MORPHOLOGICAL ANALYSIS OF HORIZONTAL SENSITIVE LOBULA PLATE TANGENTIAL CELLS OF *DROSOPHILA MELANOGASTER*

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BIOLOGY

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Contents

Zusammenfassung

Multi-Kompartiment-Modelle werden in der Neurobiologie dazu verwendet, die Morphologie und die Eigenschaften eines Systems zu beschreiben. Dabei kann es sich um einzelne Neurone oder um ein Netzwerk von Neuronen handeln. Das Ziel dieser Arbeit ist es, den Grundstein für die Erstellung eines solchen Modells der Lobula Platte der Fruchtfliege, *Drosophila melanogaster,* zu legen. Die Lobula Platte wird aufgrund ihrer wichtigen Rolle in der visuellen Informationsverarbeitung das “Cockpit” der Fliege genannt (Review: Borst & Haag (2002), Neural networks in the cockpit of the fly). Diese enthält die „Lobula-Platten-Tangential-Zellen (LPTC)“, die aufgrund ihrer Antworteigenschaften unterteilt werden und in jedem Individuum von *Drosophila* in gleicher Anzahl vorkommen. In dieser Arbeit wurden je drei Vertreter zweier unterschiedlicher Zelltypen der horizontal sensitiven HS Zellen mit einem Farbstoff gefüllt, gescannt und rekonstruiert, um ein drei dimensionales Modell zu der realen Zelle zu produzieren. Eine statistische Analyse morphologischer Parameter ergab, dass die einzelnen Vertreter der zwei Zelltypen untereinander nicht unterscheidbar sind. Zudem konnten die beiden Zelltypen an sich auf diese Weise ebenfalls nicht unterschieden werden, was unseren Erwartungen widersprach und vermutlich daran lag, dass mit dem Abschluss dieser Arbeit lediglich eine geringe Anzahl von Zellen rekonstruiert werden konnten. Die vorläufigen Ergebnisse müssen nun durch eine weitere detaillierte Analyse geprüft werden. Doch insbesondere die sehr hohe Ähnlichkeit der beiden Zelltypen führte zu dem Schluss, dass es möglich ist, virtuelle Zellen der einzelnen Zelltypen zu konstruieren, die die grundlegenden anatomischen Eigenschaften eines Zelltyps widerspiegeln. Mit diesen virtuellen, standardisierten Multi-Kompartiment-Modellen könnte wiederum der Grundstein für ein Multi-Kompartiment-Modell der Lobula Platte gelegt werden.

Introduction

Insects can be found in nearly all environments, on land, underground, in the oceans and particularly in the air. It is the animal group with the highest diversity and number of species, morphology and behavior. Due to their short life cycle and the high number of offspring, evolution has maximized during the past 400 million years the efficiency of every single cell to fit best the requirements of the insect's biological niche. A beautiful example is the nervous system of the fly and more precisely the way how visual input is processed. While flying through the environment the fly can be encountered from every direction and therefore has to effectively detect possible predators during flight. With acrobatic maneuvers the fly can respond instantly to environmental influences like gusts of winds or moving objects, maximizing its chances of survival. For instance, the neural circuit of the fly *Calliphora* computes incoming visual information very fast and precisely. It enables turning velocities of up to 3000 °/s and the initiation of compensatory flight maneuvers with a time-lag of less than 30 ms (Land and Collett, 1974; Wagner, 1986) during the chasing of mates. This is only possible with a visual system perfectly tuned to the tasks it has to perform. The fact that 70% of the 300.000 neurons in the fly are located in the optic lobes (Strausfeld, 1976) is a strong argument for the importance of the visual processing in the fly. Starting with the anatomical descriptions by Ramón y Cajal 1923, structure and anatomy of different insects have been compared and particularly parallels between nearly all dipterans have been found several times (Fischbach and Dittrich, 1989; Strausfeld and Lee, 1991).

But how does such sophisticated information processing work and how is it implemented? To analyze neuronal networks involved in motion vision, it is indispensable to have the possibility to investigate and modify their physiological properties. Therefore, the model organism *Drosophila melanogaster* with its numerous genetic tools seems to be ideal. The core of genetic manipulation is the Gal4/UAS system. It allows transgenic expression (Brand & Perrimon, 1993) of any gene of interest in specific (neuronal) cell types. The Gal4 gene encodes the yeast transcription activator protein Gal4 which binds to an Upstream Activation Sequence (UAS), a short promoter region. Binding initiates transcription and, as a result, transgenic expression of any gene of interest located downstream the UAS sequence. By crossing two fly lines, one expressing the Gal4, e.g. in a subset of

neurons, and the other one, holding the UAS section and downstream gene “X”, the two elements can be combined. Consequently, the UAS line is responsible for the desired gene and the Gal4 line for the location of expression.

The optic lobes of *Drosophila* are subdivided into the retina and the three successive layers of neuropile, the lamina, the medulla and the lobula complex, consisting of the lobula and the lobula plate. The retina is composed of hundreds of ommatidia, each containing an individual transparent lens, support and pigment cells. In the center of each ommatidium, there is a cluster of eight different photoreceptor cells R1-R8. The cells R1-R6 are responsible for motion and R7/R8 for chromatic vision (Hardie, 1984). The signals are transmitted and processed in the optic lobes, which reveal a high number of neuronal cell types, classifiable as either columnar or tangential.

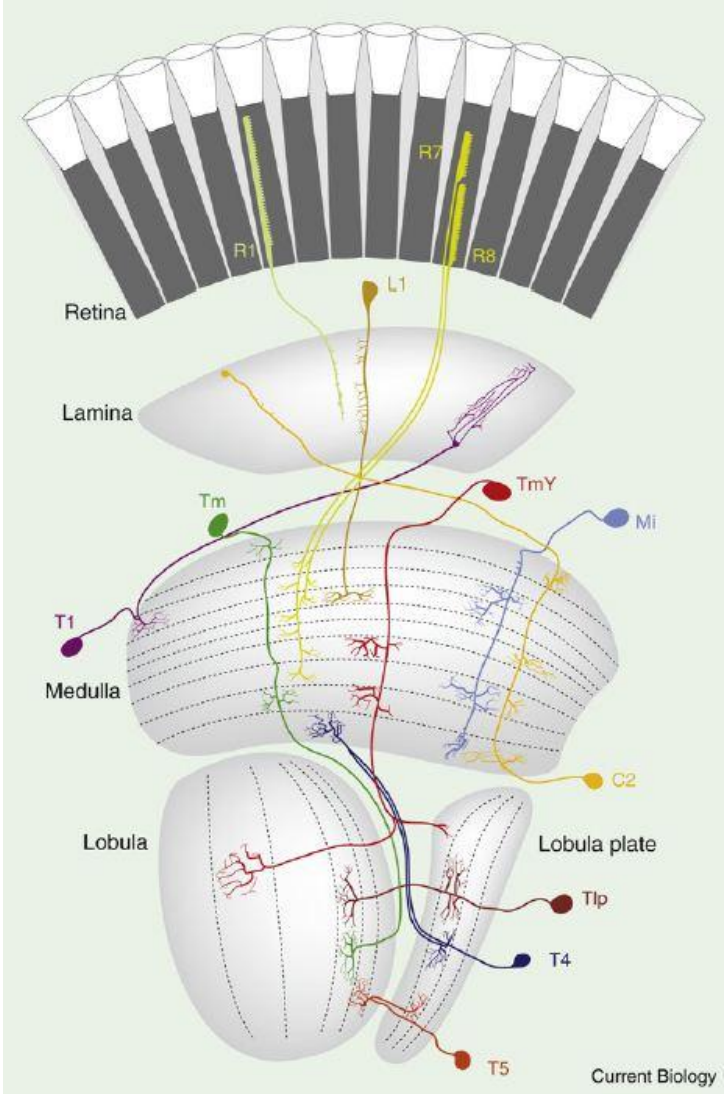

Fig. 1: Columnar cell types in the optic lobes of *Drosophila.* Schematic drawing of characteristic neuronal subtypes of the fly´s optic lobe. For clarity, only one type of cell, representative for one distinctive class is presented (from Borst, 2009).

Fig. 1 shows one representative of each neuronal cell type of the many found in each column. These are mainly named following their arborization patterns, depending on the regions where they ramify. For example the intrinsic cells (Mi) ramify only within the medulla, transmedulla interneurons (Tm) connect distinct layers of the medulla to the lobula and TmY cells, which have an additional ramification within the lobula plate.

Visual information is first processed by the columnar neurons which can subsequently synapse onto the lobula plate tangential cells. Very famous sub-groups of these cells are the horizontally sensitive (HS) and vertically sensitive (VS) cells, named by the orientation of stimuli influencing them most. In *Drosophila* 6 different VS and 3 different HS cells have been described (Heisenberg et al., 1978; Fischbach & Dittrich, 1989; Scott et al., 2002; Rajashekhar & Shamprasad, 2004; Joesch et al., 2008). Electrophysiological analyses show that moving stimuli de- or hyperpolarize these cells, depending on the orientation of movement. Interestingly, the motion responses of these cells can be described by a mathematical model of local motion detectors, the so called Reichardt detector (Reichardt, 1961, 1987; Egelhaaf et al., 1989; Borst and Egelhaaf, 1989). Reichardt detectors provide a direction selective signal by correlating the luminance levels in adjacent retinal locations, after they have been temporally filtered in an asymmetric way (See Fig. 2) over a low-pass and a high-pass filter. At each retinal location, this correlation process is done twice in a mirror symmetric way, after which the signals become subtracted from each other (for details see Fig. 2)

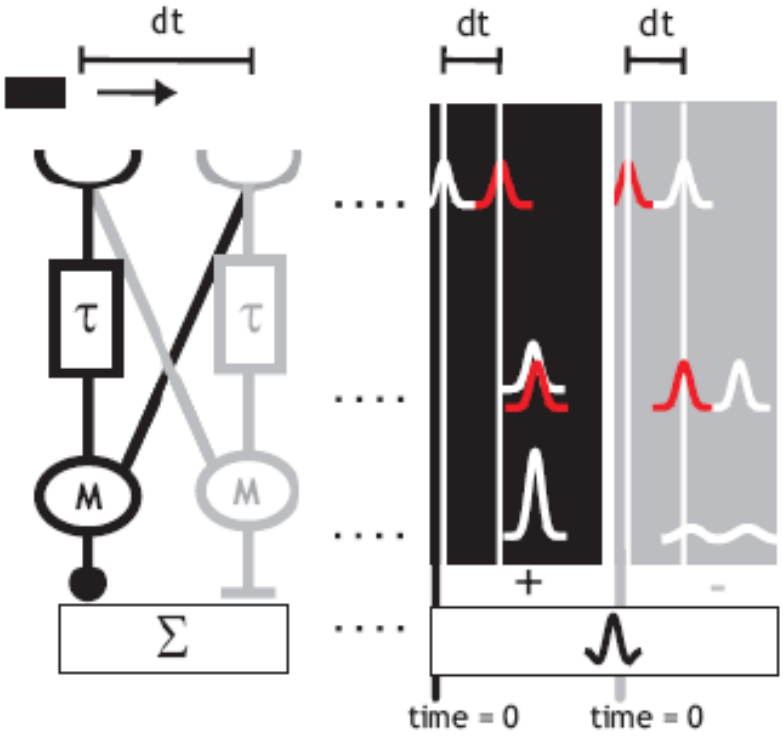

Fig. 2. Basic scheme of a Reichardt detector together with its component responses (right boxes) to a Gaussian luminescence distribution that moves with a constant velocity from left to right. The two mirror symmetric subunits (black and grey subunits) are built up by a delay (τ) and a no-delayed line that are combined in a nonlinear manner (M) and, respectively, subtracted in an integrative unit (Σ).The time of stimulation is indicated for each subunit separately (right boxes). „dt" is the time needed for the object to move from on photoreceptor to the other. The white and red distributions reflect the delayed and instantaneous signals at each processing step, respectively. At the multiplication step, the black subunit receives the inputs simultaneously while the grey subunit receives them consecutively, enhancing and weakening the input signals respectively. After subtraction of these signals, the final output shows strong direction selectivity.

The integration and extraction of global motion features from this elementary motion information is relevant for visual course control. In this respect, the lobula plate tangential cells constitute a network that achieves such processing. This is firstly done by the distinct receptive field properties of HS- and VS-cells which depend on their dendritic spanning fields in the lobula plate. Another known feature that maximizes the response accuracy is the smoothing of VS cell responses via gap junctions, expanding the receptive fields. This smoothing is thought to be essential for the correct estimation of the actual angle of rotation during flight (Cuntz. et al., 2008). Taking this information into account, the lobula plate tangential cell network has been described as the “cockpit” of the fly.

To better understand the function of this motion processing “cockpit”, a detailed analysis of their physiological response properties, as well as of their morphological characteristics, is indispensable. For this reason I set out to build realistic computational reconstructions of specified lobula plate tangential cells. To accomplish this, single dye-labeled lobula plate tangential cells were digitalized using a confocal microscope. Then, from these confocal images anatomical reconstructions with the custom written program “Milana” (Cuntz et al., 2008) were performed in order to get a three dimensional digital analogue. Although the primary responses of lobula plate tangential cells are well described by the Reichardt detector, it is still not well understood how motion vision is further processed in the lobula plate to elicit the enormous sets of visually driven behavioral responses. Therefore, the reconstruction of the complete set of tangential cells will make it possible to generate a realistic model of the lobula plate, which together with their known electrophysiological properties will be essential for the understanding of this network. Consequently, these morphological analyses are an essential step towards understanding the network computations in the visual system of the fly.

Methods

Flies:

Flies were raised on a standard cornmeal-agar medium with a 12 hr light/12 hr dark cycle, 25°C, and 60% humidity. Female flies were used one day after eclosion. The line NP 0282 (established by the NP consortium; for screening see Otsuna and Ito 2006) expresses Gal4 in two of the three HS cells (HSN and HSE, Fig. 3/4) and in unidentified neurons of the central brain. To visualize these cells, mCD8-GFP was expressed via the Gal4/UAS - system.

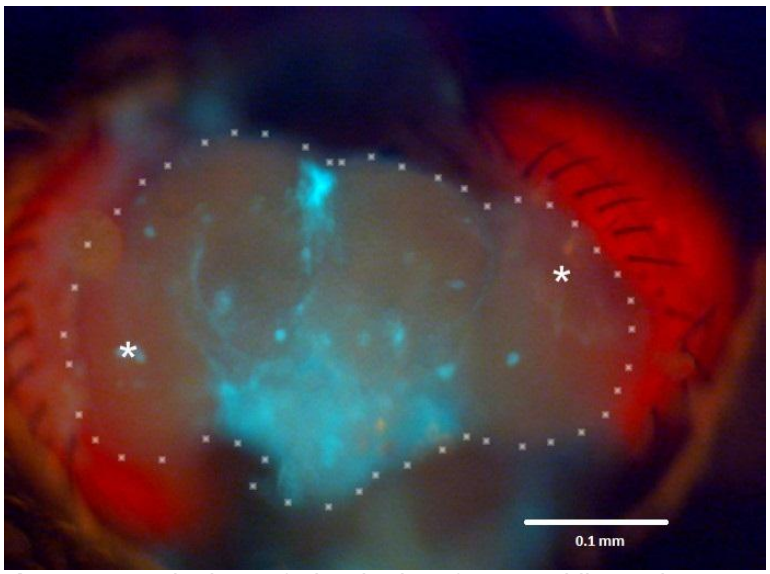

Fig 3: *Dorsal view of the brain of Drosophila melanogaster* Gal4-line NP0282 expressing GFP in 2 of the three HS cells of the lobula plate; brain *surrounded by white dots; *: optic lobe*

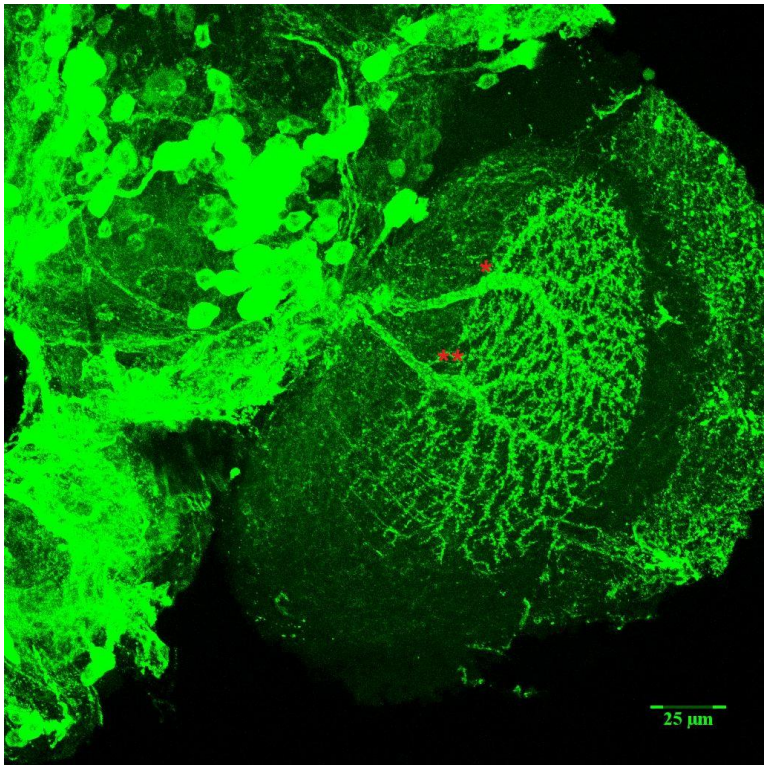

Fig. 4: *Right hemisphere of Drosophila brain (line NP0282): *: HSN; **: HSE;*

Preparation:

One day old female flies were decapitated (Fig 5A+B). The fly heads were fixed in a layer of two-component glue (UHU Plus; UHU, Baden, Germany), with the compound eyes facing downward into the glue (Fig. 5C+D). After hardening of the glue (approx. 4 min) the specimen were covered with ringer-solution. The saline composition was (in mM): NaCl 103, KCl 3, TES 5, trehalose 10, glucose 10, sucrose 7, NaHCO3 26, NaH2PO4 1, MgCl2 4 (adjusted to pH 7.2-7.4). To reduce neuronal activity and apoptotic damage, no Calcium was included in the ringer-solution. The cuticle at the back side of the fly's head was opened with sharp needles (Fig. 5E+F) (Neolus, Gx3/4 in. 0.4 x 20 mm). Finally, fat bodies and the main tracheal branches were removed to get direct access to the brain (Fig. 5G-H).

Fig. 5A: *decapitated fly (lateral view)*

Fig. 5B: *decapitated fly (frontal view)*

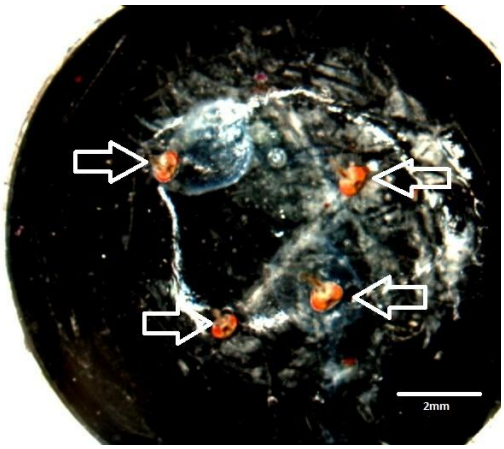

Fig. 5C: *four heads fixed with glue to a custom made specimen stage; heads marked with arrows*

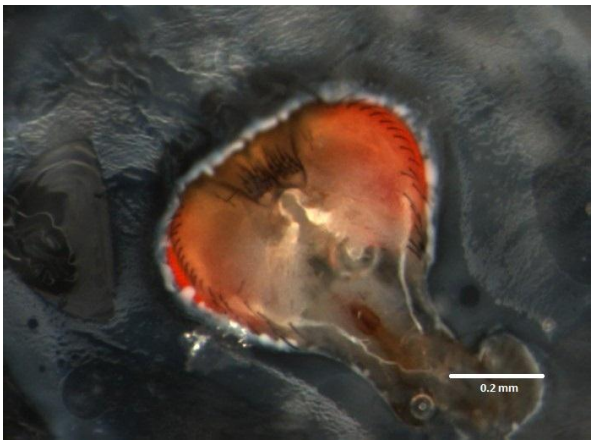

Fig. 5D: *single head in glue*

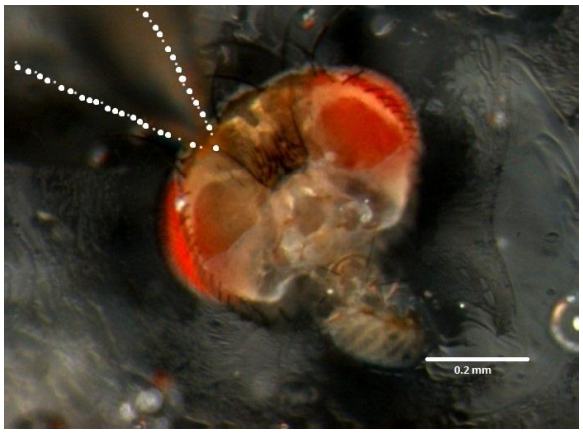

Fig. 5E: *first cut with a sharp needle (marked with white dots)*

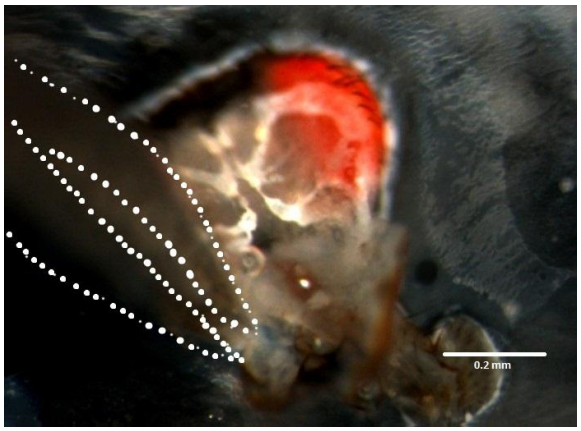

Fig. 5F: *Removal of the cuticle with tweezers (marked with white dots)*

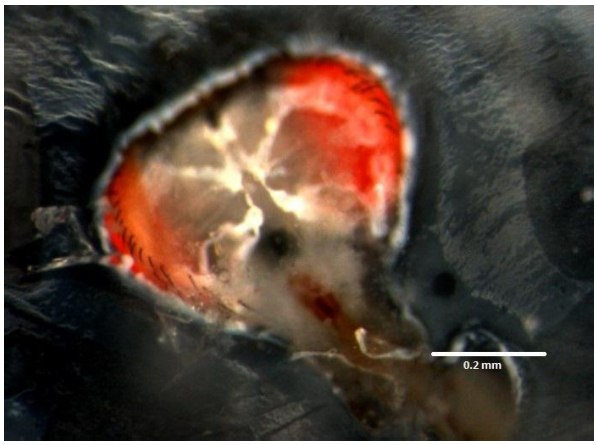

Fig. 5G: *brain with main tracheal branches*

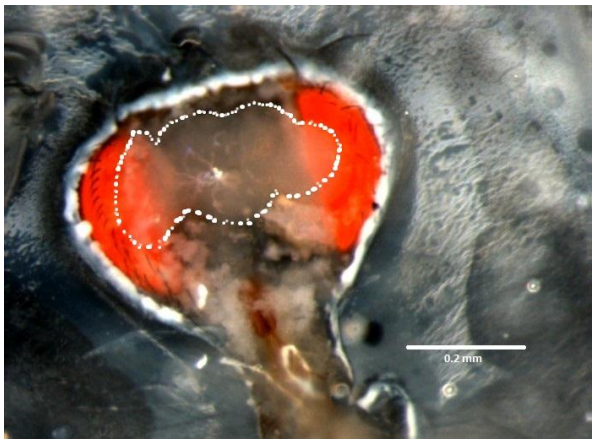

Fig. 5H: *brain without main tracheal branches; removed with tweezers*

Microscopy and intracellular dye filling:

Dye fillings were performed using quartz electrodes (QF 100-70-10; Sutter Instrument) pulled with a laser puller (P-2000; Sutter Instrument). Electrodes were filled with a 10mM Alexa Fluor 594 in 2M KCl solution (A10442; Invitrogen, Karlsruhe, Germany) and backfilled with 2 M KAc/0.5 M KCl solution.

Under a fluorescence microscope (Axiotec vario 100HD, Zeiss Jena, GER; fluorescence filterset EGFP/dsRed 1xF51-019, AHF Tuebingen, GER) with 40x magnification (Objective: Achroplan 40x/0,80 W; ∞/0; Zeiss Jena; GER), the electrode and the cell of interest were manually adjusted to the same focal level. Electrodes were positioned via a manipulator (MX-4, Narishige Micromanipulator Tokyo, JPN). Impaled cells were loaded by negative current pulses for a few

seconds. The currents were generated by an amplifier (sec 10L, npi Tamm, GER).

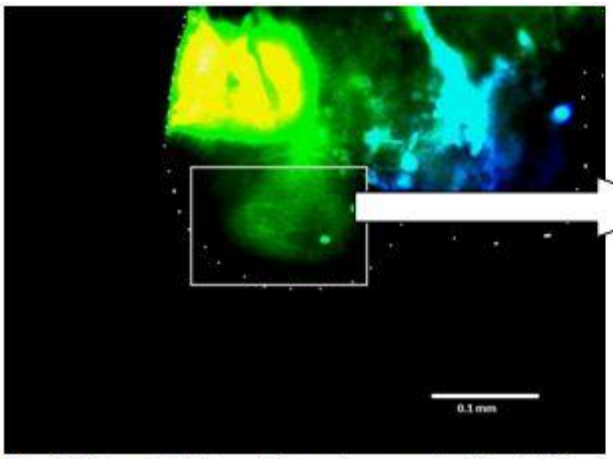

Fig. 6A1: *optic lobe of D. melanogaster line G73 under GFP2 excitation light*

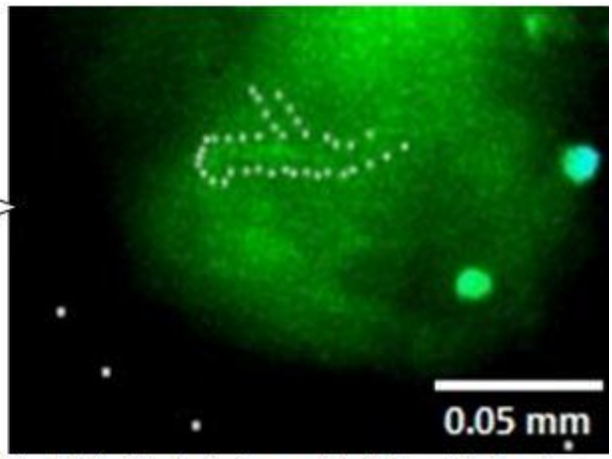

Fig. 6A2: *detailed view on Fig. 5A; pointed out: HSN-cell*

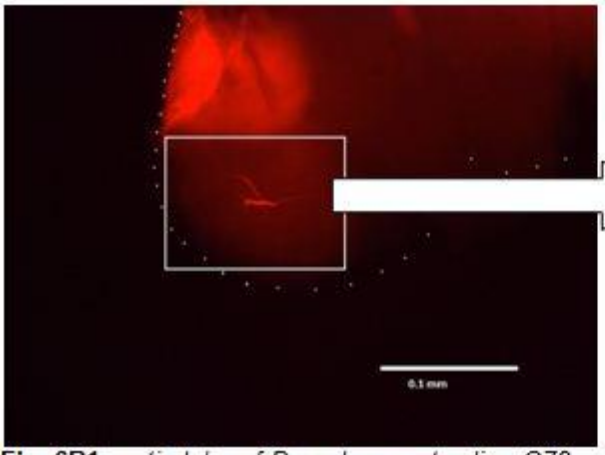

Fig. 6B1: *optic lobe of D. melanogaster line G73 under green excitation light; HSN cell filled with Alexa 594 (in white box)*

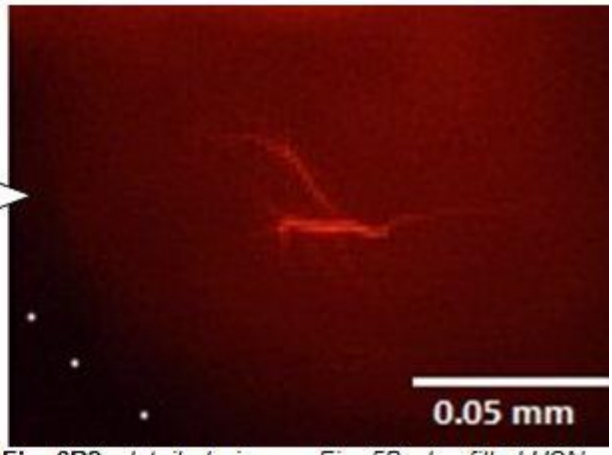

Fig. 6B2: *detailed view on Fig. 5B; dye filled HSN-cell*

Subsequently, the brains were fixed in 4% paraformaldehyde (PFA) for 30 minutes (Fig 7A). PFA binds to free amino groups of proteins and polymerizes them. Cross-linking all kinds of proteins prevents the leak of the dye from the filled cells and preserves the tissue. After fixation the brains were washed 3 times in PBS (1x) and subsequently dissected from the head capsule (Fig. 7B/C). The brains were then transferred to an object slide and embedded in mounting medium (Fig. 7D/E/F) (Vecta Shield H-1000, Vector Laboratories, Inc. Burlingame, CA). To avoid crushing the brains, 2 layers of adhesive strip (Tesa) were used as place holders between the brains and the cover slip.

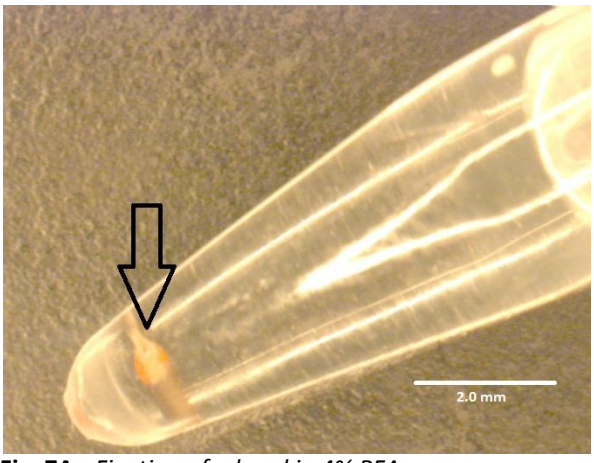

Fig. 7A: *Fixation of a head in 4% PFA*

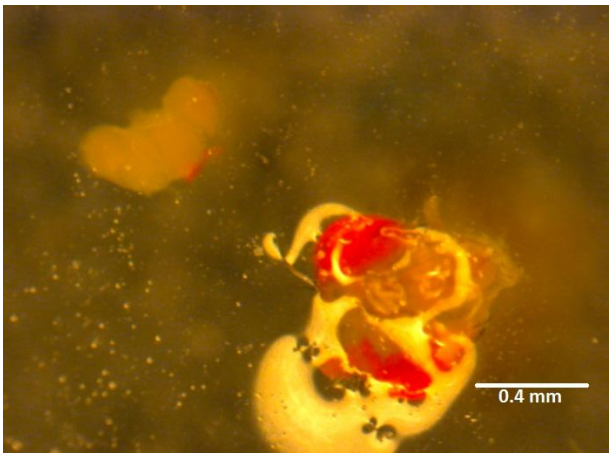

Fig. 7B: *Dissection of the fixed brain from the head capsule*

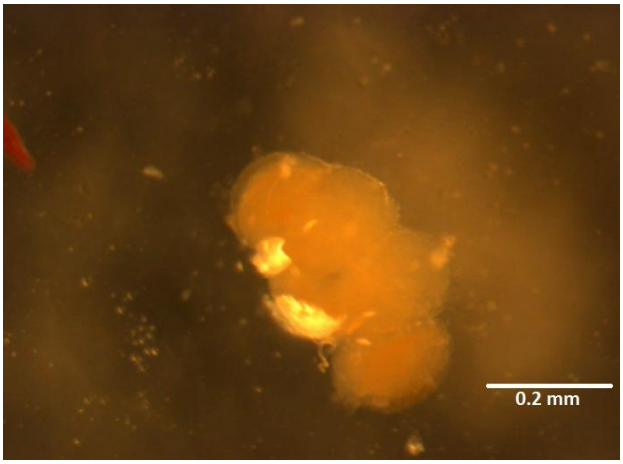

Fig. 7C: *Fixed and dissected brain in PBS*

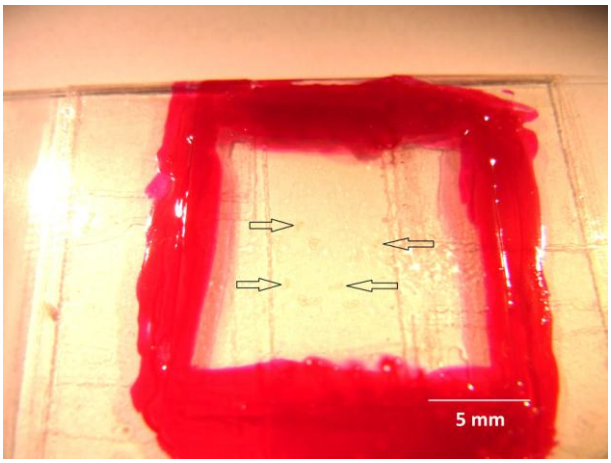

Fig. 7D: *4 brains (arrows) in mounting medium on an object slide*

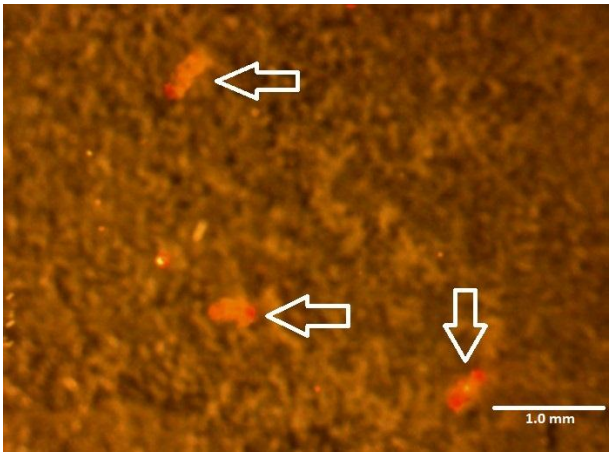

Fig.7E: *Three dissected brains in mounting medium*

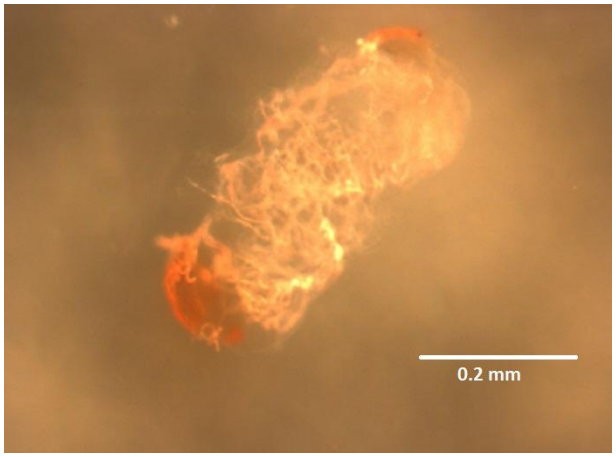

Fig. 7F: *Dissected brain in mounting medium*

Confocal Microscopy:

Serial optical sections were taken at 0.365 µm intervals with a resolution of 1024 x 1024 pixels using a confocal microscope (Leica SP2 respectively Olympus FV-1000) and oil-immersion X40 and X63 (numerical aperture [NA]=1.25) Plan-Apochromat objectives. The specialty of confocal microscopes compared to traditional wide-field fluorescence microscopes is the pinhole. The pinhole stays in an optically conjugate plane in front of the detector to eliminate out-of-focus signals. Thus, optical resolution along the z-axis is attained. Size, contrast, and brightness of the resulting image stacks were adjusted with ImageJ (National Institutes of Health, Bethesda, MD).

Reconstruction:

Cells were manually traced using Milana (Cuntz et al. 2008), a custom written software based on Matlab (Mathworks, Natick, MA) to translate the pixel based images (.tif) from the confocal scan, into a vector based model. Maps of maximum intensity and corresponding depth were computed along the Z-axis. This reduction from 3D-data to 2D images was sensible as there were no or very few 3D crossings of branches and all cells were planar. Based on these images, cylinder models of the branching structure were obtained in a semi-automated way: interactive software allowed switched viewing of either the Z-projection or an individual slice of an image stack. The cells were reconstructed as a set of connected cylinders with variable length and diameter, representing the axonal or dendritic structure of the neurons. To adjust the diameter for the cylinders, the widths of two-dimensional rectangles were fitted by gauss functions. Switching through the 2D stacks of the three dimensional image, rectangles were manually defined via their end points, representing the center of the thickest part of the neural structure and their diameter. The depth-map according to their 2D location defined the Z-value of the cylinders. The images of the reconstructed cells were adjusted and visualized using Amira (Amira 5.2.2, Visage Imaging GmbH, Berlin, GER).

Results

For this work targeted fills of around 100 HS cells were performed. It was possible to scan about 20 HSN and 10 HSE cells. The reasons why so many cells could not be scanned were at first due to initial problems with the fixative. The 4% PFA was produced by diluting 10% PFA. However, the dilution was made with H_2O_{bidest} and not with PBS, so that the quality of the fixation was reduced and the brains were destroyed easily during dissection. Secondly, electronic problems in the scanning control of the main confocal microscope forced us to use another confocal microscope (Leica SP2). Unfortunately, the excitation laser of this microscope did not allow scanning the brains previously filled with Alexa 568, since the excitation laser was tuned for Alexa 594. Hence, productive working was only possible after using correctly diluted PFA and Alexa 594.

Three reconstructions of each cell type were made for morphological analysis and comparison between z-projection and reconstruction, between the HSN and HSE cells and between the three individuals of the two cell types.

Maximum intensity Z-projections of one representative image stack for each of the three members of the HS cells were produced (Fig. 8) out of the Tiff-files obtained with the confocal microscope. The HS-cells were identified based on their position in the lobula plate. HSN-cells can be found in the dorsal, HSS-cells in the ventral part of the lobula plate and HSE-cells in between them. They were not only distinguishable by their position, but also by the form of their dendritic trees. The main branch of the dendritic tree of the HSN looked curved in direction to the central part of the lobula plate. The appearance of the HSE, however, was much more symmetrical. The shape of the HSS cell was very similar to the HSN cell, but flipped horizontally (Fig. 8).

It was possible to construct digital represantations of the cells (Fig. 8).The HS-cells were manually traced and described by a set of connected cylinders (see detailed explanation on the reconstruction procedures in the Methods section). The advantage of these digital reconstructions is that a morphological analysis

can be performed with a three dimensional model exempted from noise, because it is not possible to use the algorithms describing the morphology of the cells on Tiff-files. Furthermore, the storage space is strongly reduced. The data amount of all Tiff-files was ~10 Gb. In comparison, the data space needed for the 6 reconstructed cells (Fig. 9) was less than 1 Mb.

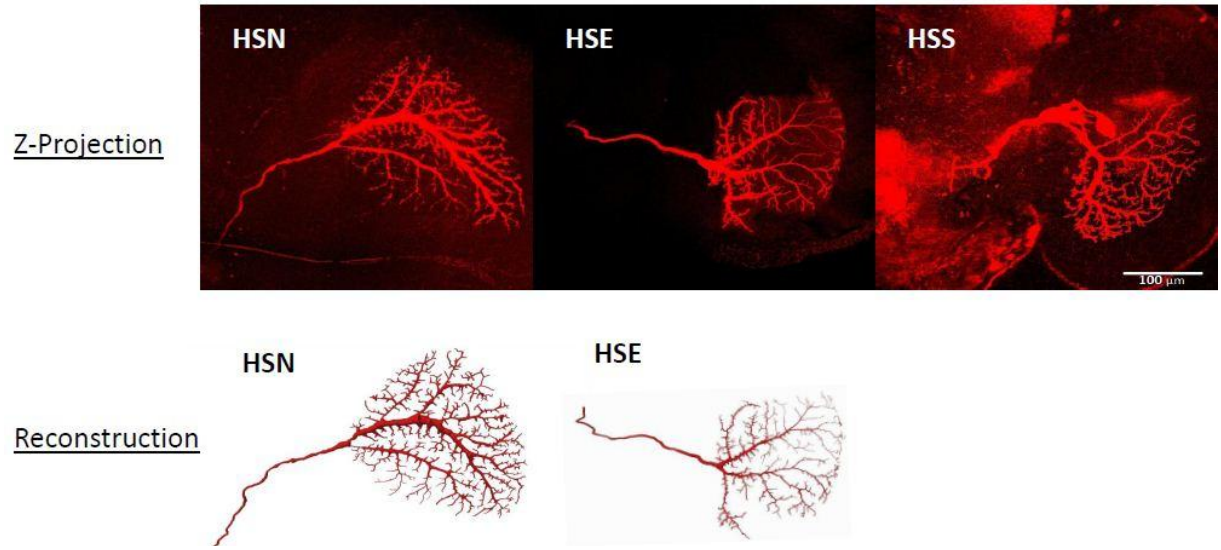

Fig. 8: Comparison between z-projections and reconstructions
upper row: r-projections with maximum intensity of the 3 HS-cells in *Drosophila melanogaster*
lower row: reconstructions of the HSN- and HSE-cell

It was just possible to distinguish the different cell types with the eye based on the form of the dendritic tree. The reconstructions show the strong planar shape of these HS-cells (Fig. 9).

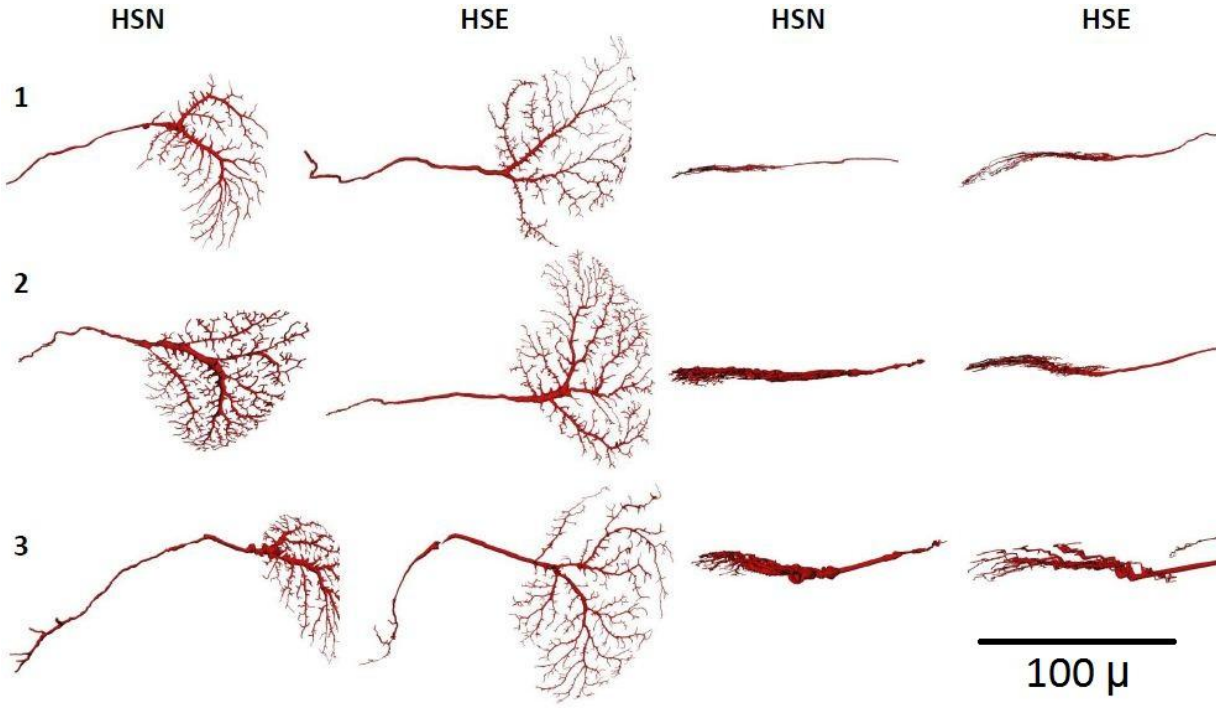

Fig. 9: Three reconstructions of HSN- and HSE-cells each
first row: view from the top on the HSNs; second row: view from the top on the HSEs;
third row: view from the side on the HSNs; fourth row: view from the side on the HSEs

To get data that allow significant comparison between the cell types, morphological parameters were calculated to analyze anatomical characteristics of the cells. For this analysis the axons of the 6 models were removed, since the axons could not be completely reconstructed for all cells. Probably lesions of the tissue in that region were the reason of the loss of the output area of some cells. In addition to that, the dendritic trees were normalized by scaling them to the width of 100 µm. The scaling corresponds to a normalization of the lobula plate and preserved the characteristic shape of their dendritic trees.

Statistical analysis of the reconstructed cells was performed by analyzing parameters of the dendritic structure. The parameters cable length, branch order and number of branches describe the branching properties of the dendritic tree. In this cases, “topological points” (branching and termination points) were used by the program to analyze the morphology of the tree. These parameters were used to describe the size of the cell, the depth of the tree and the branching complexity of the tree. Based on this analysis no differences between the cell types were found. Cable length, branch order and number of branches did not vary significantly differences between cell types (t-test results; cable length: 0.39; branch order: 0.34; number of branches: 0.15). Hence, all 6 models showed very high similarities in size, depth of the tree and complexity (Fig. 10).

The spanning area refers to the area covered by the dendritic tree, the so-called “dendrite spanning field” (Hausen K., 1982). The spanning field was defined by drawing a contour around the dendrite at a distance of 25 µm after orienting the reconstructed neuron along its axonal axis (Fig. 10). Although Cuntz and Forstner (2008) could distinguish the two cell types by the spanning field, it was not possible to find any tendency to different spanning fields under the 6 models used in this work, probably because of the low sample size.

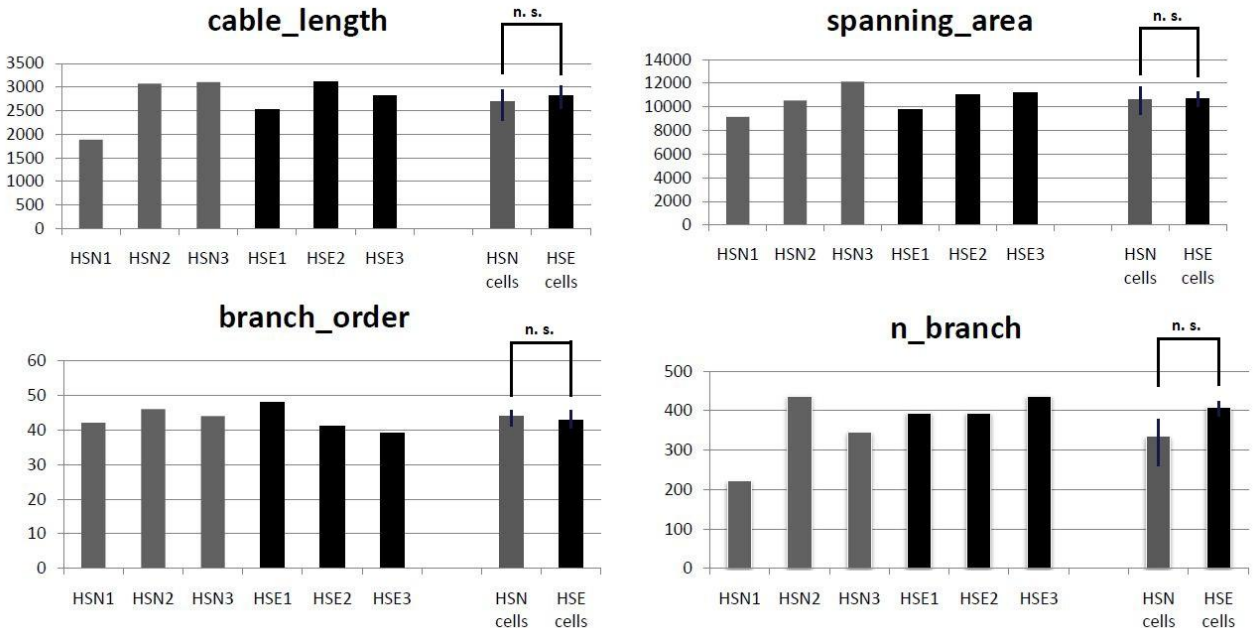

Fig. 10: Dendrite morphological statistics; clockwise: cable length, spanning area, branch order

In addition Sholl analysis (Sholl, 1953) was performed on one of the HSN and one of the HSE cells with complete reconstructed axons. The Sholl analysis traces a series of concentric circles around the end point of the axon of the neuron. Counting the number of times the neuron intersects with the circumference of these circles gives a histogram description of the dendritic tree. The histograms of both cells appeared similar, such that no significant difference could be found between the cell types.

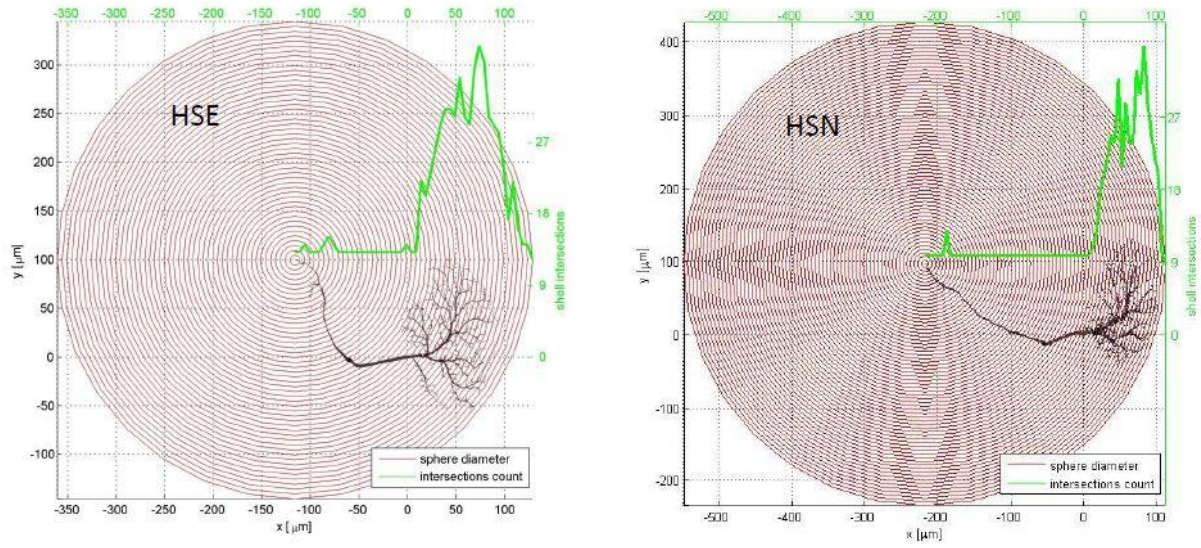

Fig. 11: Sholl analysis. Left: HSE3; right: HSN3

Finally, the three models of the HSN cells were overlaid to illustrate the similarity of the HSN cell type (Fig. 12). The statistical analysis (Fig. 10/11) shows that the shape of the dendritic trees of the 3 HSN cells is comparable. In contrast, the axons had a quite chaotic order. This was probably due to damage of the tissue, since the axonal termini could not be traced to the end.

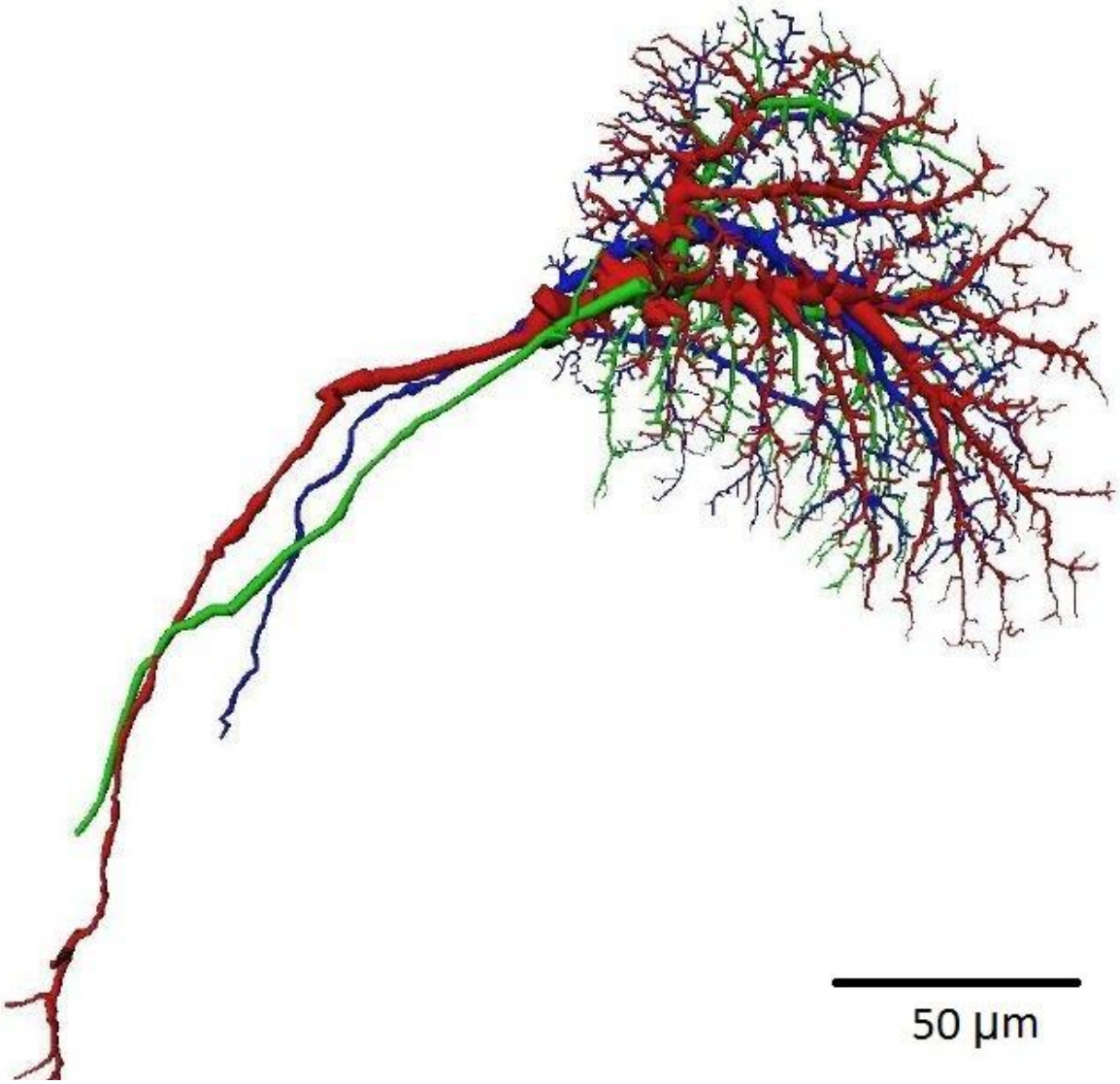

Fig. 12: cell overlay of the 3 models of the HSN-cells

Discussion

In this experimental work, a precise anatomical characterization and reconstruction of horizontal cells (HS) of the lobula plate of *Drosophila* was conducted. Fluorescently targeted dye filled HS-cells allowed exact computational reconstruction of their fine dendritic tips as well as axons. These reconstructions are the first step for future multi-compartmental models, which are essential for realistic models of the lobula plate network. Essentially, reconstructing HS-cells and presenting them in a vectorized format from confocal stacks reduces the data amount from averagely 150 Mb to less than 100 Kb. This data compression enables computational analysis of three dimensional reconstructions of hundreds of cells at the same time, reducing the computational power needed for realistic multi-cellular network models.

The conducted morphological analysis showed that the similarity of individual HS-cells of one type is close enough to propose a “standardized cell” for HSE and HSN in future computational models. This is proposed although no overlap of the axonal processes could be shown (Fig.12). This fact is most probably an artifact, since the axonal output region could not be scanned in several cells, probably due to lesions of the tissue in that region. Hence, the reconstructions of all standard cells seem to be ideal for future models of the lobula plate tangential cell network. This network has already been entitled as “the cockpit” of the fly, since it is thought to be an essential processing stage of motion vision. Therefore, it is surly key for visual course control and thus plays a great role in the acrobatic flight maneuverability of flies. Finally, once all lobula late tangential cells put together in one model of a standard lobula plate, it will be possible to test the network properties in great detail. Moreover, since *Drosophila* allows targeted genetic manipulations, these theoretical predictions can be challenged experimentally with an incomparable specificity.

This work set the ground for further reconstructions of all lobula plate tangential cells in *Drosophila* and will be further pursued in collaboration with Friedrich Foerstner. Furthermore, an extended greedy minimum spanning tree algorithm (Cuntz et al., 2007) will be used to reproduce these reconstructions. This will show if *Drosophila’s* HS-cells follow a similar branching rule as found for *Calliphora’s*, i.e., that their morphological identifier is the shape of their dendrite spanning fields.

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