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Reconstruction and Registration of three Bilateral Protocerebral Neurons in the Standard Brain Atlas of the Moth *Heliothis virescens*

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Helene Engeness Mørk

SAMMENDRAG

Adferd hos insekter er tett knyttet sammen med deres evne til å detektere viktig sensorisk informasjon fra omgivelsene. Tobakkmøllen *Heliothis virescens* benytter seg av luftbåret stimuli for å lokalisere en partner, mat og egnede vertsplanter for egglegging. I tillegg kan de detektere lyder som tillater dem å unnslippe nattlige predatorer som flaggermus. Å forstå de underliggende biologiske mekanismene for de adferdsmessige valgene insektene tar, kan bidra til utvikling av harmløs biologisk kontroll av nettopp dette skadedyret. I denne oppgaven har tre bilaterale protocerbrale nevron fra møllen *H.virescens* blitt valgt ut på grunnlag av elektrofysiologisk respons og farging. Disse nevronene ble rekonstruert med manuell merking i programmet Slicer. Videre ble segmenteringene registrert i standardhjernen med landmerkeregistrering i Amira. Nevron N1 responderte på en odorant (linalool) og nevron N2 responderte på lyden av en klirrende nøkkelring. Begge disse nevronene ble foreslått å bidra til resiprok inhibering da de projiserte i korresponderende områder i den kontralaterale hjernehalvdelen. Input-områdene til N1 lå i den høyre og output i den venstre LAL, mens N2 gikk ned fra VLP via SOG og inn til thoracalgangliet. Kontrastforsterking mellom de to sidene kan være nyttig for å lokalisere lyd eller lukt i rommet. Nevron N3 innerverte et glomerulus i hver hemisfære før det forgrenet seg inn i ventrale protocerebrum, den høyre øyeloben, og et område rett nedenfor CB. Dette nevronet responderte til ny stimulering med lys og lyd, og konsistent til stimulering med en odorant. Denne typen deteksjon av ny informasjon kan være viktig for å fokusere oppmerksomheten og å detektere viktige biologisk relevante stimuli fra bakgrunnstøy i omgivelsene.

ABSTRACT

Insect behaviour is tightly connected to their ability to detect important sensory information from their environment. The tobacco moth *Heliothis virescens* moth follows airborne stimuli to acquire partners, food and suitable host plants for oviposition, and it is able to detect sounds allowing them to escape predation by bats. Understanding the underlying biological mechanisms behind its behavioural choices may be of importance in future methods for harmless biological control. In this present study, neurons of three bilateral protocerebral neurons in the moth *H. virescens* were chosen based on their electrophysiological responses and successful staining. The neurons were reconstructed using the manual labelling software Slicer, and registered into the standard brain atlas (SBA) using landmark registering in Amira. The neuron N1 which responded to an odorant (linalool) and the neuron N2 which responded to a sound stimulus (keychain) were both suggested to participate in reciprocal inhibition. This was due to their projection in corresponding areas in the contralateral hemisphere. N1 had its input in the left and output in the right LAL, and N2 descended from the VLP through the SOG towards the thoracic ganglion. Contrast enhancement between the sides may be important for localising a sound or odor in space. The neuron N3 innervated one glomerulus in each hemisphere before branching into the ventral protocerebrum, the right eye lobe and an area below the central body. This neuron was found to respond consistently to the major pheromone component and to novel stimuli of a sound and a light stimulus. This novelty detection may be important for focusing attention, and detecting important biologically relevant stimuli from the background noise in the environment.

TABLE OF CONTENTS

ABBREVATIONS

AL – Antennal lobe

APT – Antenno-protocerebral tract

CB – Central body

GRN – Gustatory receptor neuron

LAL – Lateral accessory lobe

LALco – Lateral accessory lobe commisure

LOPG – Labial Organ Pit Glomerulus

LP – Lateral protocerebrum

MB – Mushroom Bodies

ORN – Olfactory receptor neuron

RN – Receptor neuron

SBA – Standard Brain Atlas

SOG – Suboesophageal ganglion

SP – Superior protocerebrum

VP – Ventral protocerebrum

VLP – Ventro-lateral protocerebrum

1. INTRODUCTION

1.1 The Moth and its behaviour

Insects are interesting model organisms in neuroscience as their simpler nervous systems facilitate studying connections between behaviour and the underlying neurophysiology and molecular mechanisms. Several insect species are used as models such as moths, honey bees, fruit flies and others. In our lab we have particularly studied the sensory system of the moth *Heliothis virescenes.* This heliothine moth belongs to the most important pest species on plants of tobacco, cotton, tomato, corn, soy beans and grain (Fitt, 1989). Research regarding both its behaviour and sensory perception is of importance in respect to future methods for biological control.

H. virescens is a nocturnal moth, with a rather short adult life span. In two to three weeks they have to eat, avoid predators, locate a suitable partner and mate. The females additionally need to locate a suitable host plant for laying eggs (oviposition). Selection of host plants is very important for the development of the offspring. The larva hatch on this plant, and seldom move beyond their given host. Because of this immobility, it is crucial for their development to hatch on a healthy and nutritious plant (Chew and Robbins, 1984). The moth uses several steps in selecting a host plant, which involves several senses. It searches and locates a possible plant for nectar feeding or oviposition, and then accept or refuse it (Renwick and Chew, 1994). In Lepidoptera in general, visual cues such as shape and colour are thought to be important, but less so in the nocturnal species of moths. Attractive compounds, host plant attractants, are detected in the air and guide the moths towards possible plants (Tabashnik, 1985). Similarly they localize their partners by following airborne compounds. Like many other insects, they have developed species-specific compounds, pheromones, which the female moths release into the air. By detecting this sexual pheromone in the air, the male moth flies against the wind following the pheromone plume until it reaches the female. In this way, they actively locate partners through chemical signalling. While olfactory and visual cues are important in steering the moth towards the plant, taste is the main determinant of whether the plant will be accepted. It is important to distinguish between noxious and beneficial substances. While it is advantageous to recognize nutrients and high energy food sources, it is absolutely crucial for survival to detect what is toxic. Detection of CO_2 may also be important for determining on

which areas of a chosen plant to oviposit, as it enables them to compare the metabolic activity of the different tissues (Renwick and Chew, 1994).

Although the moths do not communicate by sound, they can hear particular sounds. This is connected to protection from predators. They have a specific sound detection system to avoid bats which hunt using echo localization. These sounds have very high frequencies beyond the hearing of humans in the ultrasonic range between 20 and 80 kHz (Boyan and Fullard, 1986). Moths in general have two main strategies when encountering bats, depending on the distance to the bat. They are able to discover a bat at approximately 30 meters away, before the bat is able to notice them. At a far distance, their evasive strategy is to fly away from the bat in the opposite direction. If however the bat is very close, the moth will instead start flying in loops or dive to the ground in order to escape (Roeder and Treat, 1961). These behavioural responses in relation to host plants, partners and predation show the importance of the different senses; olfaction, taste, sound and vision.

1.2 Detection and processing sensory information

Insects acquire chemical information from their surroundings, both as taste (gustation) and smell (olfaction). The two senses are structurally distinguished by their location in vertebrates, with the olfactory cells restricted to the nasal cavity and the taste cells to the oral cavity. In addition, the stimuli are dissolved in air and in a solvent, respectively. However, in insects these senses are distinguished by different criteria as they may have sensory cell types on the same appendages, such as the antennae of *H. virescens*. In insects, the olfactory stimuli are airborne, whereas the taste stimuli have to be contacted by the gustatory sensilla, a process called contact chemosensation.

1.2.1 Olfaction

The olfactory sensory organs, the sensilla, in most insects are on the antennae. These sensilla have a thick cuticle wall containing numerous pores through which odorants pass by diffusion. The sensilla also contain the dendrites of odorant receptor neurons (ORNs) surrounded by receptor lymph (Keil and Steinbrecht, 1987). The odorants are transported to the dendritic membrane by odorant-binding proteins, where they bind to the membrane receptor proteins (Krieger and Breer, 1999). In general, each sensory cell expresses one type of receptor proteins, as well as a co-protein called olfactory receptor co-protein (Orco) (Vosshall, 2001). After reaching the receptor protein, the odorant

activates the sensory cell. Two principal methods for this transduction has been suggested, one involving an intracellular cascade, and the other an ionotropic channel (Sato et.al, 2008). Upon activation, the cell membrane depolarizes firing action potentials that are conducted via the axon into the antennal lobe, the primary olfaction centre of the moth brain.

In the antennal lobe, the primary axon projects into specific structures called glomeruli, and each glomeruli receive information from receptor neurones expressing strictly the same receptor proteins (Vosshall et al., 2000). There are in principle two glomerular structures, the macroglomerular complex (MGC) involved in pheromone information and the ordinary glomeruli involved in plant odor information. Two ventral glomeruli (one in each lobe) are slightly larger, and mediate information about CO_2. In moths, CO_2 is detected in the labial palps. The Labial Pit Organ contains a special type of sensilla with only one receptor neuron. Their axons form the Labial Palp Nerve projecting in the antennal lobe, where they terminate in the Labial Pit Organ- Glomerulus, LPOG (Guerenstein et al., 2004).

In the antennal lobe glomeruli, the primary axons form synapses with both local interneurones and projection neurones (Tanaka et al., 2004). The local interneurones innervate most of the glomeruli. The projection neurones are either uniglomerular innervating one glomerulus, or multiglomerular innervating several glomeruli (Homberg et al., 1988; Rø et al., 2007). Information is mediated from the antennal lobes by the projection neurones to higher order processing areas, like the mushroom bodies, and the lateral protocerebrum. Projection neurones relay the information through three separate tracts, the inner (medial), middle (medio-lateral) and the outer (lateral) antennocerabral tracts (Løfaldli et al, 2010; Galzia and Rössler, 2010).

The axons following the medial tract give off branches to the Calyces and then extend to the lateral protocerebrum. The Calyces are the input areas of the Mushroom bodies, which are particularly important for learning and memory, while the lateral protocerebrum is considered to be a pre-motoric area (Menzel, 2001). The axons of the lateral tract innervate the same areas, but in the opposite order. The medio-lateral tract however, first projects to the lateral protocerebrum before continuing to an area in the superior protocerebrum dorsally of the MB lobes (Galzia and Rössler, 2010, Rø et al. 2007). Other important areas of the protocerebrum include the lateral accessory lobes (LAL) and the Central Complex that contains the protocerebral bridge and the central body

(Kanzaki et al., 1991).

1.2.2 Taste

Tastants in the moth are detected by gustatory receptor neurons (GRNs) in contact chemosensilla located in different appendages, such as the wings, ovipositor, antennae, proboscis, and tarsi. The axons of GRNs placed on the proboscis and antennae project in the SOG, those on the tarsi to the thoracic ganglion and those of the ovipositor to the abdominal ganglion. The sensilla have a simple pore on the tip of the hair, through which the tastants reach the dendrite membrane of the GRNs. In *H.virescens,* GRNs were found to respond to six taste qualities and also mechanosensory stimuli. The taste qualities are sugars, amino acids, salt, water, bitterness and alcohols (Jørgensen et al., 2007). Bitter and sugar receptors are never localized together, as shown by electrophysiological and molecular biological studies (Wang et.al, 2004; Jørgensen et al. 2007).

1.2.3 Sound

The moths detect sound by RNs located in simple ears placed in each side of their thorax. The sound information is then conveyed to the thoracic ganglion, to the suboesophageal ganglion (SOG) and an area ventro-laterally in protocerebrum. The ear contains only two auditory receptor neurons, commonly referred to as A1 and A2. They are attached to the tympanic membrane, covering an air chamber. When the membrane vibrates due to sound waves, the receptor neurones will be slightly stretched, causing an action potential (Surlykke, 1984). In addition a mechanosensory neuron is present in each ear.

These receptor neurones relay information to interneurones that modulate the activity within the thoracic ganglia, and influence the wing beating and behaviour of the moths. Information is also relayed to SOG. There is a difference in the sensitivity of the two cells, A1 being more sensitive than A2 that which requires a relative loud ultrasound. In addition, A1 fires more frequently to pulses of sounds that the bats use, than to uninterrupted sounds (Roeder and Treat, 1961). This gives the moth an indication of how far it is from the bat, as the firing frequency of A1 increases proportionally to the loudness of the sound. To localize the bat in space, the moth uses the delay between sound information from the different sides of its body.

1.3 Intracellular recordings of Interneurons and the Standard Brain Atlas

The research in our lab, *Gruppe for Nevrofag* at NTNU, has focused on physiological and morphological characterisation of central neurons which forms networks involved in the sensory systems in the tobacco moth *H. virescenes.* The research is based on intracellular studies and staining of neurons within the moth brain. To be able to compare different reconstructed neurones created from confocal scans of different individuals in a common framework, the Standard Brain Atlas (SBA) was developed (Kvello et al. 2009) including a map of the antennal lobe glomeruli (Løfaldli et al. 2010). Averaged brain atlases have been created for several species, including *drosophila*, the honey bee and our model species, *H. virescens*. By displaying several different neurons in the same brain, possible networks of neurons can be studied. The atlas consists of the main neuropile of the brain, and some chosen structures that often are easily identifiable in the preparations, like the antennal lobes, the mushroom body calyces and the central complex. In this thesis, intracellularly stained neurons in the *H.virescens* brain were reconstructed in 3D and integrated into this standard brain atlas.

1.4 Aim of the Thesis and Hypotheses

The thesis had two main aims regarding 3D-reconstruction of bilateral protocerebral neurons in the tobacco moth *Heliothis virescens*. The first aim was methodological, while the second aim was related to interpretation of the data.

1. Methodological Aims

- The aim of the thesis was first to explore a method for 3D-reconstruction using the free medical imaging software Slicer of intracellularly stained neurons.
- Secondly to compare Slicer labelling to the previously used method, the Amira hexoskeleton tool.

These two aims lead to the hypothesis that:

1. Slicer is a good method for reconstruction of neurons
2. Slicer is a faster and more accurate method than previously used Amira Hexoskeleton tool
3. The segmentation created using Slicer can be registered into the Standard brain using the Amira software.

2. Aims regarding interpretation of the data

- To select neurons based on successful staining and physiological response, reconstruct them and register the 3D models into the Standard Brain.
- Identify the morphology of the three reconstructed neurons, and interpret their function.

These two aims lead to the hypothesis that:

1. Reconstructing and registering the bilateral protocerebral neurons into the standard brain allows for interpretation of their function in relation to their surrounding areas

2. MATERIALS AND METHODS

2.1 The Insects

The experimental organism was the moth species *Heliothis virescens* (Lepidoptera, u. fam. Heliothinae). The insects were received as pupae from a culture at Syngenta, Switzerland, and upon arrival they were separated according to sex. All insects were kept in a clima chamber at 22°C. They had access to a 9.15 M sucrose solution, and when emerging as adults they were kept in separate boxes.

2.2 Staining, recordings and preparations

The datasets used for 3D reconstructions consisted of stacks of images of *H. virescens* brains with successfully stained neurones of 3 different individuals. The neurones, N1, N2 and N3 were selected after investigating several rough scans of stained brains from various experiments performed by Øyvind Høydal and Bente Jacobsen in our lab. Neuron N1 has been previously described in the master thesis of Øyvind Høydal (2012) as Neuron N19, while the other two (N2 and N3) are now described for the first time.

2.2.1 Electro-physiology and preparations

These particular neurones were stained and recorded by electro-physiology routines, performed by Øyvind Høydal and Bente Jacobsen. The methods are described in closer detail in Høydal's master thesis (2012). They immobilized the insects in small tubes with dental wax (Kerr Corporation, Romulus, MI) and opened a square of the moth head cuticle. Trachea and muscles covering the brain was removed to display the brain which was kept moist in Ringer solution. The set-up can be seen illustrated in figure 1.

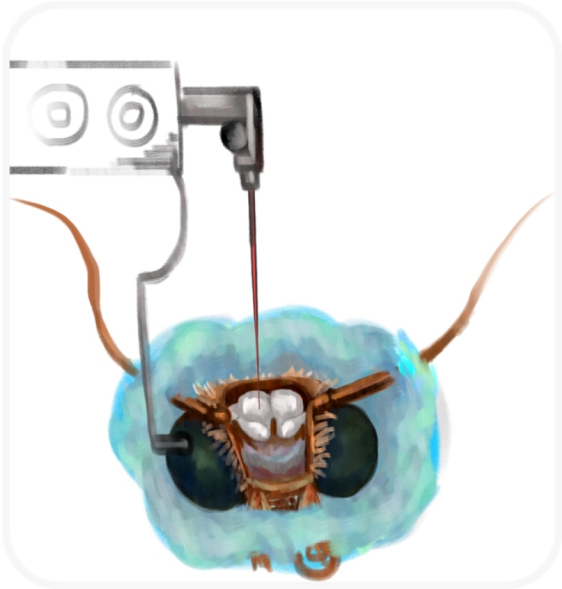

Figure 1: The set-up for electro-physiological recordings and dye injection in a moth brain.

A glass electrode containing solutions of micro-ruby and 0.2 M potassium acetate was placed into the brain. Spike activity was recorded during stimulation with sound, light, air and odorants, using a script for

Spike2. After recording, dye was released inside the same neuron using an electric current over a period of 5-15 minutes. This colouration was then left to diffuse over night. After staining, the brains were dissected and fixed over night in paraformaldehyde, rinsed in PBS and dehydrated using a series of ethanol solutions of increasing purity from 50% and up to 100%. After completion, the brains were kept and mounted in methyl salicylate.

2.2.2 Scanning with the Laser Scanning Confocal Microscope

Brains containing the different neurons were scanned several times, using a Leica confocal laser-scanning microscope (CLSM) (Leica TCS SP5; Leica Microsystems CMS GmbH, Mannheim, Germany). They were scanned with dry objectives of different strengths, including 10 and 20 (HCX PL APO CS). The laser used was a DPSS laser, which excites at a wavelength of 561 nm. As micro-ruby has a max fluorescence excitation of 550nm, this is a close match. The brain scans consisted of a stack of images in the z-direction. The photos were scanned with a resolution of 1024 x1024, and a speed of 200 and 100 Hz. The distance between slices was automatically set by the Leica software. The brain containing N1 was only roughly scanned before additional tests in the lab had degraded the material. As scanning the brain again in better resolution was not possible, the original scan was used instead. To compensate for the bigger voxel size in this dataset, it was re-sampled and re-scaled.

2.2.3 Conversion and creation of files

The datasets were initially scanned in a Leica format as .lif files, and then converted to the more common raw format using Amira. In addition to the raw-files, a separate mhd file was written for each of the neurones, containing important metadata about the dataset such as the resolution, number of images and the voxel size. These mhd-files were then loaded into Slicer, opening the datasets. The spacing was adjusted before labelling to give an approximation of the correct dimensions, but the final spacing was adjusted in the registration process.

In N1, which consisted only of a rough scan, the spacing between each slice was so big that compensating for it with voxel size alone would lead to an inaccurate and stretched model. In the rough scan, the gaps in the z direction had an inter-slice interval which was to 6 times larger than a normal scan. To fix this problem, the dataset was re-sampled in the z-direction, adding extra slices between the original slices to fill out the gap. The new slices were gradually blended from one to

the next using interpolation, which increased precision during labelling compared to only copying the slices in between.

2.3 Segmentation by Labelling using Slicer

In Slicer 4.2, a free open source program downloaded from *www.slicer.org*, a 3D segmentation was created by labelling the stained parts of the neuron in each slice of a 3D-stack. A rough labelling of the most prominent neurites were first created using a "threshold brush" which labelled everything in the marked area within a given intensity range. In the smaller and more difficult areas, a paint brush was used to label the neuron in each slice. While working, the labels were adjusted from different angles (x, y, z). Different areas of the segmentations were given different labels, allowing for parts to be turned on and off in the resulting 3D models.

Some of the most distinct anatomical features of the brain were visible in the scans in addition to the stained neurons, which appeared brighter than the surrounding brain. The thickest branches and those closest to the point of injection were brighter and easier to segment, while thinner branches farther away was more open to interpretation. The background noise in the dataset, and additional staining in other nearby neurones formed the biggest challenges. Some of the noise was reduced by adding a Gaussian blur filter as a separate layer. This allowed to easier distinguish noise from the neurons, and especially to see the different anatomical structures. Such a filter did however also remove some of the thinnest branches, and was thus only used to compare the blurred and noisy datasets with each other. Other enhancements included the light and contrast of the images in the stack.

Scanning with a confocal microscope added some additional light in the z-direction of the scan, as the laser picks up some light from the area above and below in any given slice. This "shadow of light" would cause an unedited segmentation of the datasets to appear extra thick in the z-direction. The shadow was particularly wide in the preparation of N1 due to the rough scan and re-sampling. Figure 2 shows the first rough segmentation of N1 in a frontal (A) and dorsal view (B). This "shadow of light" as clearly seen in figure 2 B was compensated for by adjusting the neuron from different angles. Assuming the cross-section of the neuron to be circular, a circular brush with the thickness of the branch was used to trace the rough segmentation in another angle, resulting in a the wanted width and thickness as seen in figure 2 C (frontal view) and D (dorsal view).

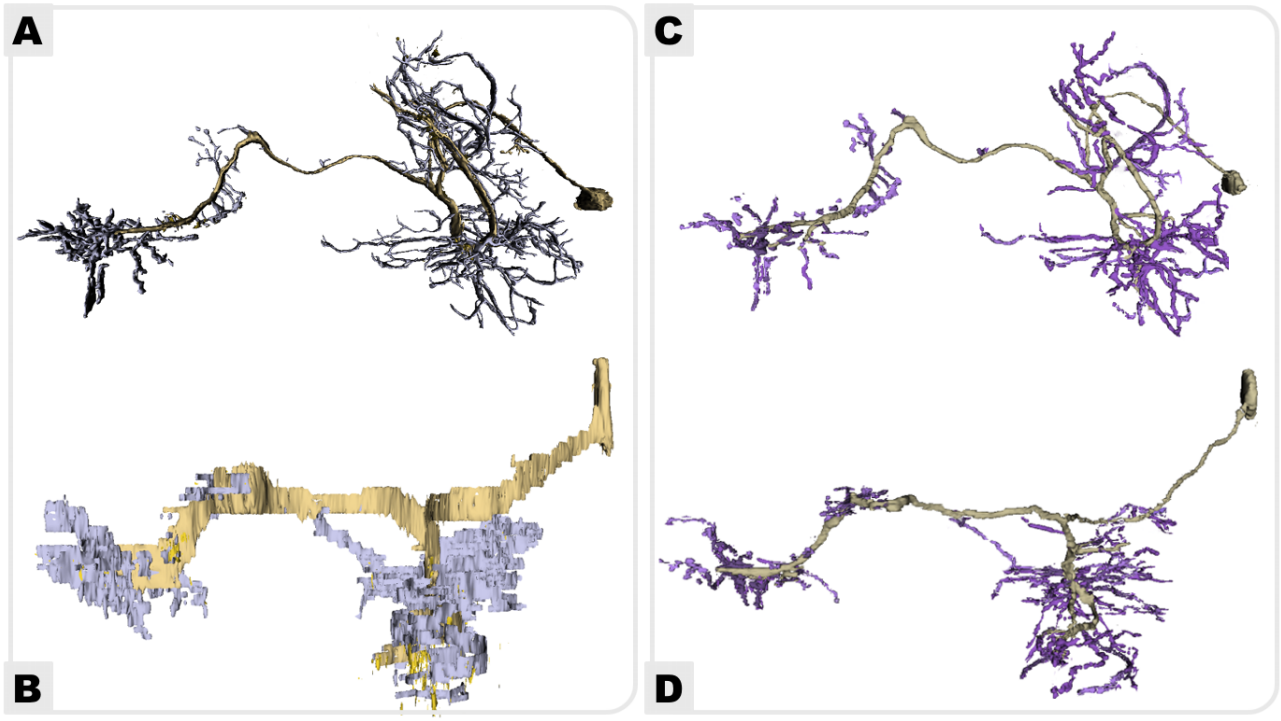

Figure 2: the direct rough segmentation of N1 with its "shadow of light" from a frontal (A) and dorsal (B) view, as well as the corrected segmentation of N1 in a frontal (C) and dorsal (D) view.

2.4 Registration into the Standard Brain

The Standard Brain was originally created in Amira in our lab (Kvello et al. 2009; Løfaldli et al. 2010), as a label-map in which different anatomical areas were given different labels. The moth brains have big individual differences and also often suffer from deformation caused by the softness of these brains leading to additional differences caused by mechanical pressure during dissection and preparation.

The registration was done in Amira, using landmark warping. At first a manual rigid transformation was performed, in which the scanned brains were moved, rotated and scaled to be roughly similar to the standard brain. When the brains were approximately the same size and rotation, they were shown in different windows where the same areas were marked with landmarks in both brains. The LPOG, central body, edges of the brain and other clearly distinguished areas were used as landmarks. The algorithm was then run, warping the labelled brain to match the form and size of the standard brain. The result was compared to the scan and labels of the brain itself to avoid too drastic changes, and the landmarks were adjusted accordingly.

2. 5 Figures

Photos of the segmented brains were created as screen-shots from Amira, while projection views were created using Image J. The elecrophysiology results obtained from the Spike software were edited in Corel Photopaint X3. All colouration, layering of images or illustrations were drawn or adjusted or edited using Corel Photopaint X3.

3. RESULTS

The results were based on 3D reconstruction of three selected neurons in *Heliothis virescens* in Slicer 4.2, followed by registration of the neurons into the standard brain atlas using Amira. The neurones were selected on the basis of successful staining and physiological responses previously obtained by Øyvind Høydal and Bente Jacobsen in our laboratory. (Høydal, 2012; unpublished data). The three neurones were bilateral protocerebral neurons and responded to a plant odorant, to unspecific novel stimuli and to sound, respectively.

3. 1 Neuron 1 (N1) responding to odor

3.1.1 Morphology of N1

One of the protocerebral neurons, N1, had its input area in the left hemisphere showing smooth arborisations spanning across the lateral accessory lobe (LAL), as well as the ventral and the medial protocerebrum. The cell body was in the left hemisphere, posterior-laterally of the arborisations and anterior-ventral to the Mushroom body Calyces. From the left hemisphere, the main neurite projected via the LAL commisure (LALco) into the right hemisphere terminating in the contralateral LAL. This area was assumed to be the output area based on the numerous blebs of the terminals. Figure 3 shows stacks of confocal images in a frontal and posterior-dorsal view as well as 3D models of the reconstructed neuron registered into the standard brain atlas.

3.1.2 Electrophysiology of N1

The neuron N1, was an odor-responding bilateral neuron, which was excited by antennal stimulation with linalool alone. However, it did not respond to a mixture containing 10 odorants, one of which was linalool as shown in figure 4.

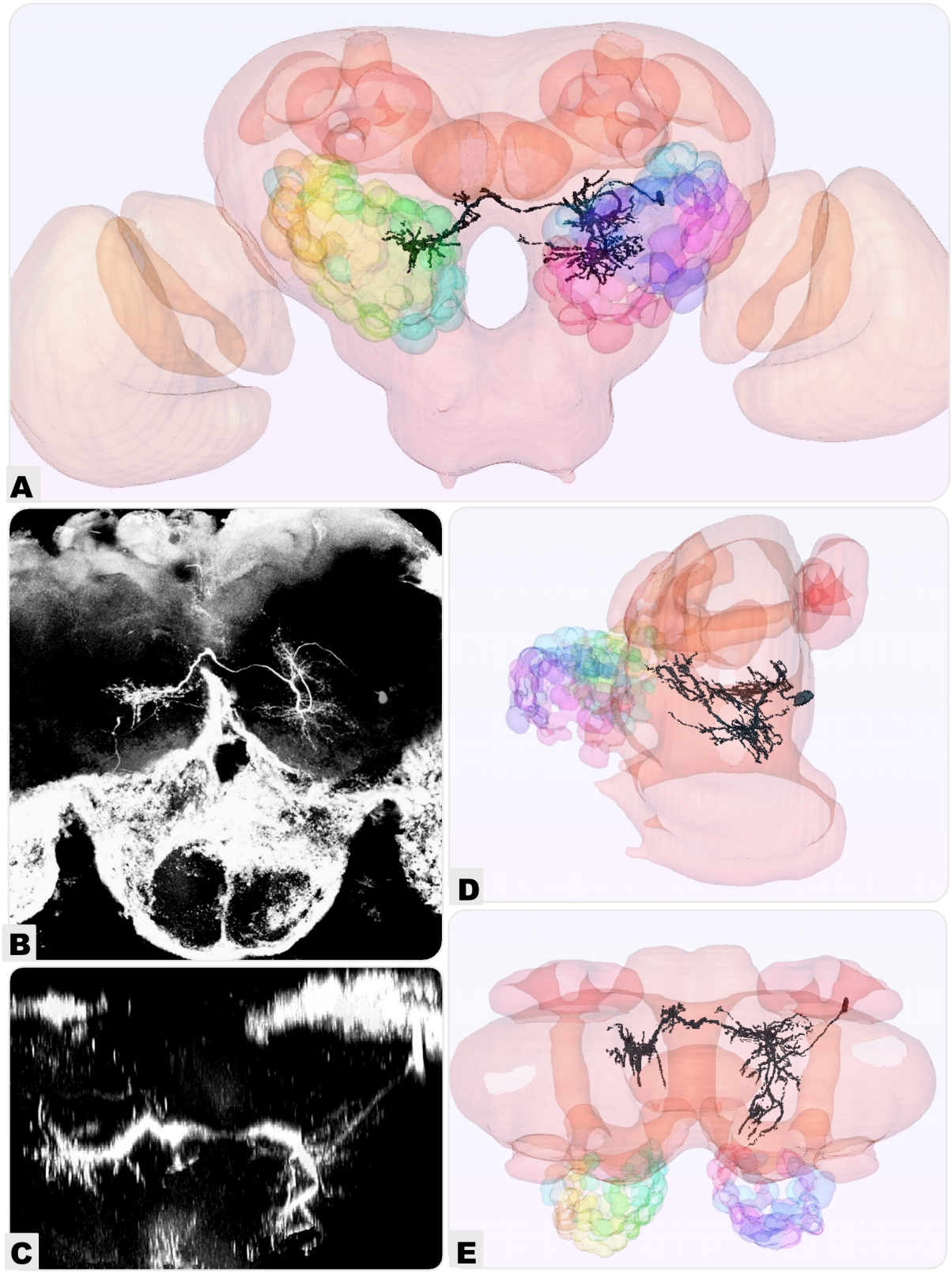

Figure 3: The neuron N1 (A) Frontal 3D-image (B) projection view of a frontal confocal scan (C) dorso-ventral 3D-view projection of a confocal scan (D) 3D-reconstruction in the standard brain from the side (E) dorso-ventral 3D-reconstruction in the standard brain

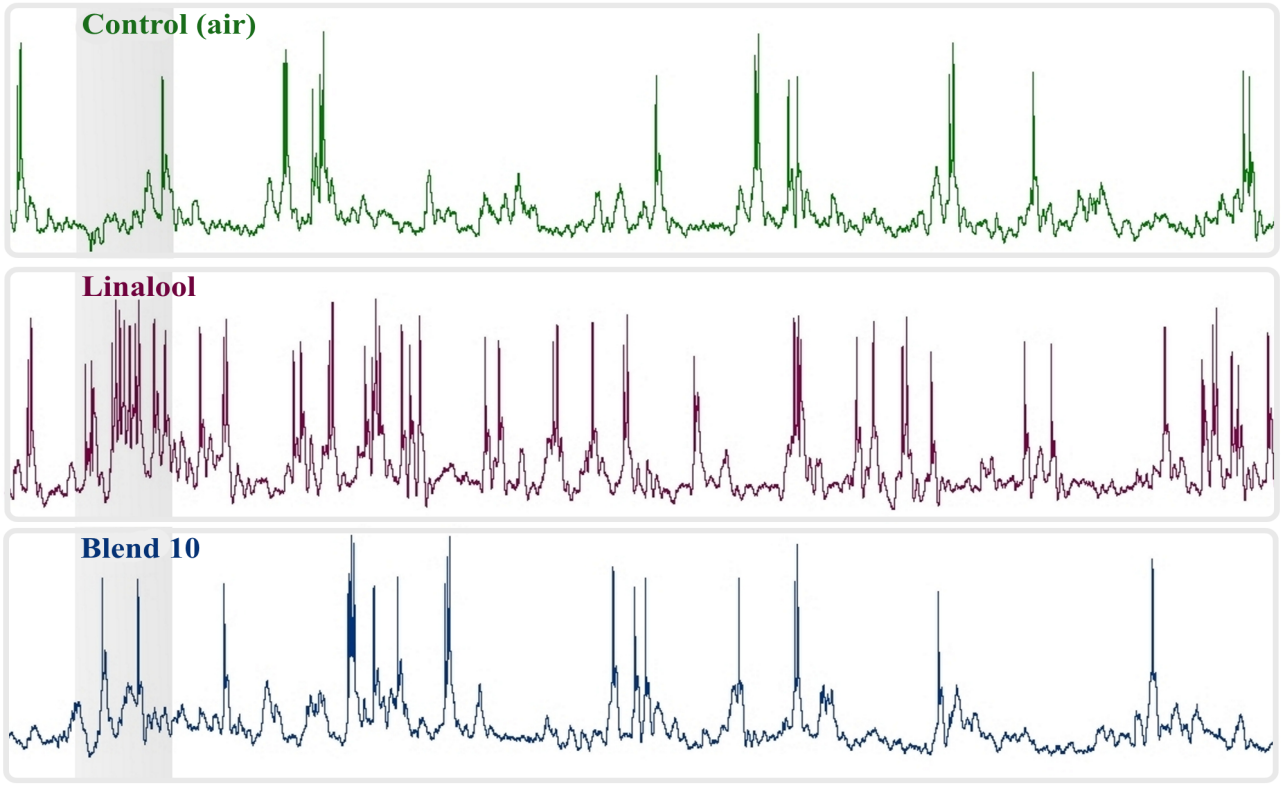

Figure 4: Recording from neuron N1 during stimulation with control (air puff), linalool, and a blend of 10 compounds, including linalool.

3.2 Neuron 2 (N2) responding to sound

3.2.1 Morphology of N2

The second bilateral protocerebral neuron has its cell body in the left hemisphere close to the antennal lobe, and the main axon running ventrally of the central body. Extensive arborisations appeared in both hemispheres. In the right hemisphere, the thick neurite ran from the protocerebrum and towards the SOG. After branching widely in the lateral protocerebrum, a neurite continued alongside the primary neurite towards the SOG, while arborising extensively all the way. A third branch extended dorsally arborising medially in the right hemisphere. All the branches in the right hemisphere contained bleb-like structures indicating output areas. In the left hemisphere, the main input area seemed to be in the ventro-posterior protocerebrum, with one branch extending towards the middle of the brain. Another branch extended in parallel to the branch with the cell body that was located anterior-dorsally of the antennal lobe. Figure 5 and 6 shows the neuron in a confocal stack (A), segmented (B) and registered into the standard brain in a frontal view (C) in a frontal and dorso-ventral view respectively.

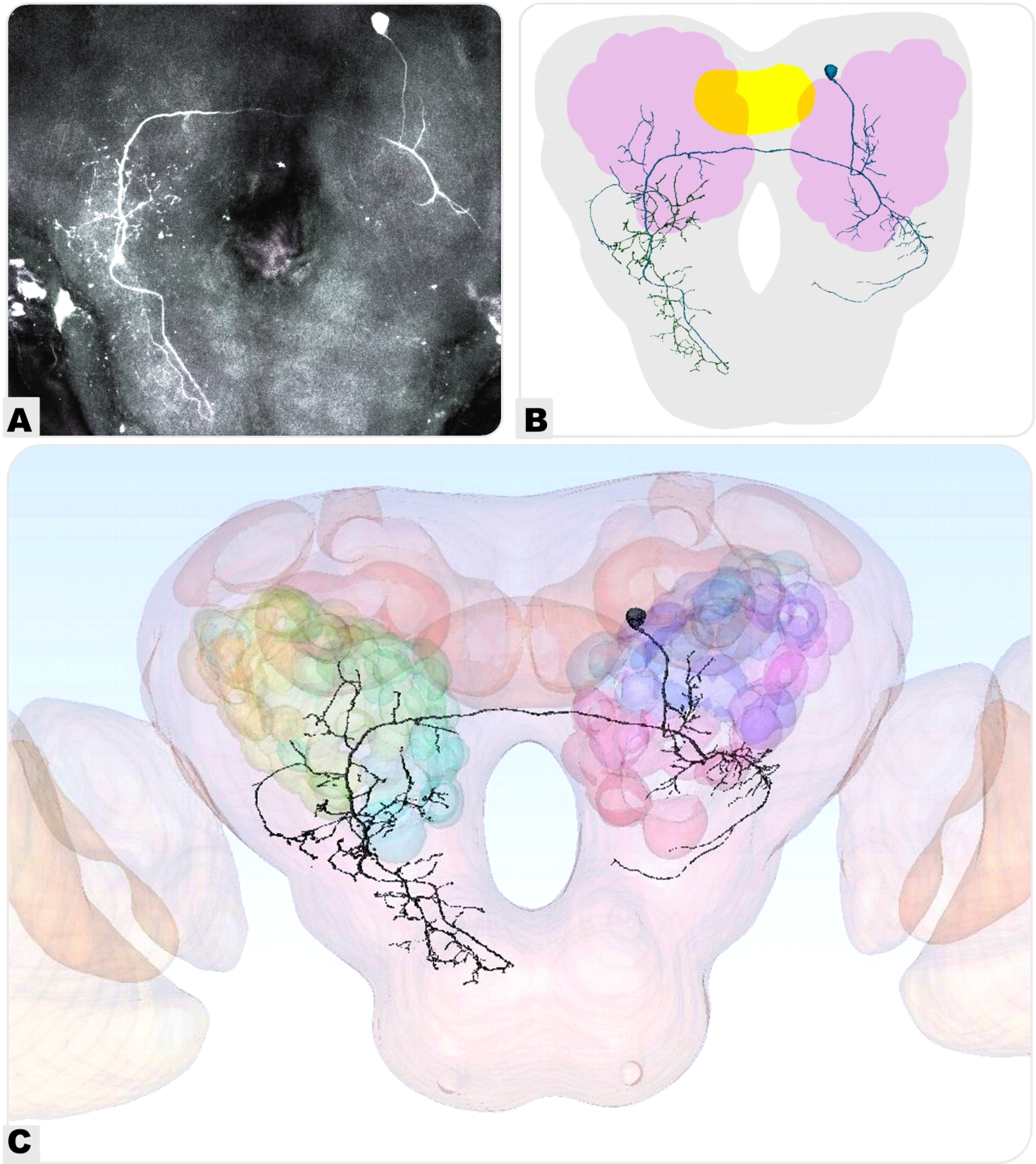

Figure 5: N2 visualised in **A:** a frontal view in a confocal image stack, **B:** reconstructed and visualised (blue, green) in an outline of the brain (grey), the antennal lobes (pink) and the central body (yellow), **C:** registered into the standard brain.

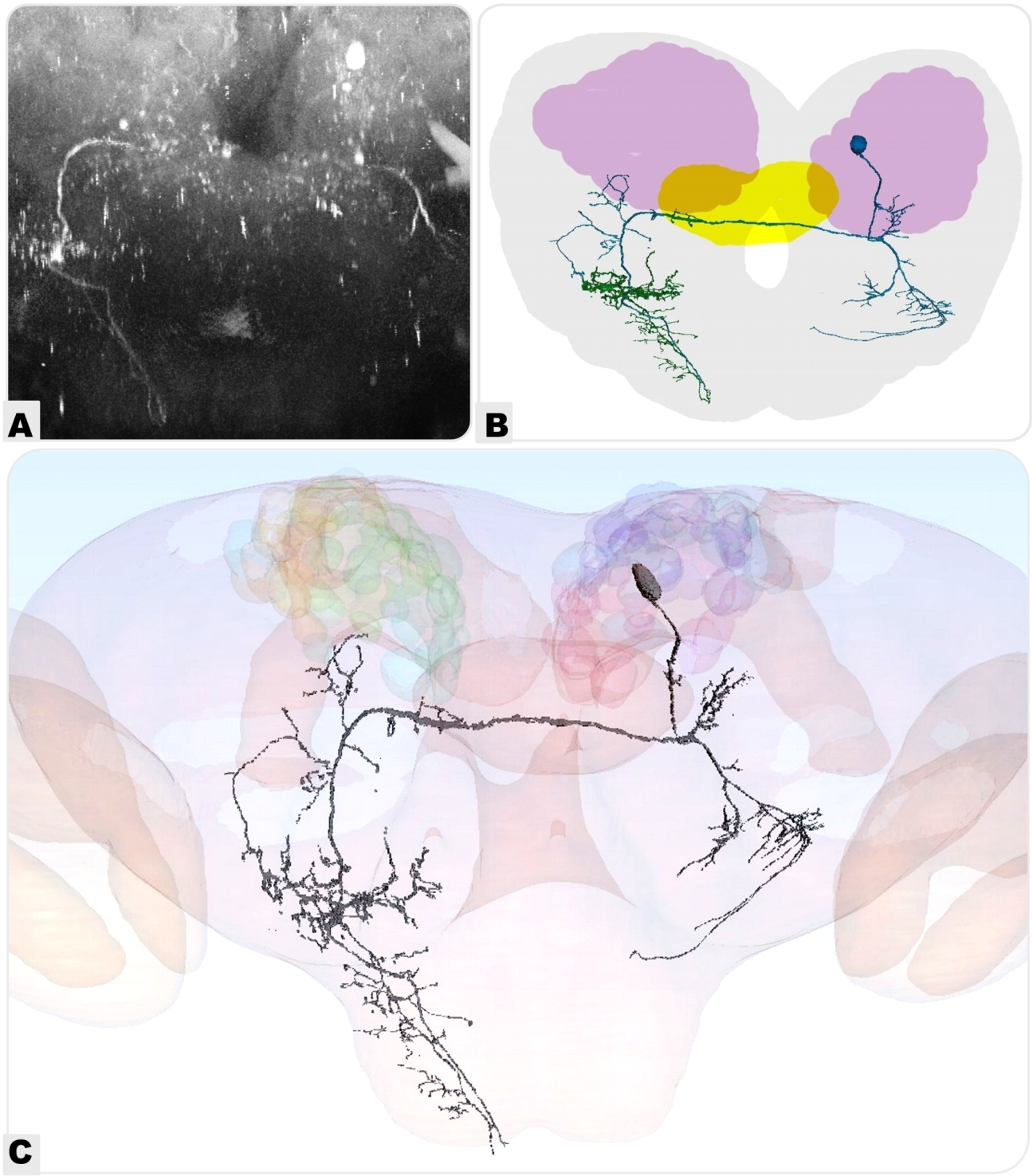

Figure 6: N2 visualised in **A:** a dorso-ventral view in a confocal image stack, **B:** reconstructed and visualised (blue, green) in an outline of the brain (grey), the antennal lobes (pink) and the central body (yellow), **C:** registered into the standard brain.

Ventrally in the SOG, there seemed to be a stained cell body, as indicated by the yellow arrow in figure 7. Although the connection of the neurite cannot be fully traced, the question is whether the branch displayed in green in the segmented figure 5B and 6B belong to this neuron. However, the staining can also be arbitrary.

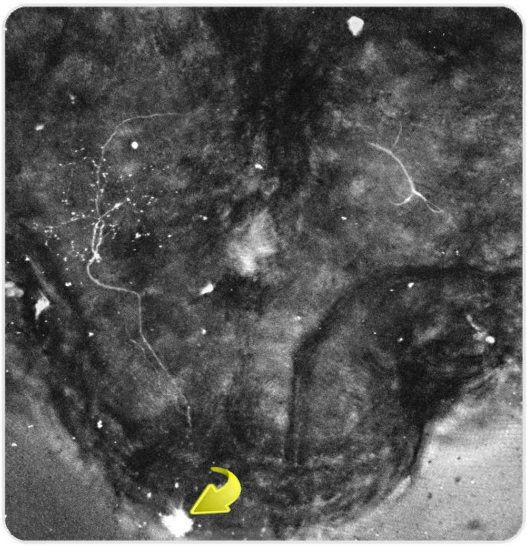

Figure 7 The arrow indicates what may be an additional stained cell body in the periphery of the brain.

3.2.2 Electrophysiology of N2

The neuron N2 responded to stimulation with sound. The neuron was silent before stimulation and fired upon the sound produced by a keychain, as shown in figure 8..

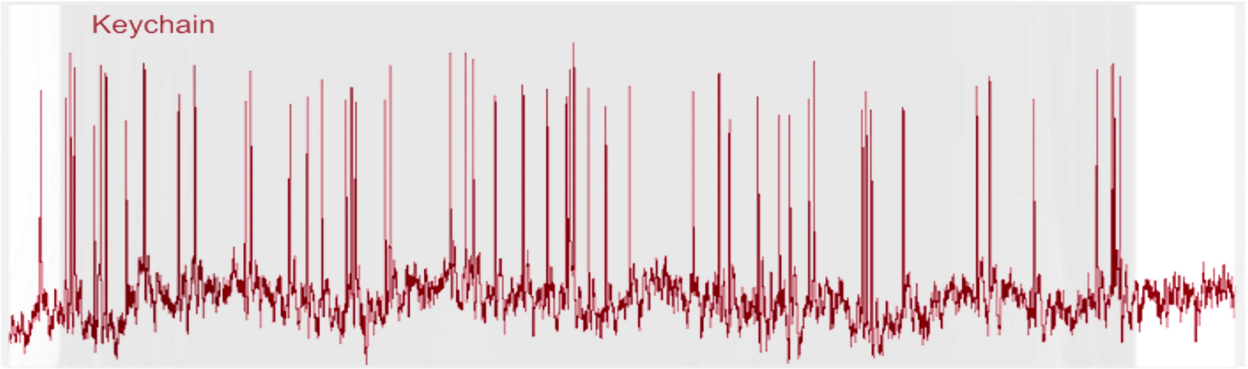

Figure 8: shows the response to the sound of a key chain.

3.3 Neuron 3 (N3) responding to novel stimuli

3.3.1 Morphology of N3

The bilateral neuron N3 is shown in figure 9 and 10 in confocal scans, segmented and registered into the standard brain. N3 had its cell body at the periphery of the right SOG and two main neurites extending to the left and right antennal lobes. Here, each of them extensively innervated one glomerulus. Initially the neuron was thought to innervate the labial pit organ glomerulus (LPOG) in both antennal lobes, but scans of higher resolution proved this not to be the case, as seen in figure 11. Instead, the glomeruli were placed posterior to the (LPOG). Exactly which one is hard to determine due to the distortion of the antennal lobes, but glomerulus 53 is a possible candidate.

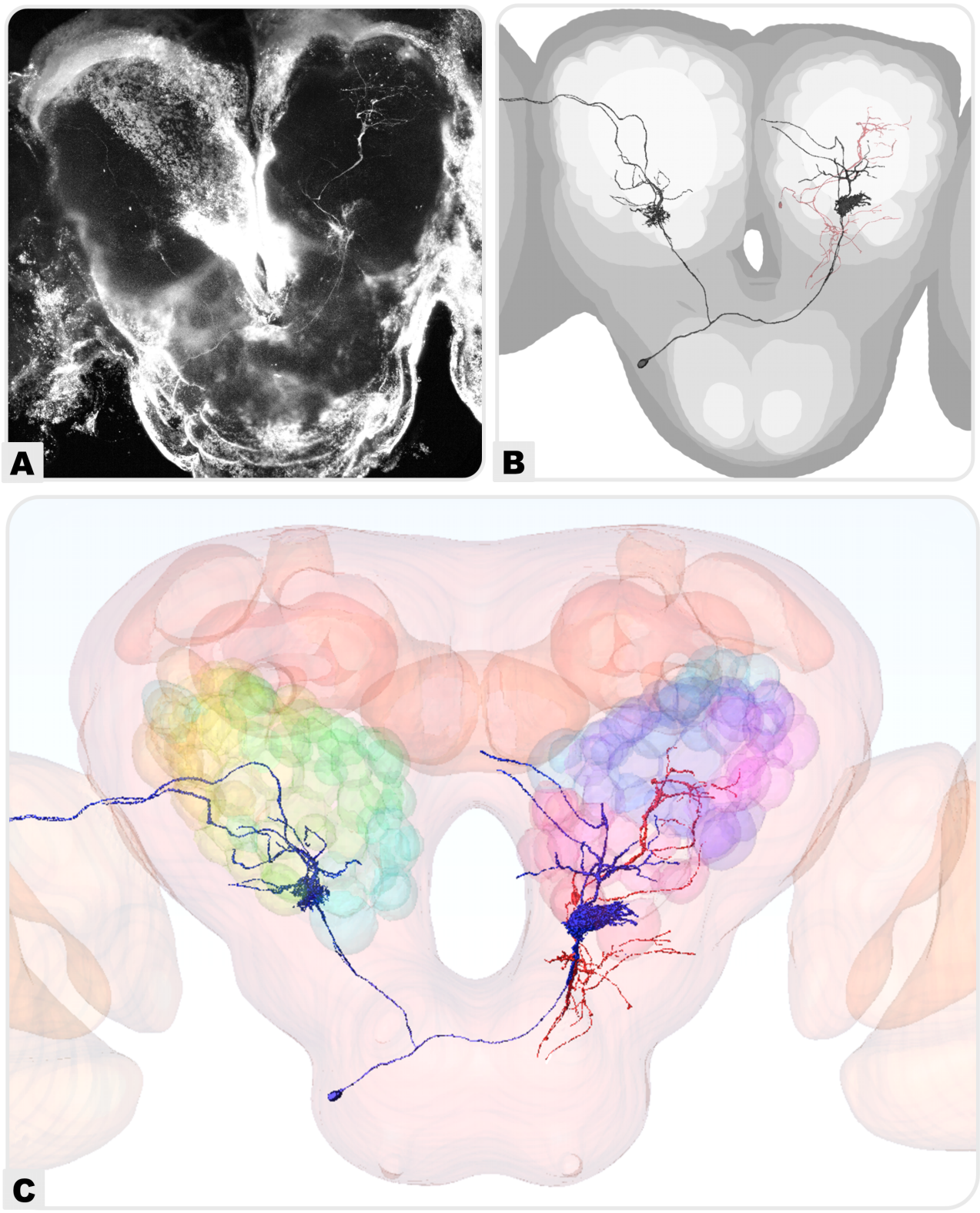

Figure 9: Neuron N3 (black, blue) and the most likely distribution of N4 (red) in a frontal view **A:** in a confocal image stack, **B:** reconstructed and visualised in an outline of the brain, **C:** registered into the standard brain.

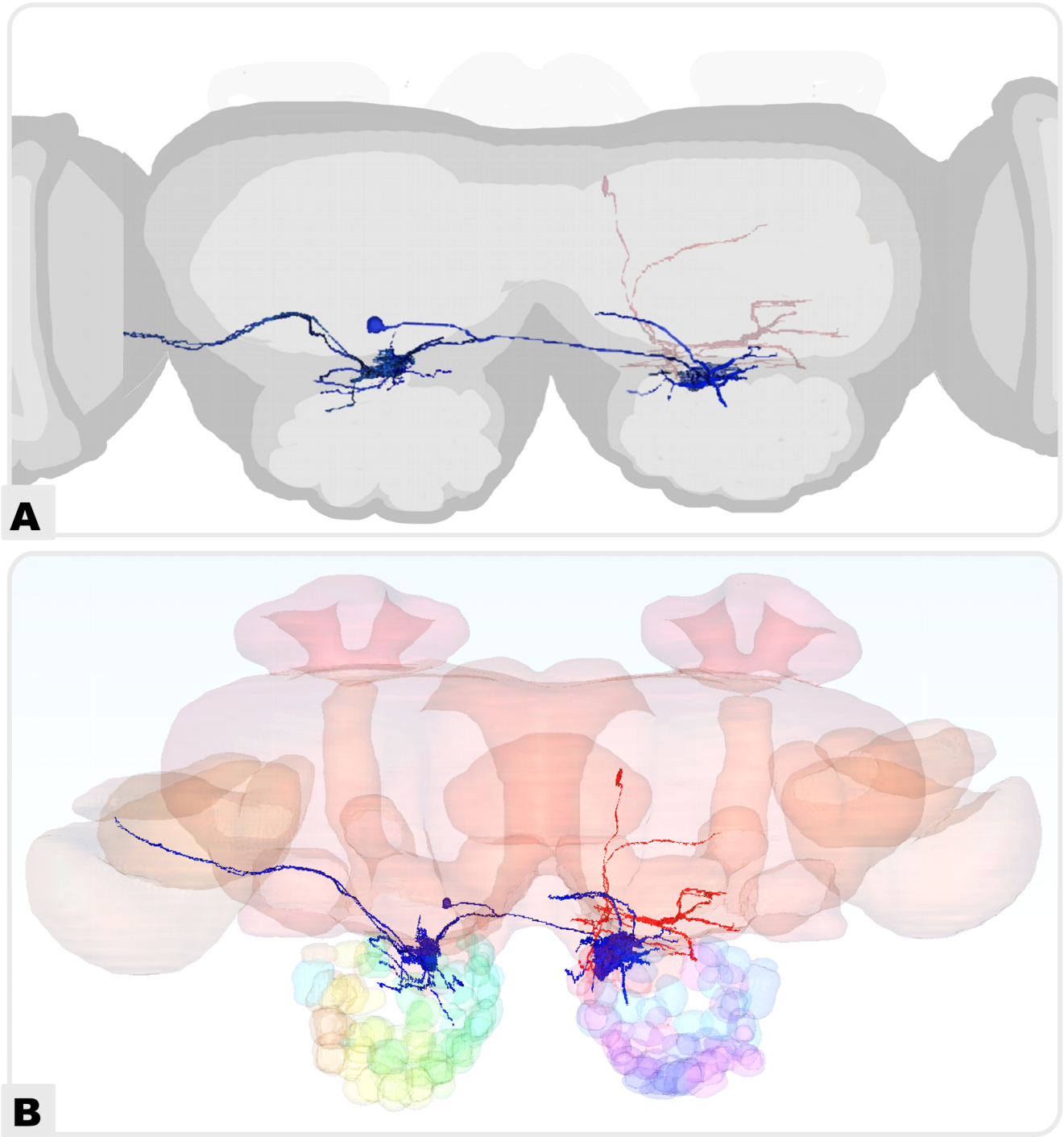

Figure 10: Neuron N3 (blue), the most likely distribution of N4 (red) in a dorsal view A: in a confocal image stack, B: reconstructed and visualised in an outline of the brain, C: registered into the standard brain.

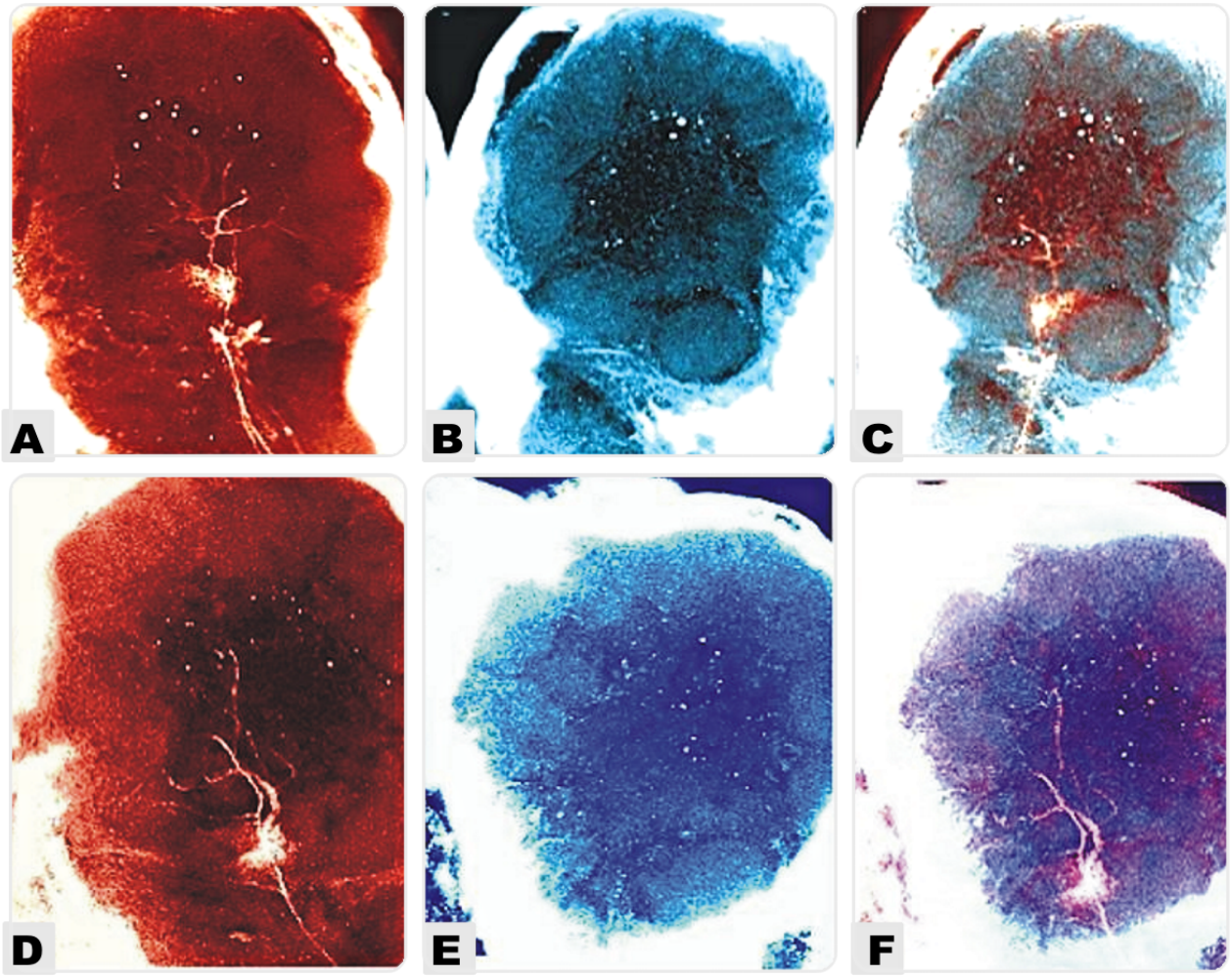

Figure 11: Two sections of stacked confocal images of the right (A, B, C) and left (D, E, F) antennal lobes at different depths. Some sections between these images were not included. The left images A, D show the neuron and its arborisation in the antennal lobe, the middle images B, E shows a section of the lobe including the distinct LPOG, and the right images C, F the two previous slices merged to visualize that the neuron location outside the LPOG.

3.3.2 The Left Hemisphere

In the preparation containing N3, two other neurones were also stained. One of these neurones, N4 was reconstructed along with N3 and is described in more details below. Both N3, N4 and additional neuron N5 is marked by the arrows in the confocal image stacks of figure 12. The borders between these three neurons were hard to determine, especially between N3/N4 and N4/N5.

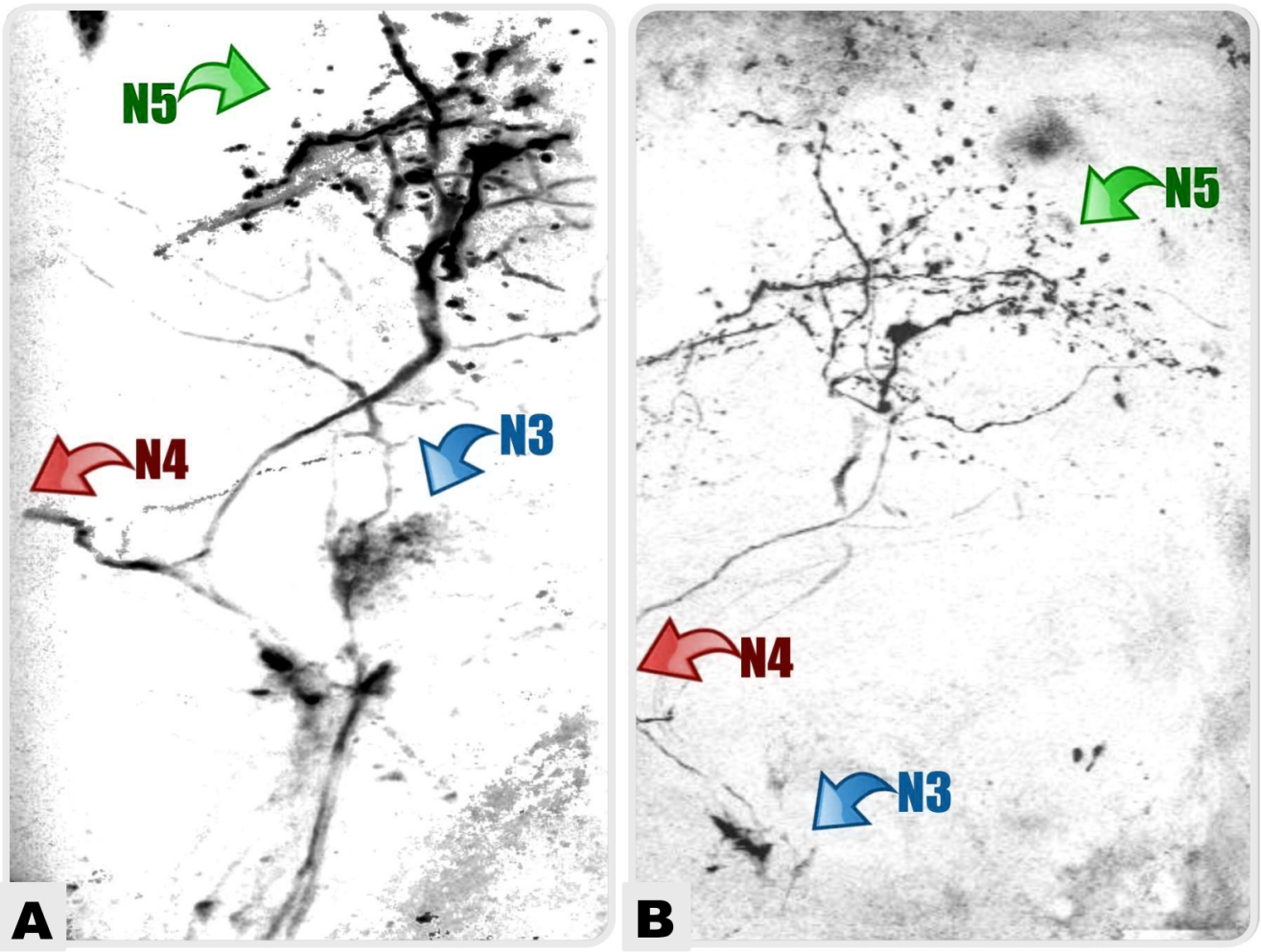

Figure 12: 3D-views of a confocal image stack containing N3, N4 and N5. The images have a slightly different angle and contrast.

In the 3D-reconstruction, both N3 and N4 were segmented as shown in figure 9. N4 (red) was entwined with the bilateral neuron N3 (blue), and in several areas they were hard to separate from each other. To explore the different possibilities for separating N3 and N4, figure 13 was created. In the first option regarding what belonged to N3 (blue) and N4 (red), only the main branch was ascribed to N3 and everything else to N4 or others. This seems to be the most likely interpretation, and is illustrated in figure 13 (A).

Another possible identification of N4 (red) was that it separated into two main neurites, both branching extensively. The first neurite extended towards N5 (not shown), and the other towards N3 (blue). One single branch from N3 would also run along the closest of the neurites from N4, before turning deeper into the brain. This is illustrated in figure 13 (B).

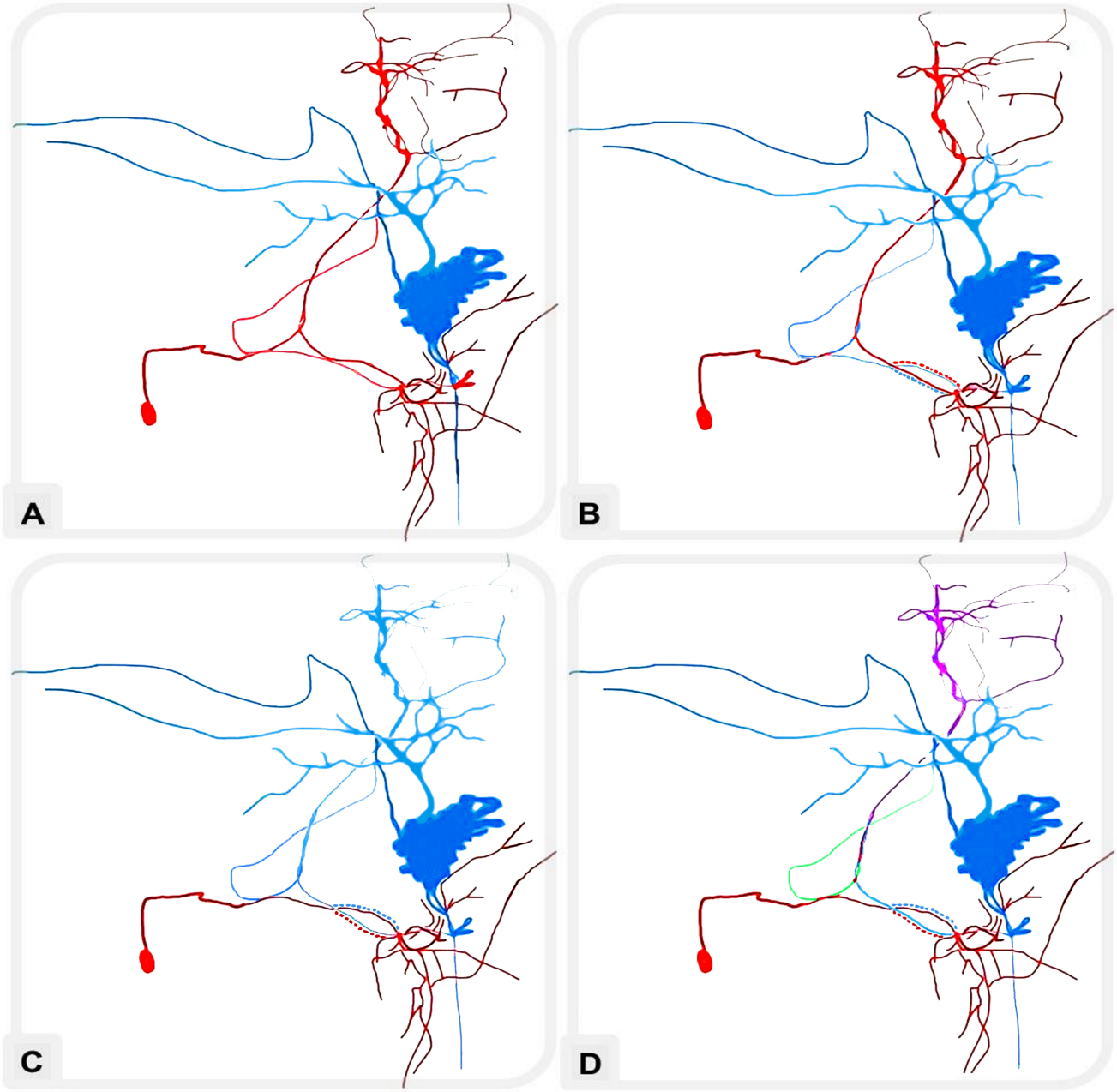

Figure 13: 4 schematic possibilities for branching patterns between N3 and N4.

A third possibility, as shown in 13 (C), is one in which the thick branch extending towards N5 belong to the bilateral neuron, N3. The blue branches would then first extend towards N5, before adding an additional branching point from which a new neurite ran deeper into the brain.

Finally, as illustrated in 13 (D) the same branch (purple) might be a combination of branches from the two neurons N3 and N4. If this is the case, the green branch would not belong to any of the two neurons, but to another weakly stained neuron. Other possible interpretations may also exist. In the cases shown in B, C and D, the dotted line indicates the two possibilities in that particular area. Either the branches may cross over each other or stay on separate sides.

3.3.3 The Right Hemisphere

In the right hemisphere, two main branches extended from the main neurite before entering the extensively innervated glomeruli. From the glomerulus, two branches further extended dorso-laterally and turned towards the eye lobe. Because of the extensive ramification in the glomerulus, it was not possible to see whether both of the main branches actually contributed to the innervation of the glomerulus or one of them just passed through. If the two branches are compared to the pattern of the left hemisphere, the same branching through the glomeruli is seen there. The images in figure 14 show a section of projections in the right hemisphere, the segmentation alone, and registered into the standard brain.

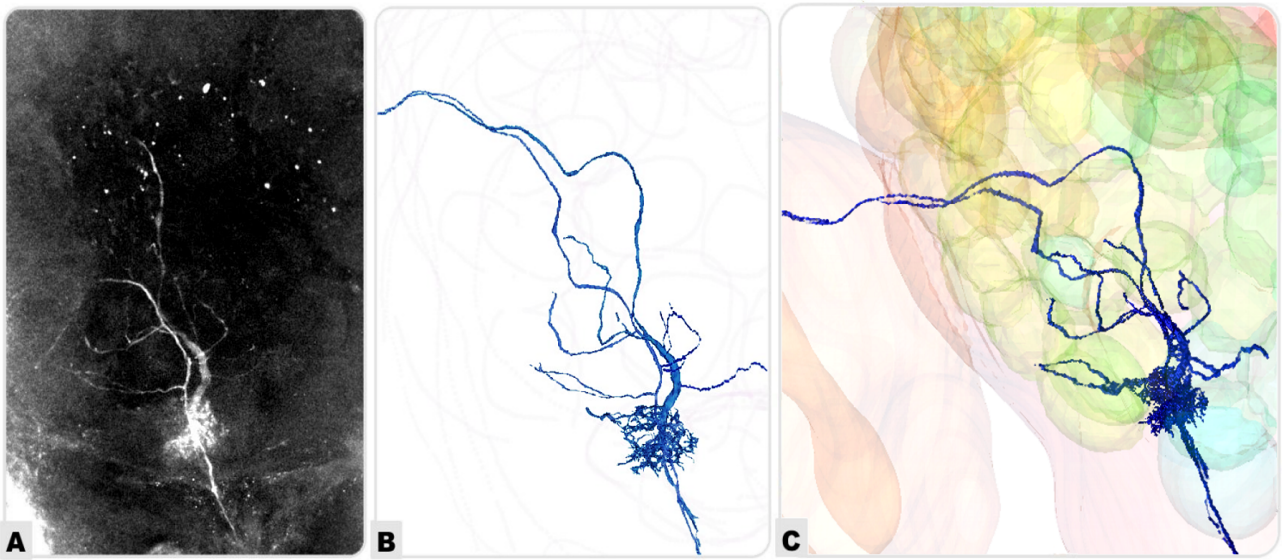

Figure 14: shows the right hemisphere as a confocal Image Stack, as a segmentation, and registered into the standard brain.

3.3.4 Electrophysiology N3

Neuron 3 responded to novel stimuli of light and sound, in which the response appeared to occur instantaneously following stimulation. In addition it responded to the major pheromone component, with a latency consistent between 120-140 ms measured over 5 trials. Figure 15 shows examples of the response of this neuron to the first stimulation with a light, an odorant, and a sound stimulus.

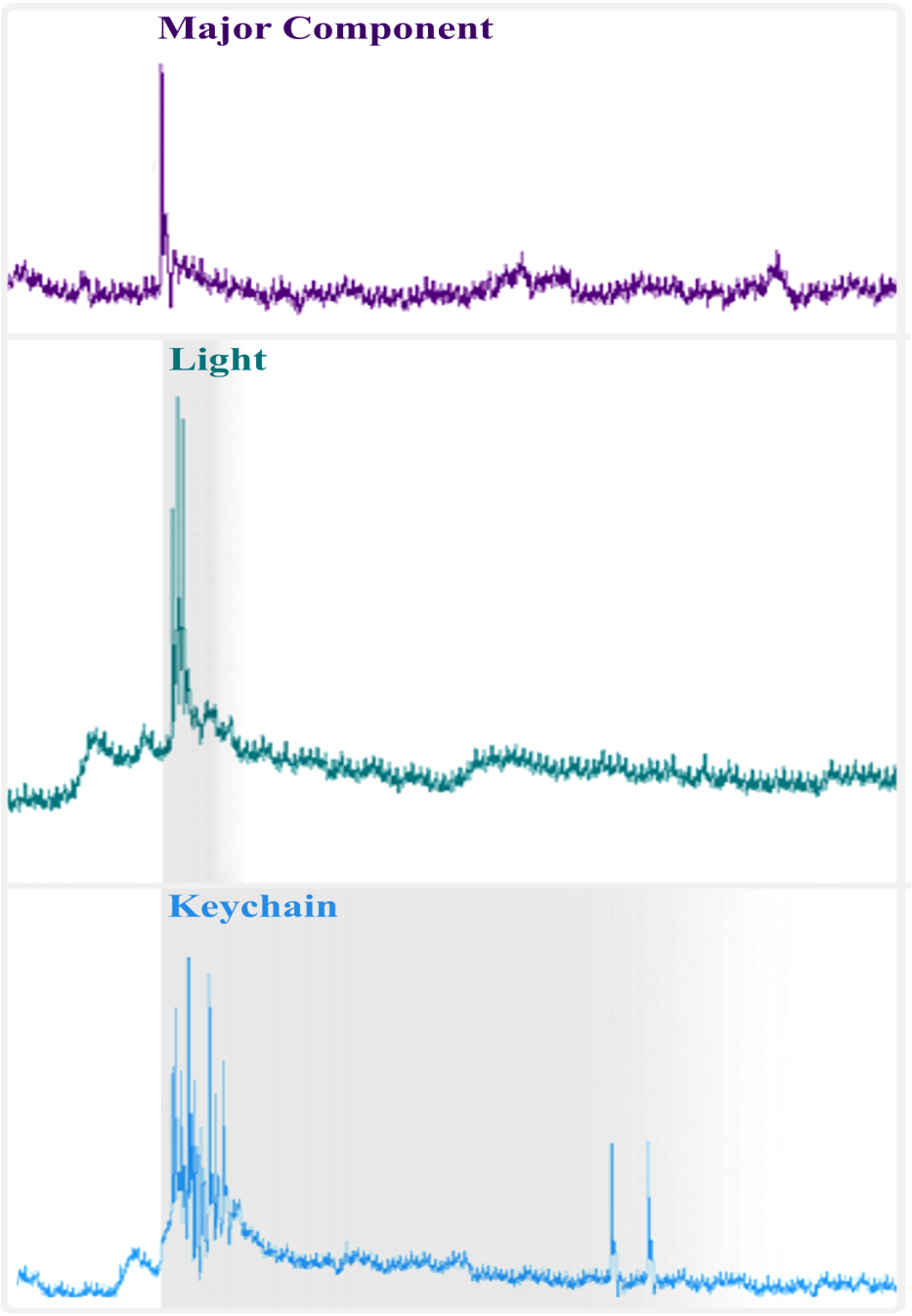

Figure 15: The response of N3 to Major component, Light, and a Keychain.

4. DISCUSSION

4.1. Discussion of the method

The major topic in the present study was to explore the use of labelling in the program Slicer for 3D-reconstruction of intracellularly stained neurons, based on the assumption that labelling in Slicer has several advantages compared to the previously used Amira skeleton tool. N1 was first segmented using the Amira skeleton tool for comparison (data not shown), and then completely segmented in Slicer. Thus, the final 3D models were created in Slicer and then registered into the Standard Brain using Amira. The Slicer method resulted in successful reconstruction of the three selected protocerebral neurons. The problems that occurred concerning the interpretation of the data were solely due to the staining quality of the preparation and not to the reconstruction method. Slicer as a program for reconstruction confirmed its advantages as opposed to the Amira skeleton tool in several aspects like the amount of time on each dataset, more manual control, and more accurate adjustments of branch attachments, curving and thickness. A main benefit from using labelling in Slicer as a method compared to the skeleton tool extension in Amira as previously used, is its manual approach which allows for more precise control of the segmentation. Although labelling is a less objective method, it gives more accurate results. Because the Amira skeleton tool relies heavily upon algorithms and objectivity, other problems arise. Labelling as a method is also available in the Amira software, but Slicer is a more intuitive program and also allows for more enhancements of the datasets and label sets.

4.1.1 The Time Aspect

The idea behind the Amira skeleton tool is that the automatic or semi-automatic generation of a neuron can be created much faster than with other programs. However, due to the complexity of these neurons and often noise in the datasets, the skeletons could in fact not be generated automatically. Instead the skeleton was created semi-automatically using a process as time-consuming as the manual Slicer labelling. Over-correcting by the automatic adjustments leads to incorrect shaping of the neuron or branches sometimes being incorrectly attached. This in turn lead to additional time spent on correcting or creating these areas anew.

In Amira the skeleton is built onwards from one point, which means that the light had to be adjusted

back and forth while working through the stacks one branch at the time. In Slicer, however, multiple branches were marked out at the time when working slice-by-slice, and thus the light and contrast did not have to be adjusted as many times. Working slice-by-slice in Slicer may seem time-consuming. However, the “threshold brush”, in which an entire area can be marked and only structures within a given intensity rage would be labelled, creates a rough labelling of the most prominent neurites. This method was not sufficient for the thinner and smaller branches, but going over these with a paint brush set to a small size was still quicker than the process in the Amira hexoskeleton tool. After creating a model that looks correct in the z view, it might be rotated and viewed from different angles allowing for corrections, as previously shown in the figure 2. As the labels are a direct translation of a 3D-model of the datasets, it does not remove the additional “shadow of light” caused by the scanning microscope. The Amira hexoskeleton avoids this problem by creating a centerline. In Slicer this problem was overcome by tracing the neuron in different angles (x,y,z) using a circular brush of the wanted size, given that the spacing of the neuron is approximately correct.

4.1.2 Centerline versus complete reconstruction

The major difference between the methods is that the Amira hexoskeleton tool creates a centerline which can be adjusted to get a proper thickness in each area, while Slicer labelling creates a complete segmentation. A centerline-model may be beneficial if different computer-analyses are to be run on the data such as quantification of branch lengths. However, these functions are less important for a purely visual purpose. Upon registering the models into the brain, the skeleton model and label reconstruction would suffer from different problems. If the model is stretched much in the registration process, or the spacing was wrong, the Slicer created label model would be overly stretched in different areas. In the skeleton tool, however, the dots will be placed further apart from each other, generating a more pixelated view. In the registrations of N1 and N2 the changes in width caused by the registration were almost unnoticeable, whereas problems appeared in N3. This was due to mechanical distortion of the brain during dissection and preparation. Thus, the antennal lobes, pushed into the protocerebrum, suffered structural deformation compared to the standard brain. When registering N3 into the standard brain, this mechanical distortion caused certain areas to be stretched a little bit more than others.

4.1.3 Automatic adjustments of the spatial distribution

Another problem encountered using the Amira semi-automatic hexoskeleton tools, was inaccuracy. When the “dots” were marked in the confocal image stack to create the structure, placing the dot accurately appeared to be of great importance. The fastest way to create a model is to go through the stack while marking out every curve and branching point of the neuron. Afterwards different parts could be adjusted to fit better. However, there were limited ways to do this adjusting beyond pressing an “adjust” button. This was however drastic, as clicking it too little tended to not correct the marked area enough, while clicking too much often over-corrected. In some very distinct areas this process worked wonderfully, but in the more difficult parts, the over-correction would at times connect branches that did not belong together, or straighten out bumps which were clearly visible to our eyes. The problem of using an objective algorithm for these purposes is that they have not yet been developed to such a degree that they can compete with a trained eye. These problems were avoided when using the manual Slicer labelling.

4.1.4 Thickness

The thickness of the dots in the Amira hexoskeleton tool could either be manually set for a selected area, or automatically set by the adjust button. The adjust button is the fastest way, but may also generate incorrect results. This is due to the algorithm basing the thickness on the nearby bright material, thus often adjusting it to match the thickness of the entire “light shadow” and not just the neurite itself. This problem caused when neurons appeared less spherical in the scan was mentioned by the developers (Evers et.al., 2005). Another problem with automatically adjusted widths was connected to the light intensity. When viewing more weakly stained areas by increasing the light, neurites would appear thicker than they actually are. By going back and forth between areas and adjusting the light, the thickness would also vary across the segmentation. In other words, the slow, manual procedure would yield a decent result, whereas the automatic tool in Amira may result in incorrect thickness. The thickness was also an issue in Slicer, but as previously mentioned it was compensated for by tracing the segmentations from another angle.

4.1.5 Different Labels

Another benefit of using labelling in Slicer compared to the Amira skeleton tool is the use of multiple labels. This enables the different labels to be viewed in multiple colours, and to be turned on and off in the 3D model. This was especially an advantage during reconstruction of N3 that contained several areas difficult to interpret. By segmenting each single part as a separate label,

different branch possibilities could be viewed from all angles as 3D-models. Correcting already labelled areas of a neuron was very easy in Slicer with drawing- and erase-tools. In the hexoskeleton tool, however, re-attaching an edited area or doing something over again was more of a challenge. The skeleton builds on what is already made, which makes the process more focused on a section at the time. This leads to less overview of the whole scene, and every area needs to be interpreted while it is being segmented.

4.1.6 Challenges

Regardless of which software was used to create the segmentation, challenges arose from noisy datasets, weaker intensity distant to the point of staining, as well as staining in nearby neurons. This was particularly the case in the third brain, where two close-by neurons (N4 and N5) were hard to distinguish from N3. In the 3D-model, N3 and N4 were reconstructed, enabling the possibility of looking at the problem in 3D. As this proved to be a very complex area, several schematic models were created in order to discuss and understand the most likely connections in the model.

4.2 The Neurons

The three neurons selected for 3D-reconstruction in Slicer were bilateral protocerebral neurons characterised physiologically and morphologically. They belonged to the sensory systems, olfaction and hearing as well as one novelty. The olfactory system of *H.virescens* has been studied extensively in our lab by characterising receptor neurons, antennal lobe neurons and protocerebral neurons. Bilaterality is an important feature of all animals, including insects. Connecting the two hemispheres is a basic feature enabling for instance depth of vision and sound localization. In insects, location of the odor source is provided by information from the two antennae. The olfactory neurons of each antennae mediate ipsilateral information to the antennal lobe and protocerebrum. In the olfaction system of moths, the bilaterality does not appear before the level of the protocerebrum.

4.2.1 N1

The protocerebral bilateral neuron N1 had both its input and output contralaterally in the LAL. These lobes protocerebral are regarded as pre-motoric output areas from the brain, and particularly for pheromone information. This particular neuron was unfortunately not tested with pheromone stimulation, but it did respond to stimulation with the plant odorant linalool. However, it did not respond to the blend containing ten primary plant odorants of which linalool was one. Protocerebral neurons responding to this blend, but not to single odorants have previously been found in our lab.

A multiglomerular antennal lobe neuron responding to only the blend and not single neurons has been found to mediate information to the lateral protocerebrum and the mushroom body (Løfaldli, 2012). Neurons responding to a blend of ten odorants were found to form a putative network between the antennal lobe, the mushroom body and the lateral protocerebrum (Løfaldli, 2012). Linalool is a biological relevant odorant typically present in flowers, and is among the identified primary plant odorants of *H.virescens* (Røsterlien et al., 2005). This odorant has been tested in many electrophysiology and behavioural studies, and is found to be important in olfactory learning in *H.virescens* (Skiri et al, 2004; Jørgensen et al., 2007). The responses of N1 to linalool suggest that LAL is also involved in mediating information about some plant odors in addition to pheromone information.

The narrow tuning to linalool seems surprising as information in higher order areas is highly processed, whereas fine tuning is more typical for first order ORNs. This selective response to linalool might be important for detecting this important odorant in a background of other odors in the environment, as suggested by Høydals master thesis, (2012). This concentration based detection of single odorants in background could be used during navigation towards the odor plume. By comparing the concentration in both sides, the moth would be able to navigate towards the source of the odor in a zigzagging pattern.

4.2.2 N2

The bilateral protocerebral output neuron N2 had its input area in the ventro-posterior protocerebrum, and the output area in the corresponding area in the contralateral hemisphere, before leaving the brain via the SOG to the thoracic ganglion. Sound information has not been as extensively studied in *H.virescens* as the olfaction and taste. Previous studies in our lab have shown the presence of sound neurones in the protocerebrum, but only a few have been identified morphologically and physiologically. Previously described neurons in the sound pathways of *H.virescens* include one sound related centrifugal neuron with input area in the superior protocerebrum and output area in all the glomeruli in the antennal lobe (Zhao et al, 2012). This neuron has been speculated to be important for shutting down olfactory information in the odor plume when the moth meets a bat. By ignoring information from the antennal lobes, it can focus more solely on sound information and thus escape from the bats. Other central interneurons identified in the sound pathways project from the thoracic ganglion and to the ventro-lateral

protocerbrum. In addition, the receptor cells A1 and A2 project from the ear to the thoracic ganglion, and one of them continued into the ventro-lateral protocerebrum. This ventro-lateral area of the brain is also found to be related to sound information in Drosophila, shown by molecular (Lai et al, 2012).

As the moths do not to produce any sound themselves, the main purpose for their sound detection system is to detect and escape from predators. The output neuron N2 indicates a simple and fast sound information circuit to enable efficient responses leading to a successful escape. N2 was an output neuron mediating directly to the motor neurons in the thoracic ganglion via the SOG.
The output in the controlateral protocerebrum suggests reciprocal inhibition may be involved in coordinating information laterally. In this process, one neuron inhibits its mirrored partner to increase the contrast between the two sides, enabling the insects to localise sound in space. A similar neuron, the omega neuron, exists in the cricket thoracic ganglia, and is used in sound localization (Wohlers et. al, 1982).

In this preparation with neuron N2 there was also an additional stained cell body in the SOG close to the ouput area. This might be an artificial staining, but if it did belong to a part of the segmentation, it would most likely be the neurite displayed in green in figure 5 and 6 B. This would constrict the output area of N2 between the ventro-lateral protocerebrum and the SOG. As there was no sign of any clear connection between this additional cell body and the neuron, they may not be connected at all.

4.2.3 N3

Neuron N3 was a novelty neuron with its cell body in the right peripheral SOG. The neurons main neurites ran in each hemisphere where they both innervated one glomerulus. N3 initially appeared similar to previously stained neurons innervating the CO_2-glomeruli as seen in *H.virescens* (Dahl, Master thesis 2013) and drosophila (unpublished). Because of this, N3 was originally thought to innervate the LPOG. However, closer scans revealed that this was not the case, as the neurites instead innervated a close by glomerulus. Mechanical distortion of the antennal lobes during the preparation process causing the antennal lobes to be pushed into the protocerebrum lead to difficulties in interpreting which glomeruli was innervated. The distortion caused the antennal lobes to look quite different from each other and also from the SBA, but as the innervated glomerulus was

lying posteriorly to the LPOG, glomeruli G53 may be a possible candidate. Another effect of the distortion of the brain was that registering it to the SBA caused certain areas to be more stretched than others, especially the area between the protocerebrum and antennal lobes.

Two neurites extends to each hemisphere where they both innervate one glomerulus before branching narrowly in the antennal lobe, and the ventral protocerebrum. From this point on, the paths in each hemispheres differed. In the right hemisphere, two prominent neurites entered the eye lobe, while the neurites in the left hemisphere faded into an area below the central body. Entering the eye lobe may be connected to the response to novel light stimuli by a flash light. The ventral protocerebral arborisations of N3 may be connected to the response to sound by a keychain and the projections in the antennal lobe corresponds to the response by the major component odor stimuli. Several difficult areas appeared during reconstruction of this particular neuron, due to staining of additional neurons in the left hemisphere near what is thought to be the point of dye injection. Different possible origins of the branches in that area are shown in figure 13. The first and probably most likely option ascribed most of the branches (red) to a nearby stained neuron N4. If different parts of the additional branches belong to N3 as shown in the examples 13 B, C and D, the protocerebral branching of N3 would be increased. Some possibilities (C, D) includes a branch which was closely connected to both N4 and the output area of a neuron N5, which lied in the superior lateral protocerebrum as shown in figure 12, an area often connected to processing of visual stimuli.

Responding to novel information is important for the moths in relation to predator avoidance, mating and feeding. To avoid spending energy on processing a continuous flow of information in the environment, a strategy is to increasing the contrast and adapt to the background noise in the form of odors, sounds and visual stimuli. Responses to different types of stimuli can be up- and down-regulation of responses by modulatory neurons in different contexts. This is an important feature for adaptation, learning and memory. One particularily well studied neuron in regards of up-regulation of responses to conditioned odor stimuli in the honey bee is the modulatory ventral unpaired medial neuron of the maxillary palp (VUMmx), which innervated the antennal lobes, the lateral protocerebrum and the mushroom bodies (Hammer, 1993; Hammer and Menzel, 1998). In *H.virescens,* similar innervation patterns have been described by Rø et al. (2007), and neurons with suggested modulatory functions innervated the antennal lobes in both *H.virescens* and *Helicoverpa*

armigera (Zhao & Berg, 2009; Zhao et al., 2012).

The responses of N3 were to important stimuli regarding moth behaviour. The bilateral innervation of the antennal lobe glomeruli and the response profile together suggests a modulatory function for N3. The responses of the odor information differed from the sound and light stimulus. The latter by instantaneous spiking to novel stimuli, while the response to the major pheromone component consisted of repeated and consistent spiking with a couple of spikes within a latency range of 120-140 ms. These differences could be attributed to the behavioural needs connected to the different types of stimuli. Light and sound is both related to detection and avoidance of predators and thus needs a fast circuit compared to the more slow process of pheromone signalling.

4. 3 Limitations of the study and future studies?

Reconstruction of neurons stained with intracellular electro-physiology is a time-consuming process regardless of which method was used, much because it requires interpretation of complex areas. The resulting reconstruction will be strongly affected by the image resolution of the confocal scans, and also personal experience and interpretation. Challenges were caused by noise in the datasets, and weak staining of thinner branches causing some areas to be hard to separate from the background and surrounding structures. While some of these branches could be traced through the stacks after enhancing the image quality, they were hard to visualise in the confocal image stacks. In addition to the problems caused by weak staining, too much dye was also an issue. This was particularly the case in N3, where additional branches (N4, N5) near the point of dye injection had been stained. Because of this conflicting area in addition to mechanical distortion of the brain, it was hard to understand the exact extent and thus complexity of this particular neuron.

Much is still left to be studied in relation to both protocerebral networks, modulatory and bilateral neurons. Our lab currently runs a project on sound neurons in *H. virescenes,* which may give important insights to their pathways and processing. In addition, it would be very interesting to see behavioural experiments in relation to these neurones, which could shed light on their biological relevance.

5. CONCLUSIONS

The reconstructions of three bilateral protocerebral neurons in *H.virescens* created with manual labelling in Slicer were successful. This method had several benefits compared to the semi-automatic Hexoskeleton tool for Amira, but were more influenced by personal interpretations and thus less objectivity. Some of the benefits included more control over the thickness and branch attachments leading to increased accuracy. Slicer was found to be a faster method for an inexperienced user for reconstruction than the hexoskeleton tool. This was especially the case for complex datasets with areas open for interpretation. Using labelling instead of a skeleton tool allows for easier corrections and the use of multiple labels. This in turn enables the possibility for testing out different options in complex areas in 3D by turning different labels on and off. Regardless of method or software used, there were challenges caused by the staining quality, noise in the dataset and mechanical distortion of the brain. The segmentations were registered into the standard brain.

The bilateral protocerebral neurons responded to three different modalities, namely an odorant, a sound stimulus and novelty. The neurons N1 and N2 both projected from one area to the same area on the contralateral side. This bilaterality was suggested to indicate reciprocal inhibition, and thus contrast enhancement between the two hemispheres. This could enable the insects to orient towards the odorant and sound in space. That N1 responded to linalool stimulation, but not a blend containing linalool, further support the suggested contrast enhancement. Both neurons resided in output areas, N1 in the Lateral Accessory Lobe and N2 in first in the ventro-lateral protocerebrum before leaving the brain through the SOG. The last neuron, N3, was found to respond to novel stimuli of sound and light, and consistently to the major pheromone component. This novelty detection may be important for focusing attention, and detecting important biologically relevant stimuli from the background noise in the environment. The neuron innervated one glomerulus in each hemisphere before branching into the ventral protocerebrum. From there the neurites turned into the eye lobe in the right hemisphere and fading into an area below the central body in the left hemisphere.

6. REFERENCES

Boyan, G S., Fullard, J H. (1986) Interneurones responding to sound in the tobacco budworm moth *Heliothis virescens* (Noctuidae): morphological and physiological characteristics. J Comp Physiol A (1986) 158:391-404

Chew, F., Robbins RK. (1984). Egg laying in butterflies. In Vane-Wright RI, Ackery P, eds. 1984. The Biology of Butterflies, Volume 13:65-79

Dahl, I M., Master thesis (Not yet published)

Evers, J F., Schmitt, S., Sibila, M., Duch, C. (2005) Progress in Functional Neuroanatomy: Precise Automatic Geometric Reconstruction of Neuronal Morphology From Confocal Image Stacks. Journal of Neurophysiology. 93:2331-2342

Fitt, G P. (1989) The ecology of Heliothis species in relation to agroecosystems. Annual Review of Entomology 34:17-52

Galizia C. G., Rossler W. (2010). Parallel Olfactory Systems in Insects: Anatomy and Function. Annu Rev Entomol 55:399-420

Guerenstein, P G., Christensen, T A., Hildebrand, J G. (2004) Sensory processing of ambient CO_2 information in the brain of the moth Manduca sexta. J Comp Physiol A (2004) 190: 707–725

Hammer, M. (1993). An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. Nature 366:59–63

Hammer, M., Menzel, R. (1998) Multiple sites of associative odor learning as revealed by local brain microinjections of octopamine in honeybees. Learning & Memory 5(1-2):146-56.

Homberg U, Montague RA, Hildebrand JG. (1988) Anatomy of antenno-cerebral pathways in the brain of the sphinx moth Manducta sexta, Cell Tissue Res 254:255-281

Høydal, Ø. A. (2012). Central Processing of Plant Odor Mixtures and Single Odorants in the Moth Heliothis virescens. (Master thesis). Norges teknisk-naturvitenskapelige universitet Institutt for biologi.

Jørgensen, K., Almaas, T. J., Poll, F. M. and Mustaparta, H. (2007), Electrophysiological characterization of responses from gustatory receptor neurons of sensilla chaetica in the moth

Heliothis virescens, Chemical Senses, 32:863-879.

Kanzaki, R., Arbas, E A., Hildebrand, J G. (1991) Physiology and morphology of descending neurons in pheromone processing olfactory pathways in the male moth Manduca sexta. Journal of Comparative Physiology. A. 169:1-14

Keil, T., Steinbrecht, R.A., (1987), Diffusion barriers in silkmoth sensory epithelia: Application of lanthanum tracer to olfactory sensilla of Antheraea polyphemus and Bombyx mori, Tissue & Cell, 19: 119-134

Krieger, J., Breer, H. (1999), Olfactory reception in invertebrates, Science, 286: 720-723

Kvello, P L., Løfaldli, B B., Rybak, J R., Menzel, R., Mustaparta, H. (2009) Digital, three-dimensional average shaped atlas of the heliothis virescens brain with integrated gustatory and olfactory neurons. Front. Neural. Circuits. 5:7

Lai, J S-Y., Lo, S-J., Dickson, B J., Chiang, A-S. (2012) Auditory circuit in the *Drosophila* brain. Proceedings of the National Academy of Sciences of the United States of America 109(7): 2607-2612

Løfaldli, B.B., Kvello, P., Mustaparta, H. (2010), Integration of the antennal lobe glomeruli and three projection neurons in the standard brain atlas of the moth Heliothis virescens, Front. Syst. Neurosci., 4:5

Løfaldli, B B, Kvello, P., Mustaparta, H. (2012) Activity in neurons of a putative protocerebral circuit representing information about a 10 component plant odor blend in *Heliothis virescens*. Front. Syst. Neurosci. 6:64

Menzel R. (2001). Searching for the memory trace in a mini-brain, the honeybee. Learn. Mem 8, 53–62

Renwick J A A, Chew F S., (1994) Oviposition behavior in lepidoptera, Annu. Rev. Entornot. 39:377-400

Roeder, K D., Treat, A E. (1961) The detection and evasion of bats by moths. American Scientist 49:135-148

Rø, H., Müller, D., Mustaparta, H. (2007), Anatomical organization of antennal lobe projection neurons in the Moth Heliothis virescens, J. Comp. Neurol., 500:658-675

Røstelien T, Stranden M, Borg-Karlson A-K, Mustaparta H, (2005) Olfactory receptor neurones in two heliothine moth species responding selectively to aliphatic green leaf volatiles, Chem Senses

30: 443-461

Sato, K. et al. (2008), Insect olfactory receptors are heteromeric ligand-gated ion channels, Nature, 452:1002-1006

Wang Z, Singhvi A, Kong P, Scott K. (2004). Taste representations in the *Drosophila* brain. Cell. 117:981–991.

Skiri, H., Galizia, C G., Mustaparta, H. (2004) Representation of primary plant odorants in the antennal lobe of the moth Heliothis virescens, using calcium imaging. Chem. Senses 29:253-267

Surlykke A. (1984) Hearing in Notodontid Moths - a Tympanic Organ with a Single Auditory Neuron. J Exp Biol 113:323-35

Tabashnik BE. (1985). Deterrence of diamondback moth (Lepidoptera: Plutellidae) oviposition by plant compounds. Environ. Entomol. 14:575-78

Tanaka, N.K., Awasaki, T., Shimada, T., Ito, K. (2004), Integration of chemosensory pathways in the Drosophila second-order olfactory centers, Curr. Biol., 14:449-457

Vosshall LB, Wong AM, Axel R, (2000) An olfactory sensory map in the fly brain Cell 102:147-159

Vosshall LB (2001) The molecular logic of olfaction in Drosophila, Chem. Senses 26:207-213

Wohlers, D W., Huber, F. (1982) Processing of sound signals by six types of neurons in the prothoracic ganglion of the cricket *Gryllus campestris* L. Journal of comparative physiology. 146:161-173

Zhao, X-C., Berg, B G. (2009); Morphological and Physiological Characteristics of the Serotonin-Immunoreactive Neuron in the Antennal Lobe of the Male Oriental Tobacco Budworm, *Helicoverpa assulta* Chemical Senses 34(5):363-372

Zhao, X-C., Pfuhl, G., Surlykke, A., Tro, J., Berg, B G. (2013), A Multisensory centrifugal neuron in the olfactory pathway of Heliothine moths. The Journal of Comparative Neurology*521:152-168*