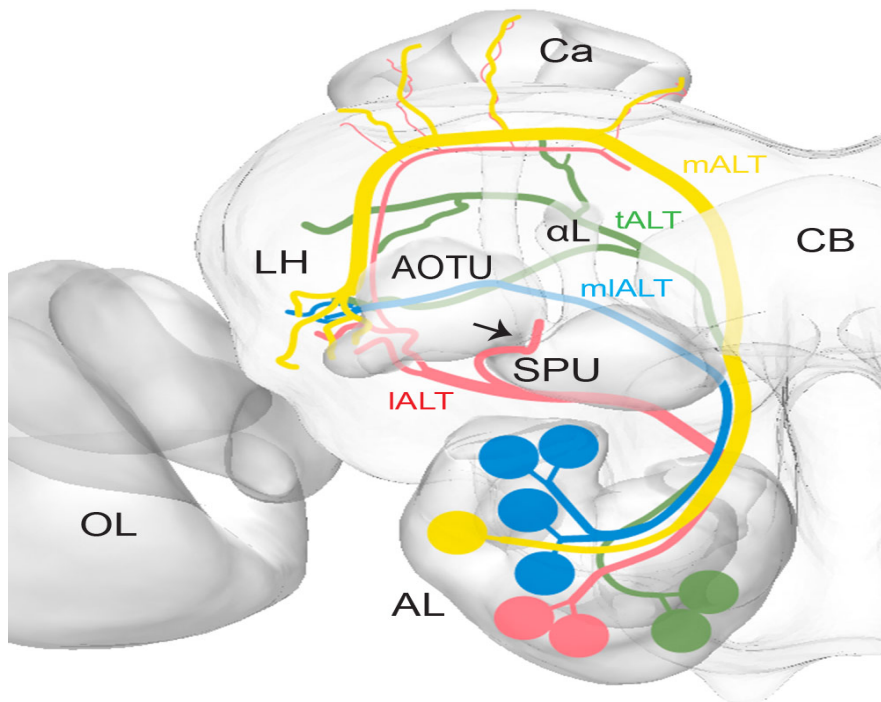
Figure 1: An illustration of the moth brain and the antennal-lobe tracts (ALTs) connecting the Antennal-lobe (AL) to higher processing areas as the calyces (CA) in the mushroom bodies (MBs) and the Lateral Horns (LH). The medial ALT, lateral ALT, medio-lateral ALT are all depicted. (Adapted from Ian et. al. under revision for publication in Cell and Tissue).

Antennal-lobe Tracts

From the AL the olfactory information is carried along three main antennal-lobe tracts (ALTs) to the higher brain centers. An illustration of these tracts adapted from Ian et. al (under revision), can be seen in figure 1. The ALTs are made up of the axons of the PNs having their dendrites in the AL. The most prominent tract is the medial ALT (mALT), which has been found in all insect species studies (Hansson B., 2010). It exits the AL medially and continues posteriorly close to the edge of the central body. It then turns laterally and innervates the calyces of the mushroom bodies before terminating in the lateral horn. The

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mALT consists of uniglomerular PNs which branch in a single glomerulus within the AL. The lateral ALT (lALT) exits the AL ventrally and immediately projects laterally innervating the inferior part of the lateral horns. It consists of both multi and uniglomerular PNs. The third tract - the medio-lateral ALT (mlALT), which is the thinnest tract, runs together with the mALT, but it bends laterally close to the central body and terminates in the lateral horns (Hansson B., 2010). A fourth tract worth mentioning is the *antennal-subesophageal tract* (AST), which connects the AL to the SEG. This tract consists of both ascending axons from neurons located in the SEG as well as sensory input from the mouthparts of the moth (Hansson B., 2010) (Bogner, Boppre, Ernst, & Boeckh, 1986). A shared feature of these tracts is the fact that they project mainly ipsilaterally; innervating higher processing areas located on the same side of the nervous system as the AL they project from.

The Calyces

The mushroom bodies (MBs), so called because of its particular shape, is an olfactory processing area in the insect brain. It is additionally divided into the calyces and the pedunculus as well as the alpha and beta lobes. The calyces, which are cup shaped parts of the MBs, are formed by the dendrites of Kenyon cells (KC), plus the AL PNs. These KCs are intrinsic to the MBs and their cell bodies surround the calyces. The axons of the KCs funnel together into the anteroventrally located pedunculus. The lobes, located more distal to the peduncules are the primary output area of the MBs (Farris, 2005). The MBs have been found to be implemented in intersensory memory formation and retrieval (Galizia & Sachse, 2010) (Dubnau, Grady, Kitamoto, & Tully).

Lateral Horns

The Lateral Horns (LH) is located in the lateral protocerebrum within the insect brain. It is one of the two main integration areas for olfactory information processing. It receives information from all the ALTs. Studies on fruit flies have found evidence that the LH is divided into two networks. One is located antero-ventrally and is connected to pheromone specific PNs, whilst plant odors are represented in a posterior-dorsal part (Berg, Zhao, & Wang, 2014) (Jefferis, et al., 2007). It is likely that the LHs are implicated in extracting general stimulus features, such as the intensity of an odor and in integration of other sensory stimuli. I.e. there are cells within the LH that respond to both olfactory and visual stimuli (Gupta & Stopfer, 2012). Furthermore, it has been proposed that the LHs are involved in

innate behavior linked to olfactory stimulus (Heimbeck, Bugnon, Gendre, Keller, & Stocker, 2001)

The mammalian olfactory system

The AL resembles in many ways the *olfactory bulb* (OB) in humans and other mammals. The OB is also made up of spherical neuropils that receive direct input from the OSNs. These OSNs are bipolar like the OSNs in moths. The OSNs dendrites are located in the mucus of the nasal cavity and they express g-coupled receptor proteins that interact with molecules in the environment. Both insects and mammals primary olfactory processing areas are organized as glomeruli. However, contrary to the ALs the OSNs cell bodies are located within the OBs, whilst in the ALs the cell bodies are located on the outside of the glomeruli. Furthermore the OBs are laminarly organized in layers unlike the AL (Ache & Young, 2005). Studies have found that OSNs in mammals also project to one or at most a few glomeruli within the OB, much like in the insect AL. This way, odorants can be recognized by complex integration areas in the brain by means of topographic patterns of activity within the bulbs (Vassar, Chao, Sitcheran, Nunez, Vosshall, & Axel, 1994).

The *olfactory tract* (OT) connects the OB with cortical regions that process olfactory stimuli, such as the amygdala, piriform cortex and entorhinal cortex. It exits the OB laterally and from there it routes on the ventral surface of the forebrain before innervating the anteromedial portions of the temporal lobe on the ipsilateral side of the brain (Bear, Connors, & Paradiso, 2007) (Zald & Pardo, 2000).

Contralateral projections in olfactory systems: previous findings

In a few insects, like the fruit fly, *Drosophila melanogaster,* a substantial amount of contralateral projections within the olfactory system have been found (Stocker, Singh, Schorderet, & Siddiqi, 1983). In these insects, OSNs innervate both the ipsilateral and the contralateral AL (Hansson B., 2010).

Also in moths, neurons that innervate both ALs have been found, but these connections are in higher order systems compared to the fruit fly. In example a large paired uniglomerular projection neuron located in the SEG has two axons that innervate the same glomeruli in both ALs. These neurons seem to be involved in mechanosensory processing by responding to puffs of air independently of odorants (Kanzaki, Arbas, Strausfeld, &

Hildebran, 1989). Additional neurons that innervate both ALs are the serotonin-immunoreactive neurons. These neurons cell bodies are located within the ALs. They project long axons that run parallel to the mALT, innervating the calyces, LHs and the ALs, both ipsilaterally and contralaterally (Homberg, Montague, & Hildebran, 1988) (Zhao & Berg, 2009). Different from the last two examples, a third unpaired neuron located in SEG has been found to innervate both ALs as well as other higher olfactory processing areas in the moth brain (Rø, Müller, & Mustaparta, 2007). However, these neurons are thought to be of modulatory importance rather than to provide immediate contralateral projections of sensory information.

Earlier studies on moths suggest that there are no contralateral projections in the lower parts of the olfactory system in moths. In other words, OSNs from the antenna does not have direct connections to the contralateral located AL. However, as mentioned previously, there is evidence of contralateral projections further up the olfactory processing system, and this study searches for such contralateral connections by applying dye to the ALs of two species of moths and scanning the contralateral brain hemisphere. If found, what is the structure of these contralateral connections and what functions may they serve? Furthermore, we know that the olfactory system is primarily an ipsilateral organized sensory system, and this must also be considered in relevance to such contralateral projections in order to further understand their function.

Materials and Method

Specimens

Moths of the two heliothine species *Helicoverpa armigera* and *Heliothosis virescens* were used during this study. The *Helicoverpa armigera* are bred in China and imported as pupae by mail to the laboratory at the Norwegian University of Science and Technology (NTNU) in Trondheim, Norway. *Heliothosis virescens* are bred in the laboratory at NTNU Dragvoll using eggs or larva shipped from Bayer Crop Services in Germany. The pupae are then separated based on sexes and kept in different climate chambers at 22 degrees Celsius until they mature as full grown moths. The climate chambers have a phase-shifted photoperiod LD of 10-14 hours. The specimens are fed 10% saccharose solution and they have access to distilled water.

Equipment

All equipment for performing the staining procedure is available at the Chemosensory Laboratory located at NTNU Dragvoll. Cut plastic pipettes and dental wax were used to restrain the specimen. Scalpel, scissors, and forceps are utilized to cut open the head capsule and access the brain. A micro needle is used to apply the dye crystals. Finally, a Zeiss confocal microscope, available at the Department of Physics, NTNU, is used to image the stained preparations.

Staining procedure

When the pupae have successfully transformed into fully grown moths they are put in a plastic tube and covered in dental wax so that the head is tightly restrained. Small incisions are made to open up the head capsule, exposing the brain. Fine paper is used to absorb fluid surrounding the brain. This is done in order to prevent the dye crystals from dissolving before they are in contact with the neuronal tissue. Crystals of the fluorescent dye *Micro-Ruby* are then applied into one of the ALs using a micro needle. Ringers solution (in mM: 150 NaCl, 3 CaCl2, 3 KCl, 25 Sucrose, and 10 N-tris (hydroxymethyl)-methyl-2-amino- ethanesulfonic acid, pH 6.9) is subsequently applied to the brain in order to prevent dehydration. A small piece of paper is soaked in Ringers solution and subsequently sheathed around the exposed brain in order to prevent it from dehydrating. The specimens are then kept in a container, away from any light sources, in a fridge for four hours, or overnight, for the Micro-Ruby to be carried retrograde through the neurons.
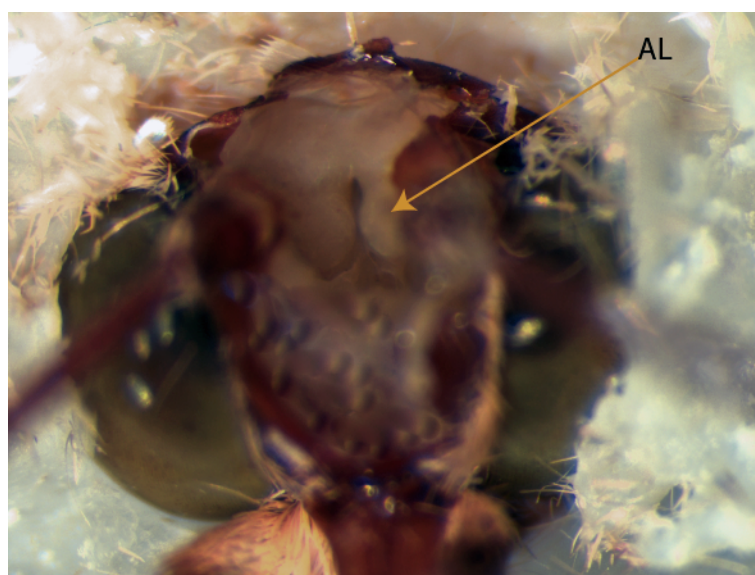

Figure 2: A close up view of a restrained moth where the head capsule has been removed exposing the brain. Both the antennal lobes (AL) can be seen.

Dissection

Following this procedure, the brains are dissected out of the head capsule and fixated in 4% paraformaldehyde for one hour at room temperature situated on a platform shaker, or overnight in a fridge at 4 degrees Celsius. It is always covered from light in between working sessions. Next the brain is washed in 0,1M Phosphate-buffered saline (PBS) pH 7,2 for ten minutes while situated on a platform shaker. The brain is thenceforth dehydrated in ethanol in series of 10 minutes in 50%, 70%, 90%, 96% solutions, and two times 10 minutes in a 100% ethanol solution. The brain is subsequently positioned in methyl salicylate between two covering glasses on an aluminum plate with a small hole in it. This creates a compartment where the brain is stored and light can travel through, which is necessary to obtain pictures using the confocal microscope. All preparations where subsequently analyzed in a light microscope containing fluorescence filters (Aristoplan). Successfully stained brains were further analyzed via confocal microscopy.

Confocal microscopy

Pictures are obtained using a confocal microscope located at Realfagsbygget at NTNU Trondheim, Norway (LSM 5 META Zeiss, Jena, Germany). A 20x objective is used (Plan-Neofluar 20x/0,5). Resolution was set to 1024x1024 pixels and z-axis mean is set to 4. A Helium-Neon laser (HeNe1) is used for the visualization of the Micro-Ruby dye.

Data Analysis

The LSM image browser is used to view and analyze the specimens. This program allows one to observe the scanned brain both in stereo and projection views. Here, specific Z-intervals can be selected, making it possible to remove interference, allowing optimal pictures of neurons or neuronal pathways. Adobe Photoshop and Adobe Illustrator CS6 are used to edit pictures for an optimal presentation of the illustrations.

Results

The total number of specimens used this study is 29. Of these, 19 were dissected, fixated and positioned on to an aluminum plate, ready for inspection via light microscopy. Out of the 19, four (4) were found to be suitable for further analysis by means of confocal microscopy. A

number of scans were necessary to obtain good pictures of the specimens. Figure 3 pictures a successfully stained AL in the ipsilateral hemisphere.
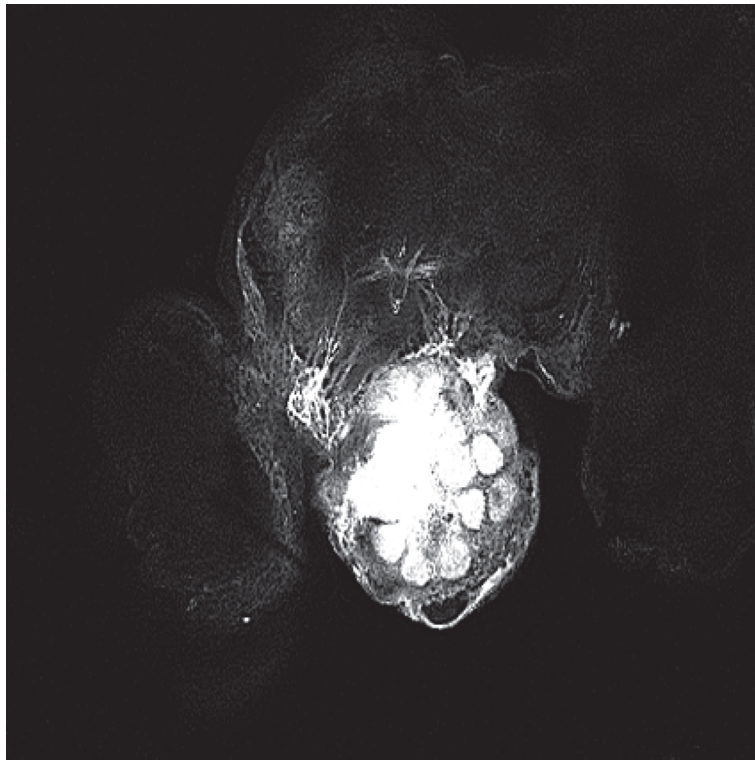
Figure 3: A picture of a stained Antennal lobe (AL) in the ipsilaterally located hemisphere. The glomeruli of the AL can be seen.

Staining in the ipsilateral hemisphere

As expected, successful staining visualized the three main ALTs projecting to the two main integration centers, the calyces and the LH in the ipsilateral hemisphere. The mALT projects posteriorly, innervating the calyces of the MBs before terminating in the LH (Figs. 4 and 5). The considerably thinner mlALT projects laterally after splitting off from the mALT. On its course to the LH, the mlALT splits into two sub-branches. The lALT appears as a fiber bundle leaving the antennal lobe slightly more ventrally and laterally then the other tracts. As shown in figures 3 and 4, it projects laterally and targets the LH. The preparation shown in theses figures includes a handful of thick lateral-tract fibers projecting to the most ventral region of the LH. Each of these axons terminates in a characteristic club-like structure. Also, a distinct pastry-shaped structure formed by two fused circles appears in the LH in the preparation presented in these figures. Finally, in addition to the three classical ALTs mentioned above, a fourth tract can be seen in figures 3 and 4. This tract is the tALT. Similarly to the mlALT, it deviates from the mALT, however more posteriorly. On its lateral

course it splits into several sub-branches. In addition, staining an ipsilateral AL results in visualization of the globular forms of the glomeruli within the AL (fig. 5).

Staining in the contralateral hemisphere
In general, visualization of the main ALTs in the ipsilateral hemisphere was defined as a criterion for successful staining as regards identification of contralateral projections. The four successfully stained preparations analyzed in this study comprised projections in various brain regions of the contralateral hemisphere including the antennal lobe, the SEG, and the protocerebrum. In addition, one labeled axon projecting into the ventral cord from the contralateral AL was found.

Staining in the contralateral antennal lobe
Dye applied into one AL resulted in visualization of a large cell body located within the contralateral AL (fig 6a and b). This cell body belongs to a particular bilateral serotonin-immunoreactive centrifugal neuron. This neuron has an axon that extends into the ipsilaterally located AL. An equivalent neuronal body is located in the ipsilateral AL, and its axon projects into the contralateral located AL. In addition, neural processes innervating several glomeruli were stained. The outlining and form of some of the glomeruli within the AL are also visualized.

Staining in the contralateral region of the SEG
In total, three cell bodies within the contralateral region of the SEG were stained (figure 7a). Two of the cell bodies were located close to the midline in the SEG (7a), where as, the third was located most laterally. As shown in figure 7a, the laterally located cell body was considerably larger compared to the two others. The confocal images also showed the primary neurite connected to the large cell body (figure 7a, 7b).

Staining of a contralateral projection in the protocerebrum
An axon that runs contralaterally immediately after leaving the ipsilateral AL was stained (figure 8). It appears to project both to the LHs and the posterior parts of the protocerebrum in the contralateral hemisphere. However, due to the relatively weak staining, its difficult to determine the exact projection pattern.

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Staining within the ventral cord

A stained projectional axon running contralaterally immediately upon exiting the AL is visualized. This is at the same z-axis level as the mALT. After crossing the midline it travels ventrally and posteriorly before innervating the ventral cord of the moth (figure 9a and 9b).

*Staining of cell clusters*

One cell cluster located close to the brain midline and the border of the posterior part of the AL was stained. These somata appear to have connections to both the contralateral and the ipsilateral AL (figure 10 a, b).
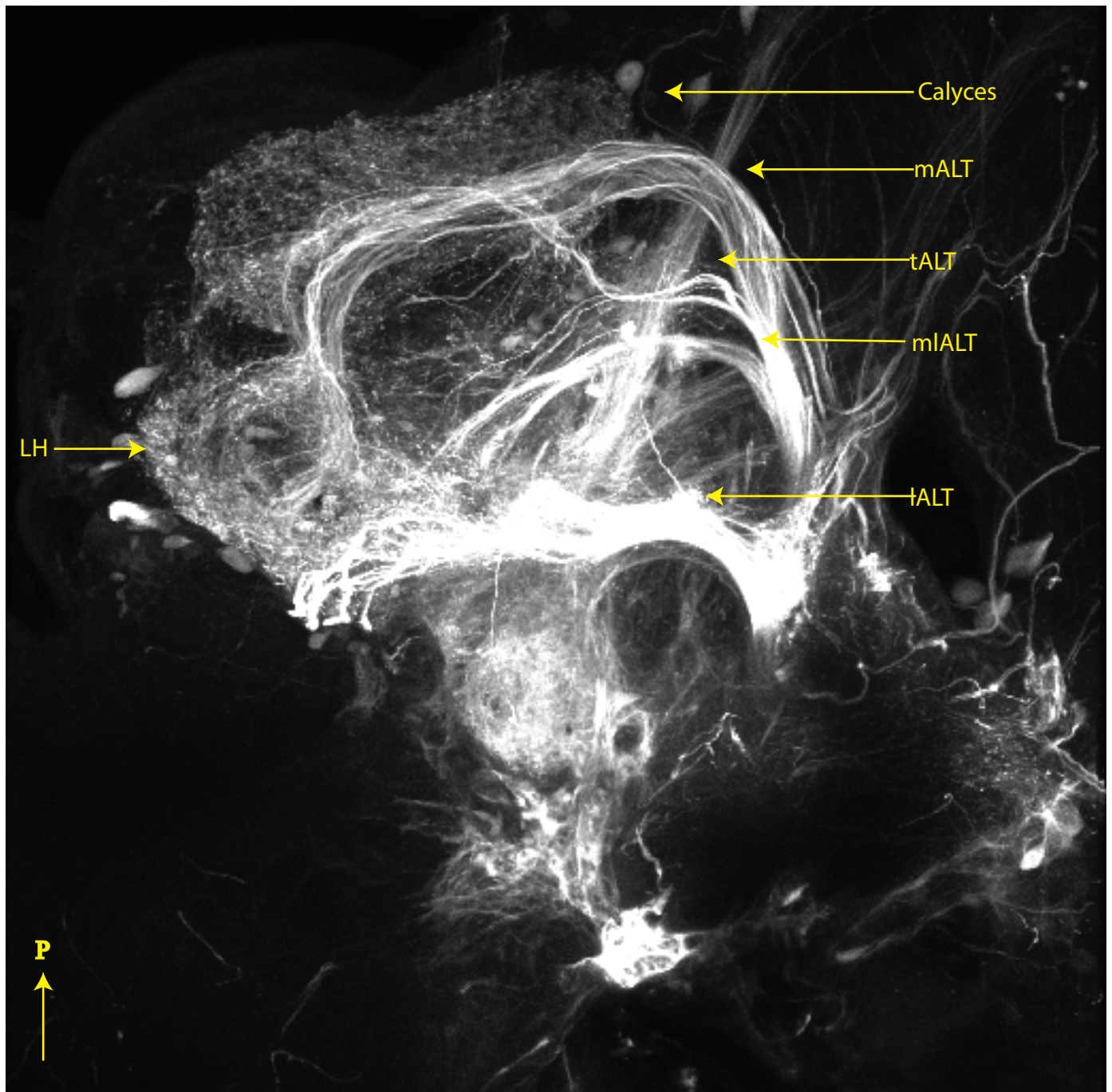
Fig 4: Projectional view of the ipsilateral hemisphere of the moth brain, which shows projection neurons originating in the stained antennal lobe (AL). These projections neurons form tracts to higher processing areas in the brain. The lateral antennal-lobe tract (lALT) exits the AL ventrally projecting laterally innervating the inferior protocerebrum, the calyces of the mushroom bodies (MB) and the lateral horns (LH). The medial antennal-lobe tract (mALT) exits the AL medially innervating the protocerebrum and the calyces in the MB. The medio-lateral antennal-lobe tract (mlALT) and transverse antennal-lobe tract (tALT) are also stained. P=posterior
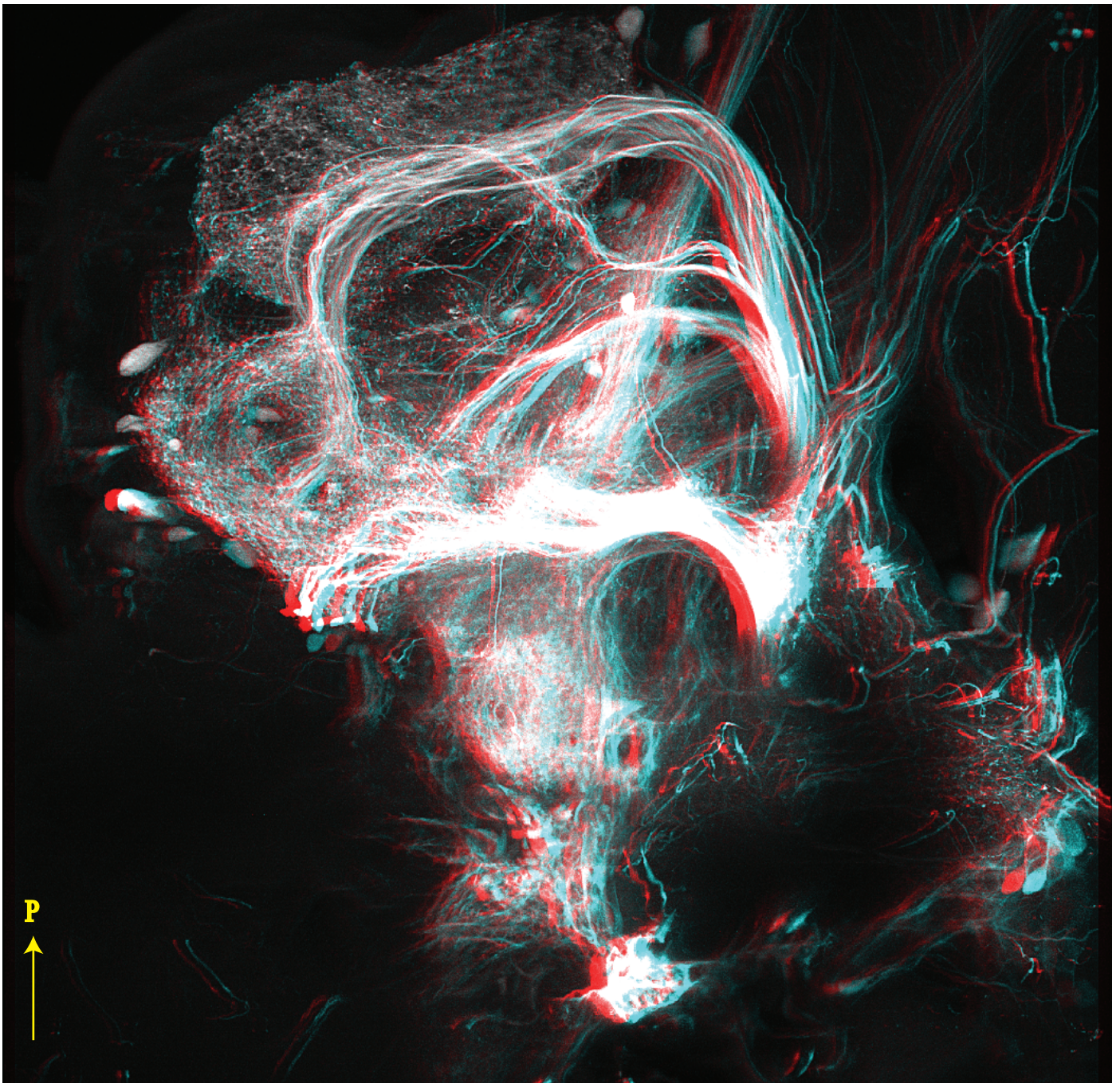
Figure 5: Stereo view of the ipsilateral hemisphere of the moth brain, which shows projections that originate in the AL. The tracts are named in the previous figure (Fig 4). P=posterior (Use glasses provided in the front side folder).
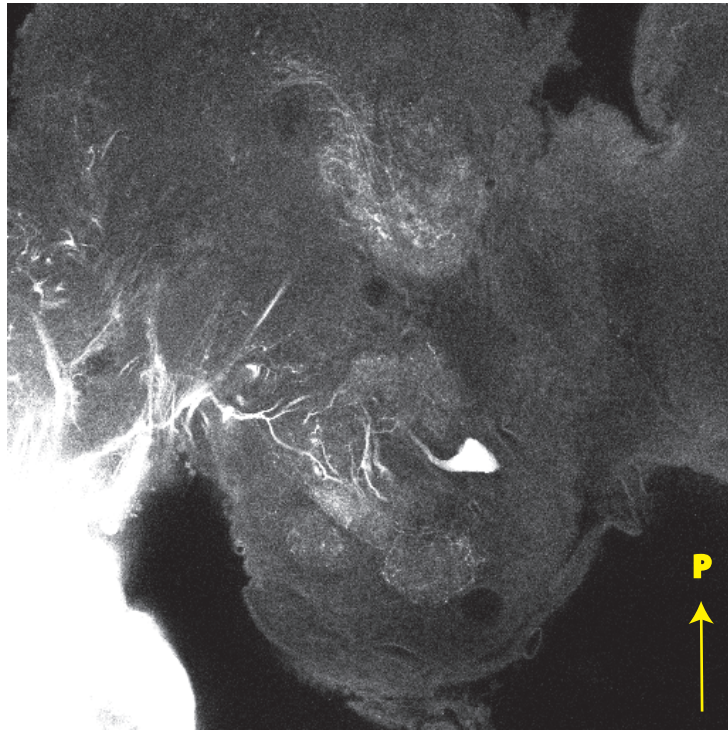
Figure 6: Confocal image showing a serotonin-immuno-reactive centrifugal neuron in the contralateral antennal lobe (AL). This neuron is mapped in previous studies. P=posterior
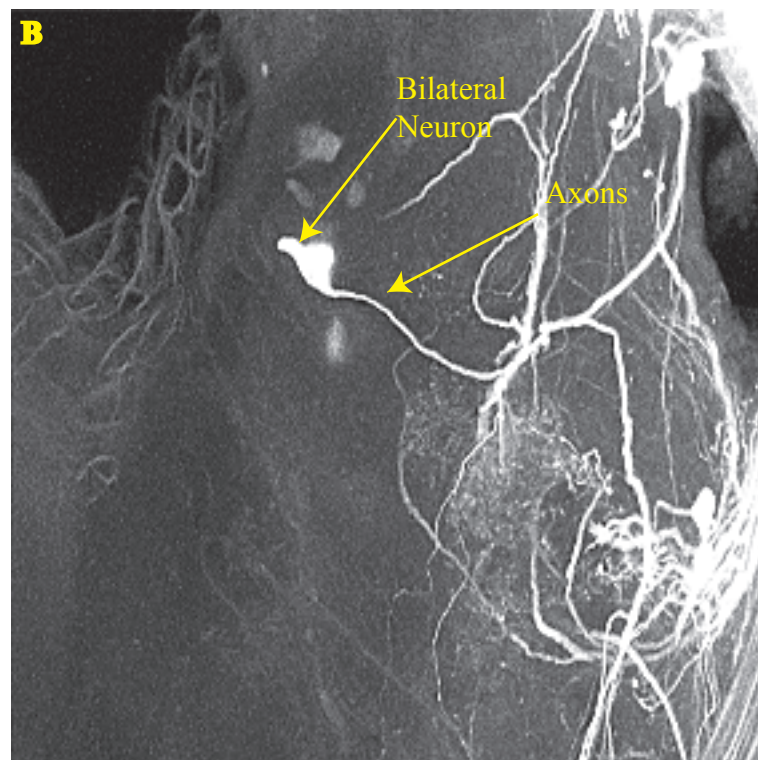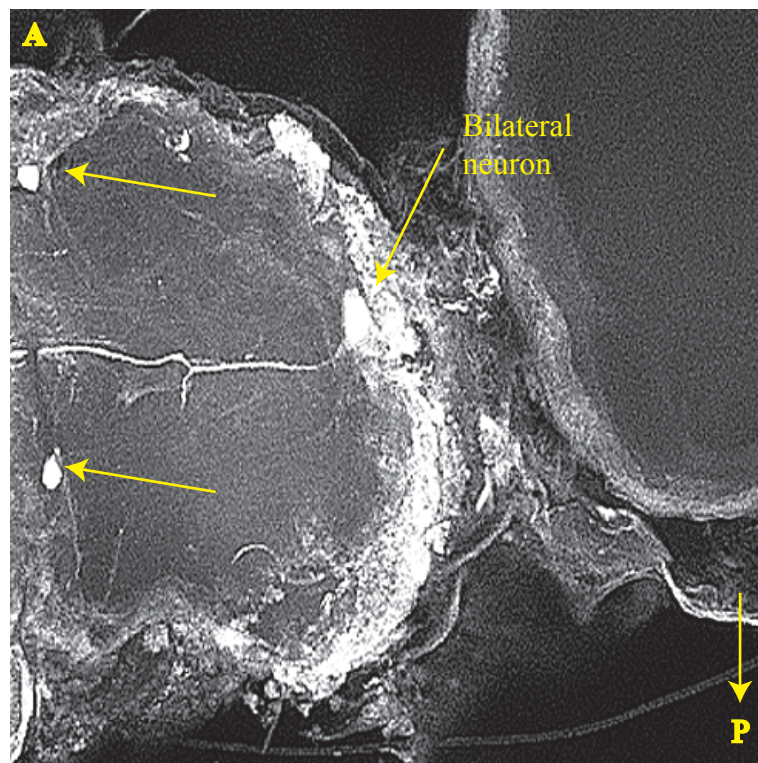
Figure 7: Confocal image showing the sub-esophageal ganglion (SEG) in the contralateral hemisphere. **(A)** A bilateral neuron located laterally in the SEG is stained. In addition, two cell bodies located close to the midline is staiend. **(B)** Another picture of the bilateral neuron and its axon. P=posterior.
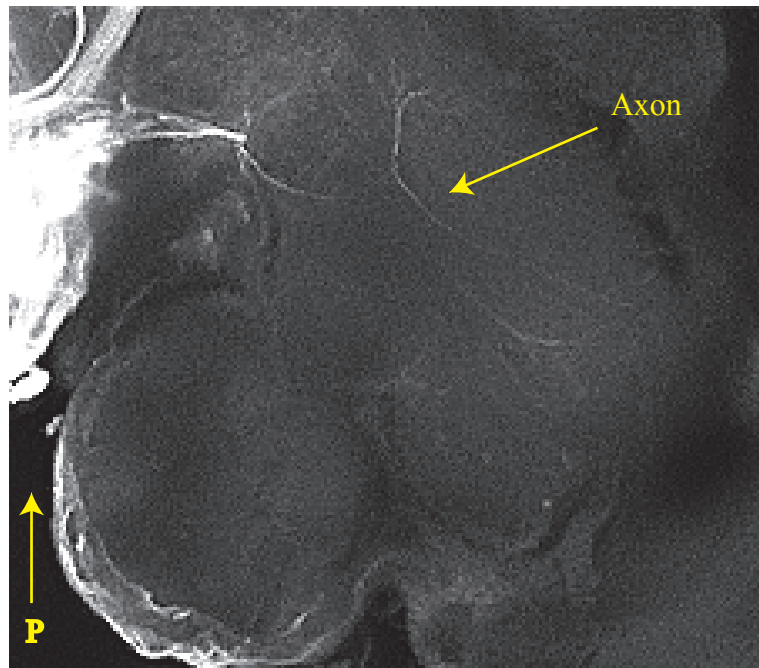
Figure 8: Confocal image of a contralateral projectional neurite in the protocerebrum. P=posterior.
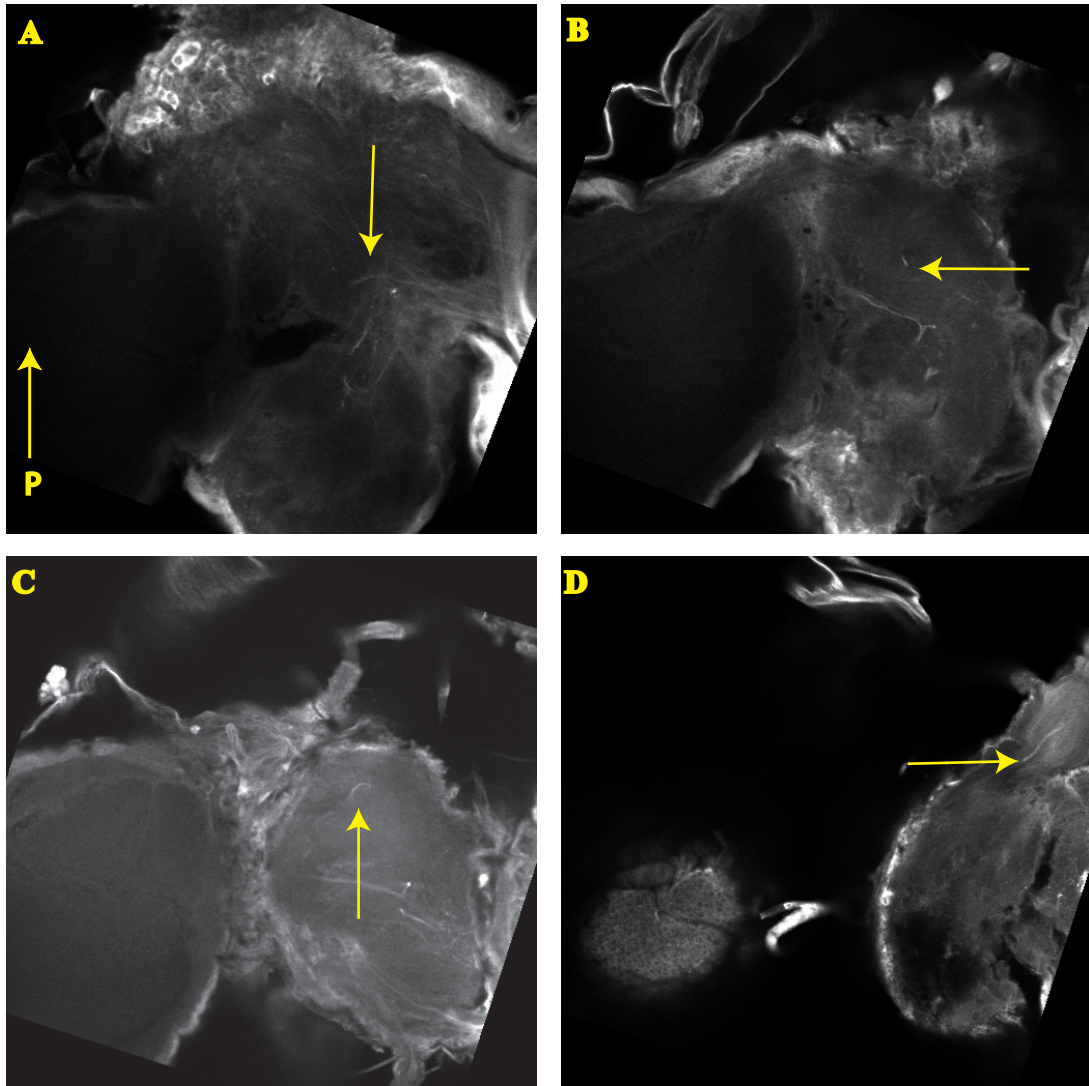
Figure 9 A-D: Confocal image showing a contralateral projection to the ventral cord from the ipsilateral protocerebrum. The projectional axon crosses the midline at the same level as the medial antennal-lobe tract, before it travels ventrally, innervating the ventral cord. P=posterior.
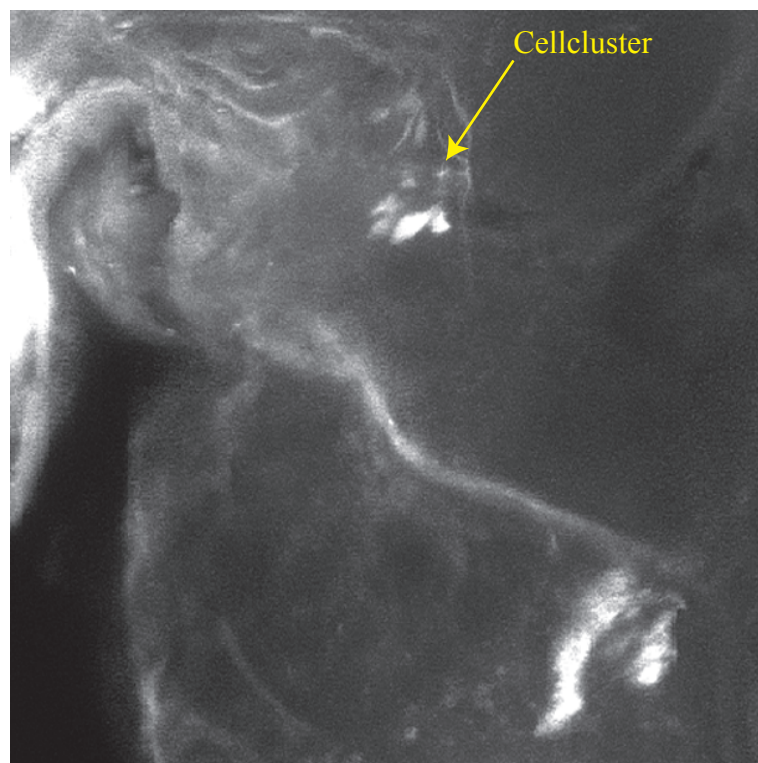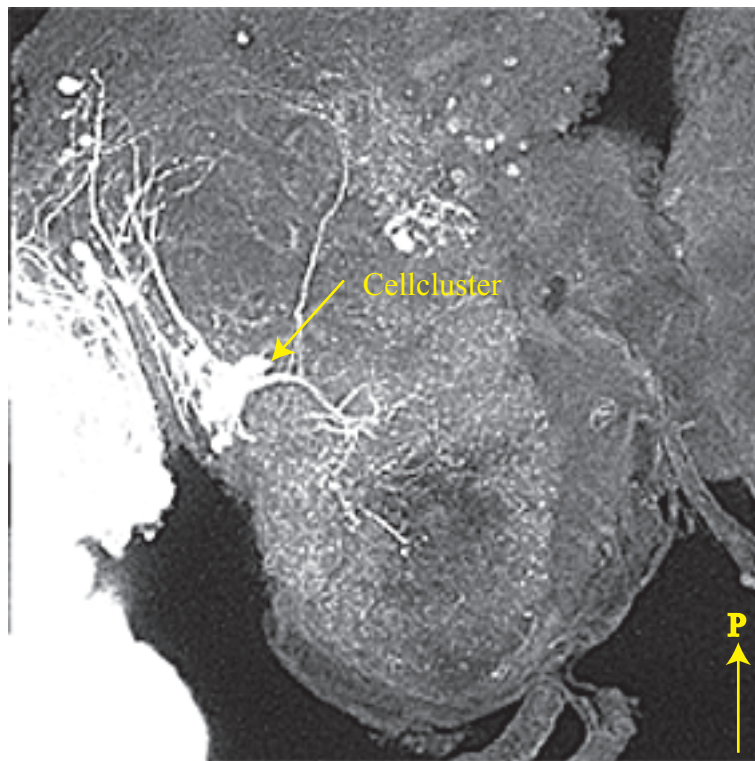
Figure 10 A-B: Confocal image showing cell clusters located outside the posterior parts of the AL.

DISCUSSION

The purpose of this study was to map contralateral connections at a particular level of the moth olfactory pathway. Thus, by applying dye into the primary olfactory center, the AL, in one brain hemisphere, and thereafter obtaining confocal images of labeled projections in the other hemisphere, contralateral connections were revealed. The results indicate that the majority of second order olfactory neurons projects ipsilaterally, which is in agreement with previous research (Homberg et al. 1988; Rø et al. 2007). However, the findings demonstrate that there are contralateral neuronal pathways as well, obviously serving a particular role in integrating and modulating olfactory information.

The subsequent text starts with a short discussion about the method. Then, the results obtained in the current study are commented on. Finally, a more general discussion concerning ipsilateral and contralateral organization of sensory systems is included. Since the thesis presented here is part of an educational pathway for clinical psychologists, I have taken the opportunity to reflect on the olfactory system of humans especially.

Methodological considerations

The current pilot study provided successfully stained preparations demonstrating projection patterns of antennal-lobe neurons both in the ipsi- and contralateral hemisphere. The mass staining technique resulted in relatively consistent findings regarding the ipsilateral projections; the three classic ALTs, the medial, the medio-lateral, and the lateral, were always visualized in successfully stained brains. Staining of contralateral projections, on the other hand, varied substantially. This fact may be due to arborization patterns of the various neuronal categories within the antennal-lobe glomeruli. Thus, if PNs projecting contralaterally are linked to particular glomeruli, for example, they may be stained occasionally. Recently, a contralateral ALT originating from selected glomeruli located posteriorly in the AL was discovered in the lab (Ida C. Kjos, master thesis, 2016). In order to collect reliable data on the entire assembly of contralataleral projections, a sufficient number of specimens therefore have to be obtained.

Staining of the ipsilateral hemisphere

The consistent staining of three parallel tracts, the medial, the mediolateral, and the lateral ALT, connecting the AL with higher integration areas in the ipsilateral protocerebrum is in

accordance with previous reports (Homberg et al. 1988; Rø et al. 2007). Furthermore, the staining of two main target regions for these tracts, the calyces and the LH, corresponds with the former findings. In general, these results demonstrate the prominence of ipsilateral connections between the AL and the higher integration regions in the moth brain.

Two particular issues deserve more detailed comments. The first is the visualization of an organized neuropil in the LH. As shown in figure 4, the axon terminals of AL PNs form a particular structure including two fused toroids in this area. Actually, the pastry-like structure was recently discovered in our lab (Ian et al. submitted) and its appearance in the study presented here is therefore in full agreement with this new report. The finding contradicts with previous publications characterizing the LH as an unorganized neuropil. The appearance of the structure seems to require a substantial amount of stained axon terminals, plus a dorsal orientation of the preparation – which may explain why it was not uncovered before.

The second interesting issue concerning the staining pattern in the ipsilateral protocerebrum includes a group of unique axon terminals in the LH; as shown in figure 5, assemblies of characteristic club-line endings in the LH are connected with PNs confined to the lALT. These PNs seem to correlate with the POc neurons in *Manuca sexta* (Homberg et al. 1988). Interestingly, an individual PN belonging to the same type was recently identified in our lab; this lateral-tract neuron projected to the antero-ventral part of the LH where it terminated in a few short branches one of which form a club-shaped ending (Ian et al. pers. comm.)

Staining of the contralateral antennal lobe

The large cell body visualized within the contralaterally located AL is connected to one of a pair of serotonin-immunoreactive (SI) neuron, which has been mapped in several moth species in previous studies (*M. sexta*: Kent et al. 1984; *Bombyx mor*i: Hill et al. 2002; *Helicoverpa assulta*: Zhao et al. 2009). Each of these neurons has one large cell body located posteriorly in the AL. From the cell body, the axon run along the mALT ipsilaterally before crossing the brain midline posteriorly of the central body and passing along the other mALT to the contralateral AL where it projects to seemingly all glomeruli. In addition, the axon innervates regions in the protocerebrum both ipsilaterally and contralaterally. The functional roles of these two serotonergic neurons are unknown, however, they show some interesting properties: studies in *H. assulta* have found that they display two spike amplitudes, one small and one large. It is hypothesized that the small spikes act locally in the AL whereas the larger spikes act globally, which may indicate that they are capable of encoding signal information

in a specific manner depending on the odours being present at different events (Zhao & Berg, 2009)

Staining in the contralateral region of the SEG

The stained cell body located laterally in the SEG (Figure 7 A,B) might correspond to one of a pair of bilateral AL neurons formerly identified in the heliothine moth (Rø et al. 2007). As shown by Rø et al. (2007), the primary neurite connected to the cell body of this neuron splits and each of the two axons projects to one AL via the antennal subesophageal tract (AST). Within each AL, the neuron innervates one glomerulus, known as G28 (Rø et al., 2007). Some of the axons visualized close to the cell body are in fact from the contralateral equivalent neuron (fig 8b). The contralateral axon projects into the protocerebrum where it becomes a part of the mALT on the contralateral side. (Rø, Müller, & Mustaparta, 2007). The other stained cell body in the SEG, which was located anteroventrally, close to the midline of the SEG, is part of a so-called unpaired neuron. Interestingly, Rø et al. (2007) identified one unpaired neuron with cell body in the SEG and bilateral branches in the two Als. This neurons also innervated the calyces, the LH, and in the medial protocerebrum. Within the ALs the neuron innervated the majority of glomeruli.

The functional roles of these neurons are unclear, however, it is hypothesized that they are involved in integrating bilateral information from the ALs (Rø, Müller, & Mustaparta, 2007).

Staining of a contralateral projection in the protocerebrum

Staining in the contralateral protocerebrum was sparse in the preparations obtained in this study. However, PNs projecting from the AL to the contralateral protocerebrum have been found in heliothine moths. Several output neurons originating from the labial pit organ glomerulus (LPOG) are for example reported to innervate the contralateral protocerebrum (Ingrid Moe Dahl, master thesis 2013). Also, as previously mentioned, a population of PNs originating from the posterior part of the AL project to the contralateral ptotocerebrum (Ida C. Kjos, master thesis, 2016). The reason why these PNs were not stained in the study presented here, is assumingly caused by the fact that they are relatively difficult to access via general mass staining experiments. The contralateral projection that was stained here seems to innervate the LH. Also, it projects at the same depth as the mALT. These two facts may suggest that it is a part of the contralateral fiber bundle mentioned above.

Staining within the ventral cord

The stained axon passing via the contralateral part of the SEG to the ventral cord is an interesting finding. There are previous findings of such projections in moths. This kind of connection may be involved in simple odor-evoked responses, i.e. orientation and reaction to, or localization of an odor stimulus. However, the functional meaning of this type of connection needs further investigation (Iwano, et al., 2010).

Staining of cell clusters

The cells depicted in figure 10a and b are located posteriorly to the ALs and may be implemented in the visual system of the moth. Studies on locust visual system has shown that there are cell bodies of neurons that make up the anterior optic tubercle residing on the anterior dorsal surface of the AL. Such cell may have been accidentally stained if the needle penetrated too far upon staining (Homberg, Hofer, Pfeiffer, & Gebhardt, 2003).

Olfaction; an ipsilaterally organized sensory system

Even though there is some evidence of contralateral projections from the ALs to higher processing areas in the moth brain, the olfactory system is predominantly an ipsilaterally biased system. This is contrary to other sensory systems, as i.e. the visual system, where information is processed in both the ipsi- and contralateral hemisphere of the brain. Which advantages does a contralateral projectional set up provide compared to an ipsilaterally system?

      The olfactory sensory system is spatially agnostic in the sense that the moth cannot automatically determine in which direction the stimulus originated from[1], whilst this is not true for other sensory systems. This is because it allows delay line cross correlation which is only made possible if two different sound perceiving organs are located some distance away from each other. Studies have found that moths do have this ability (Yager, 1999) (Michelsen, 1998). This phenomenon is also described in higher order animals like mammals. The ears are at a different distance from the source of the sound, and the central nervous system makes use if this delay from one ear to another to determine which direction the sound originated. Thus contralateral projections allow other senses properties olfaction does not have. In example, determining the source of a sound (echolocation) is made possible because of the fusion of the stimuli from both ears via the thalamus.

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[1] This is possible only if the moth moves along an "odor track", as for example a pheromone plume.

Another example of this is the human visual system. Here, the visual signals originating from the two eyes is perceived as one object and allow for in-depth vision and the phenomenology of having a continuous field of vision. So a contralateral projectional setup clearly has its advantages. The moth is very dependent on its sense of smell in most interactions, from finding food to finding a mate. However, joined by most species on planet earth, it also has an ipsilaterally biased olfactory system. This leads to an intriguing question of why this sensory system is the only one that projects primarily on the ipsilateral side, and why this is recurrent in almost all species. Could it be a connection between this sensory system being the most primitive sensory system and this unique observation?

If one imagines the most simple single cell organisms that evolved millions of years ago, an adaptation to respond to neighboring molecules would not only be a necessity, but a precursor. Chemosensory systems existed before life as we think about it even existed. In this regard, the word "sensory" is misguiding. Sensory, as in "to sense" necessitates an entity that observes or reacts to a given stimulus. By language and definition, we are already misguided. If life as we know it, began on earth by long chains of molecules merging in the evolutionary soup, chemoreceptors would not be an adaptation but rather a consequence of molecules attaching to one another. The end of a long molecule chain acts as a receptor for another molecule that matches a certain set of characteristics. Single cell organisms that developed an enclosed phospholipid layer would be cut off from the world around them, losing the ability to react to changes in a dynamic environment. This would not be an adaptive feature and any such organism would surely die within a short period of time. On the contrary, an organism with receptor proteins pointing out in to the environment could always have a reaction to the changes, and some of these reactions would in the long run increase the fitness for some. As these simple organisms advanced further, integrating more and more molecules, and finally becoming multicellular organisms, new sensory systems adaptive to survival on this planet would emerge. However, they would be a product of chemosensory systems. Actually, chemo sensation is the arrangement upon which all other systems are built.. Over a long time, environmental pressures would favor organisms with receptor molecules that allowed the organism to take advantage of the information molecules in the surrounding environment expresses.

Even though we like to think that olfaction has lost its importance among humans, its anatomical nature tells another story. First of all, it's the only sensory where the sensory cells have a direct connection to the brain. Second, it is the only sensory system where stimuli information is processed primarily ipsilaterally. A third observation is its close connections to

brainstem reflexes and the limbic system (especially the amygdala). On the basis of these observations, it seems as though olfaction is central in quick reactions and decision making in situations where the mammal's life may be in danger. It might be easily overlooked and underrated in a world full of other stimuli.

CONCLUSION

The data presented here demonstrate that contralateral projections at the second order level of the olfactory pathway in moths do exist, but that they are few in number compared to ipsilateral connections. The results show that the method of mass-staining the AL is suitable for mapping contralateral connections at this level of the olfactory system. However, in order to conclude about more detailed projection patterns in the contralateral hemisphere, further experiments have to be implemented. The contralateral connections included neural processes in the AL, the protocerebrum, the SEG, and the ventral cord. Some of these connections have been described in previous studies, like the centrifugal IS neuron, whereas others, like the axon projecting to the ventral cord, is not previously reported. The staining in the ipsilateral hemisphere included two interesting findings, previously rarely reported: 1) an organized neuropil in the LH and 2) a distinct population of lateral-tract PNs forming unique terminal endings in the LH. The fact that the second order level of the olfactory pathway is predominantly ipsilaterally organized in moths as well as in mammals, humans included, demonstrates the conservation of basic neural principles residing within this sensory system during evolutionary time. Regarding the question of why olfaction is predominantly processed ipsilaterally. It could be related to its phylogenetically primitive nature, being a sensory system that developed before organisms even had a central nervous system and two bilateral symmetric sides of a body. The evidence of some contralaterally projectional neurons that is implemented in the olfactory system would in that case be developed later in evolutionary history, and thus being related to higher processing systems in the moth brain, i.e. memory processes, muscle reflexes or integration into other sensory systems.

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