
WHOLE CELL PATCH-CLAMP RECORDINGS
FROM LOBULA PLATE TANGENTIAL CELLS
IN *DROSOPHILA MELANOGASTER*



Bachelor's Thesis

BIOLOGY

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Zusammenfassung

Das visuelle System ist dafür verantwortlich, optische Reize zu verarbeiten, zu integrieren und damit motorische Vorgänge zu unterstützen. Das Verhalten eines Organismus, der durch visuelle Bewegungsreize beeinflusst wird, kann mit Hilfe eines mathematischen Modells simuliert werden. Der sogenannte Reichardt Detektor sagt das Verhalten eines einfachen Insekts (Käfer) in Reaktion auf visuelle Stimulation voraus. Untersuchungen an bewegungssensitiven Neuronen im visuellen System von Fliegen haben ergeben, dass die Voraussagen dieses Modells auch auf neuronaler Ebene angewandt werden können. Die mathematischen Eigenschaften des Bewegungsdetektors sind weitgehend bekannt, welche neuronalen Strukturen jedoch diesem Phänomen zugrunde liegen ist bislang noch größtenteils im Verborgenen. Mit Hilfe genetischer Manipulationsmöglichkeiten im Modellorganismus *Drosophila melanogaster* wurde bereits dargelegt, dass mindestens zwei Bewegungsdetektoren nötig sind. Durch transgenes Ausschalten von L1 beziehungsweise L2 (Lamina Monopolar Zellen) wurde herausgefunden dass das ursprüngliche Signal in einen ON und einen OFF Signalweg getrennt wird. Mit diesem bahnbrechenden Ergebnis wurde gleichzeitig der neuronale Eingang zu den Bewegungsdetektoren beschrieben: L1 und L2. Um die weiteren Bestandteile des Reichardt-Detektors zu entschlüsseln soll als nächster Schritt die Ausgangs-Ebene auf die Lobula Platten Tangentialzellen bestimmt werden. Nachdem anatomische Arbeiten T4 und T5 Zellen als mögliche Kandidaten vorschlagen, soll die Beteiligung dieser an der Bewegungswahrnehmung untersucht werden. Hierzu ist es nötig per Patch-Clamp Ableitung an Lobula Platten Tangentialzellen die Antworten dieser auf Bewegungsstimuli zu testen. Gleichzeitig können kolumnäre präsynaptische Neuronen – in diesem Fall T4 und T5 Zellen – genetisch ausgeschalten werden. In dieser Arbeit soll die Technik des elektrophysiologischen Ableitens an Tangentialzellen gelernt und in Fliegen mit inaktivierten T4 Zellen praktiziert werden.

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Introduction

Motion detection

Sensory systems provide the interface between us and our environment. We hear noisy objects approaching with our ears, feel the structure of objects with touch receptors, but the visual sense contributes perhaps most strongly to our perception of the outside world. One important function of the visual system is the detection of self-motion which is critical for the coordination of movements. When we turn our head from left to right, the image of the environment on our retina shifts from right to left. The apparent-motion of the surrounding is called optic flow. The mechanism of using optic flow fields to detect self-motion and to compensate for it by changes in movement is known as optomotor response. Optomotor responses can be examined in various species in order to elucidate the computational basis for motion detection in the visual system. Based on behavior in a pioneering study, Werner Reichardt and Bernhard Hassenstein came up with a mathematical model that predicts the turning behavior of a beetle (*Chlorophanus viridis*) on a Y-maze in response to visual stimulation (Hassenstein & Reichardt 1956). The model they

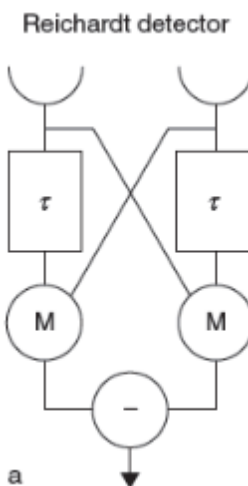


Figure 1 Reichardt Detector (from Borst 2009)

concluded from these experiments very nicely explains the behavior of insect and is also believed to underlie vertebrate motion detection and optomotor-responses. The Hassenstein-Reichardt correlation detector (Fig. 1) consists of two mirror symmetrical subunits (review in Borst 2009). For each subunit, the input signal is delayed by a temporal filter (τ) and subsequently multiplied (M) with the unfiltered signal. The final output signal of the detector results from the subtraction (-) of one subunit output from the other. Nevertheless it is only a theoretical prediction and does not give any hints about the neural network properties that are necessary for its computation. For the past decades, scientists have been trying to

elucidate the biological implementation that forms the basis for this important theoretical model.

Visual system of flies

Flies have been found to be ideal to investigate motion detection. Flies or invertebrates in general have, compared to vertebrates relatively low numbers of neurons (review in Borst & Haag, 2002). Neurons are often uniquely identifiable by their anatomical and physiological properties allowing to study the same neurons with particular response-properties in different animals. Furthermore the handling of flies (i.e. raising, feeding etc.) and the accessibility to the neuropils of interest such as the optic lobes is relatively easy. Thus in vivo electrophysiological recordings und visual stimulation can be performed effectively (Borst & Haag, 1996; Joesch et al., 2008). Due to these features, the visual system of flies has been studied intensively over the last decades and much is known about both anatomy and physiology (Hengstenberg et al., 1982; Fischbach & Dittrich, 1989; Scott & Raabe, 2002). Differently from vertebrate visual systems, insects have compound eyes consisting of a large number of facets, also called ommatida. The retina of a fruit fly for example consists of approximately 700 facets (Chapman, 1998). The visual system of flies is organized in a columnar fashion, i.e. every column contains generally the same set of cells with as many columns as there are facets. Each ommatidium is composed of eight photoreceptor cells (R1-R8) that are divided in an inner (R7&R8) and an outer group (R1-R6). The inner group of photoreceptor cells is thought to be primarily involved in color vision whereas the outer group has been shown to play an important role in motion detection. The fly's optic lobes consist of four different neuropils. Five different monopolar cells (L1-L5) in the lamina receive input from the outer photoreceptor cells. The medulla, the next neuropil is quite complex and comprises many different cell types such as intrinsic cells (Mi), transmedulla interneurons (Tm) that connect distinct layers of the medulla to the lobula plate and TmY cells (Fischbach & Dittrich, 1989). The lobula complex consisting of the lobula and the lobula plate forms the last neural structure in the optic lobes. In the lobula

plate, the prominent group of large wide-field cells called lobula plate tangential cells is located (review in Borst & Haag, 2002). These neurons are characterized as directionally selective to optic flow and are considered to be involved in course control (Borst & Haag, 2002). Motion in the preferred direction (PD) of lobula plate tangential cells causes activation, i.e. depolarization of the membrane potential or an increase in firing rate, whereas movement in their anti-preferred direction (null-direction, ND) causes a hyperpolarization or a decrease in firing rate (Hengstenberg et al., 1982). The lobula plate tangential cells are grouped – according to their preferred orientation – into two major groups; horizontal motion sensitive (HS) and vertical motion sensitive cells (VS). Interestingly, the response properties of these giant neurons can be modeled by motion detectors of the correlation type, such as the Reichardt detector (Borst & Haag 1996). Thus, the mathematical model based on the turning behavior of a beetle also holds true for the electrical responses of motion sensitive cells in the lobula plate. Still, this does not yet answer the question about the neural location of the detector. All facts presented so far have mainly been found in experiments in blow-flies (*Calliphora*) and house-flies (*Musca*).

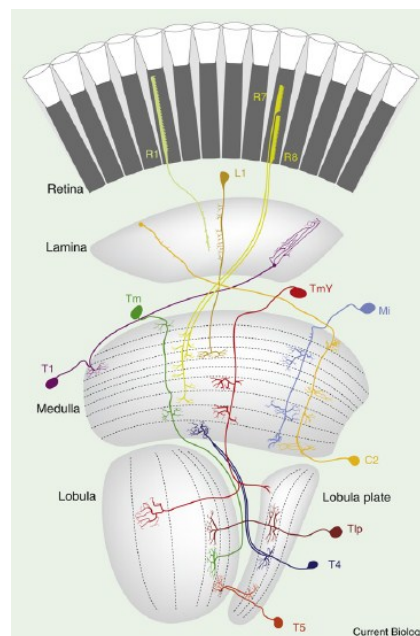


Figure 2 Columnar cell types in the fly visual ganglia (from Borst 2009b)

These two species have the advantage to be relatively big so that electrophysiological recordings to investigate functional properties of single wide field cells have been performed with great success. In order to learn more about network properties of columnar neurons, electrical recordings from single neurons, presynaptic to lobula plate tangential cells are necessary. The density of neurons in the lamina and medulla is very high and at the same time their size is very low. This combination makes it very difficult to record reproducibly from single cells. Therefore new techniques had to be introduced to help scientists to manipulate the neural network.

Genetics

With the fruit-fly *Drosophila melanogaster* an entirely new sophisticated toolbox has entered the field: genetic manipulations. Using the so called Gal4/UAS system (Fig 3), one can express any gene of interest specifically in certain cell types (Brand & Perrimon, 1993; review in Luo et al., 2008). The Gal4 gene derived from yeast, codes for a transcription activation protein. Gal4 can be expressed under control of regulatory sequences (enhancers), and thus in specific cell types in the organism. The upstream activation sequence (UAS) is a binding site for the Gal4 protein that controls a gene linked to it. Thus the binding of Gal4 to the UAS activates the transcription of the gene downstream of the UAS. Fluorescent proteins derived from the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* have played a major role in the development of knowledge about the network in the optic lobe. Genes, encoding for fluorescent proteins can be fused to the UAS to label certain cells. A modification of the Gal4/UAS system is the so called split Gal4 (Luan et al., 2006, review in Luo et al., 2008). For this method, the Gal4 gene is split up into two parts: the activation-domain (AD) and the DNA-binding-domain (DBD). These two fragments can now be fused to different enhancers, for instance specific for certain neurotransmitter classes. The transcription of the gene of interest is only active in cells,

where both AD and DBD are present and can be used to restrict initially broad expression patterns. Besides anatomical studies, the genetic toolbox can be used for diverse other purposes. There are several UAS-gene constructs that lead to inactivation of neurons e.g. by apoptosis or by expressing mutant, non-functional proteins. One commonly used method to manipulate cells is the temperature sensitive dynamin-mutant *shibire^{ts}* (Kitamoto 2001). The phenotype of *shibire^{ts}*-expressing flies is the inability of endocytic vesicles to be separated from the membranes and thereby the depletion of vesicles especially in the synaptic terminals, resulting in a loss of synaptic transmission. The temperature sensitive nature of the mutated protein allows the conditional silencing of genetically targeted cell types. All these previously described genetic tools can be applied in *Drosophila* and help functionally dissect the neural implementation of the Reichardt-detector.

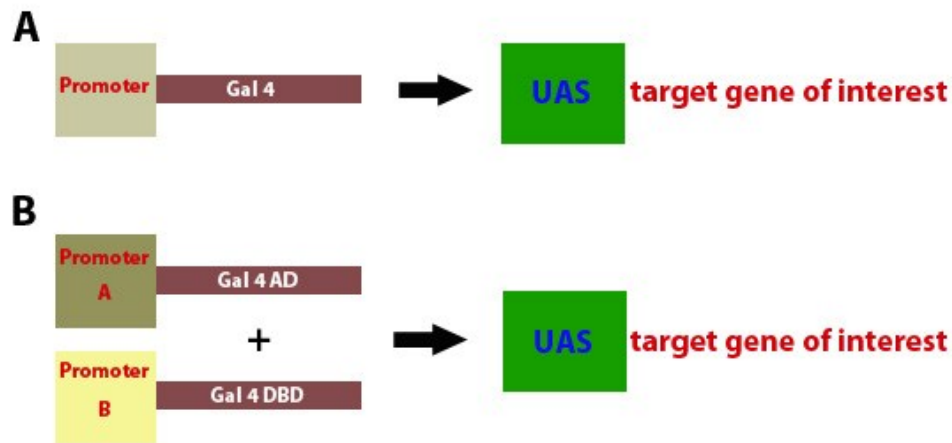


Figure 3 Schematic representation of the Gal4/UAS System expressed in transgenic *Drosophila melanogaster*.
A: Gal4 UAS system **B:** Split Gal4 system AD = activation domain; DBD = DNA binding domain

T4/T5

In 2010, Maximilian Joesch et al. have used these genetic tools to identify the input channels to the motion detector (Joesch, Schnell et al. 2010). By silencing individual cell types in the lamina and simultaneously recording from lobula plate tangential cells they found that the input signal is split into an ON (via L1) and an OFF (via L2) pathway. This groundbreaking finding suggests that there is not only one motion detector responsible for the optomotor responses but at least two. At the same time it points out once again the functional similarities between invertebrate and vertebrate sensory systems. The On- and Off-center visual fields of vertebrate retinal ganglion cells are known for a long time. Now that we know the input to the motion detector one could ask what are the output-cells that are supposed to be presynaptic to the LPTCs. Anatomical studies suggest possible candidate neurons for this role (Strausfeld & Bassemir, 1983; Buchner & Buchner, 1984). Both T4 and T5 cells ramify in the lobula plate and are assumed to provide the main synaptic input to the tangential cells. T4 connect the medulla to the lobula plate and is thought to get input indirectly from L1 via medulla interneurons. T5 has dendrites in the lobula, and is thought to get input from L2 via Tm cells (Fischbach & Dittrich 1989). Based on these anatomical properties, their roles can be logically connected to either the ON or the OFF pathway. With the same approach as Joesch and colleagues have used to investigate the role of lamina monopolar cells, one can also examine the contribution of T4 and T5 cells to the motion-detector. In order to test the effect of silenced columnar neurons on tangential cell responses, patch-clamp recordings have to be performed. In the following study the establishment of patch-clamp recordings on lobula plate tangential cells will be presented.

Methods

Flies & Genetics

Most flies were provided from Gerald Rubin's lab at Janelia Farm who generated an extensive collection of cell type-specific Gal4 lines. These flies were raised on a standard cornmeal-agar with a 12 hours light/12 hours dark cycle, at 20°C and 60% humidity. We used female experimental flies one to two days after eclosion. For the first experimental setup, transgenic flies were used, expressing GFP predominantly in the somata of lobula plate tangential cells (fly line DB 331 CB, *tab1*). These flies have been used to practice the patch-clamp technique as the somata of the cells of interest are easily detectable. In order to generate flies that specifically express genes of interest in T4 cells the split Gal4 approach is used. Therefore flies are combined, using a DBD fragment fused to a promoter that is only active in cholinergic neurons (*Cha-DBD*) and the AD domain of a line that shows a high level of expression in both T4 and T5 cells (*42FOG*). As T4 cells, in contrast to T5, are probably mostly cholinergic (Raghu et al., 2011), this split Gal4 combination should only express in T4 cells. The split T4 line is subsequently crossed with lines expressing either UAS-GFP or UAS-*shibire*^{ts}. The UAS-GFP is used to control the expression level of the driver lines and additionally to check its specificity. Flies that expressed *shibire*^{ts} were used in two different conditions. To activate the dynamin mutation in the neurons, flies have to be heat-shocked prior to the experiment. To this end they were kept at 37°C for 60 minutes and experiments were then performed within one hour after induction. Control flies, with the same genotype did not undergo heat-shock and thus T4 cells remained functional.

Table 1 Fly-lines used for experiments

Abbreviation	Genotype	Phenotype
DB331-CB	DB331 ; mCD8-TN-XL-8aa; +	GFP-labeled somata of LPTCs
Split T4-shi²	shi ^{ts} /w ⁺ ; 42FOG-AD/+ ; shi ^{ts} /Cha-DBD	T4 cells silenced by shibire ^{ts} (two insertions)
Split T4-shi¹	w ⁺ ; 42FOG-AD/+ ; shi ^{ts} /Cha-DBD	T4 cells silenced by shibire ^{ts} (one insertion)
Split T4-GFP	w ⁺ ; 42FOG-AD/+ ; MCD8-GFP/Cha-DBD	GFP-labeled T4 cells

Immunohistochemistry

To control the expression level and the specificity the Gal4 lines are crossed to UAS-GFP flies. The brains of the flies are dissected, fixed in Paraformaldehyde (PFA, 4% in PBS), transferred in mounting medium (Vecta Shield H-1000, Vector Laboratories, Inc. Burlingame, CA) and scanned under the confocal microscope (Leica SP5). Confocal stacks are post-processed with ImageJ (National Institutes of Health, Bethesda, MD). In order to visualize the background structures to identify the different neuropils immunohistochemical stainings of the fly-brains are produced. Therefor antibodies against the postsynaptic protein “disc large” specifically bind neural tissues. A secondary antibody that is connected to the fluorescent dye Alexa 568 then binds to the primary antibody and thereby stains the neuropil red.

Preparation

Flies were waxed onto plastic holders with their legs fixed. The head was bent in order to access the brain posteriorly at the region of interest. The fly was then covered with aluminum foil covering everything but the backside of the head to maintain accessibility. Subsequently the cuticle covering the right hemisphere of the optic lobe was removed carefully keeping all structures, including trachea untouched. To prevent the fly-brain from drying and osmotic shock the whole dissection procedure was performed in Wilson-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, CaCl₂ 1.5 (adjusted to pH 7.2-7.4). The different steps in the preparation procedure can be seen in figure 4. The final arrangement of the fly under the microscope is shown schematically in figure 5.

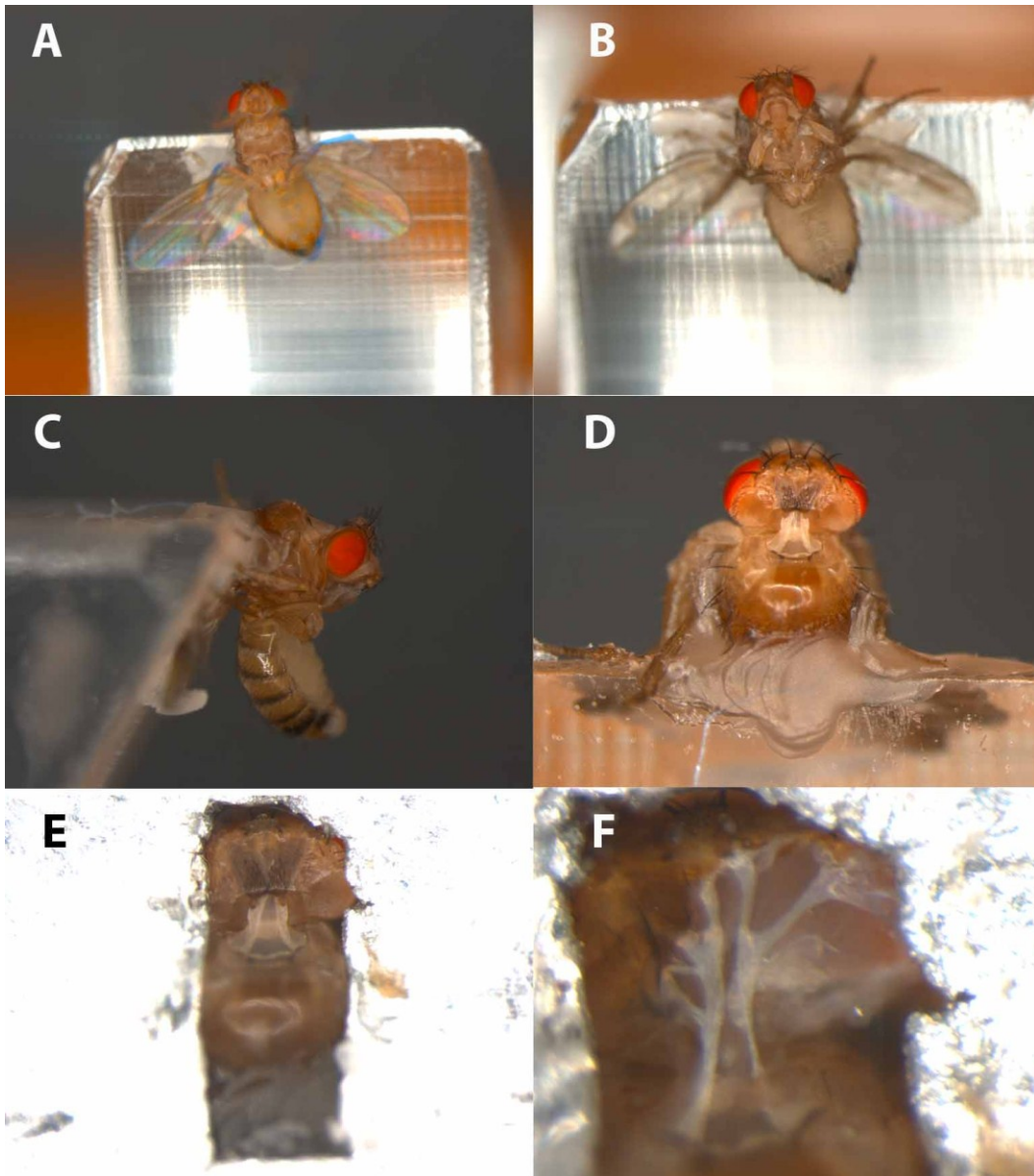


Figure 4 Preparation of a fly; **A:** dorsal view on fly waxed to a Plexiglas holder, moving legs freely, **B:** dorsal view on fly with legs waxed to the holder, **C:** lateral view on fly with proboscis waxed onto the throax, **D:** dorsal view on fly with fixed head, **E:** dorsal view on fly head partially covered with aluminum foil, **F:** dorsal view on fly brain (cuticle partially removed)

Electrophysiology

The experimental fly was placed under a fluorescent 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; $\infty/0$; Zeiss Jena; GER), facing the center of a custom designed LED arena (Joesch et al., 2008) in an angle of 90°. To achieve accessibility to the somata of the lobula plate tangential cells, the neurolemma had to be removed locally. This was done by applying Collagenase (0.5 mg/ml in Wilson-Ringer) with a glass pipette (cleaning pipette, WPI 4IN Thinwall 1.50 OD) to the region where the cells were expected. The removal of the surrounding tissue made the cells visible using polarized light contrast microscopy. The clean somata could now be approached with the patch-clamp electrode. Both the cleaning and patch-clamp (WPI 4IN Thinwall 1.50 OD) electrodes were pulled on a filament puller (Sutter Instruments P97). The electrical resistance of the patch-clamp electrodes was between 6 and 10 M Ω . The cleaning electrodes have an average diameter of 5 μ m. The patch-clamp electrode is filled with intracellular solution (Tab. 2) that mimics the composition and properties of the cytoplasm in order to keep the cell alive as long as possible during recording. The fluorescent dye Alexa 568 was used to visualize and characterize the neurons after recording. Once the recording electrode is filled with intracellular solution it is connected to a head stage and then to an amplifier (npi BA-1s) and both, the reference- and recording-electrode are placed in the bath. To keep the extracellular solution (Wilson Ringer) from entering the recording electrode and mixing with the intracellular solution, slight air-pressure is applied. To this end the patch-clamp-electrode is connected to a flexible tube where pressure and suction can be introduced orally. The establishment of patch-clamp recordings can be divided into two parts; the first part is to form a so called “giga-seal” between the tip of the electrode and the plasma-membrane of the neuron. The successful formation of the giga-seal can be detected by permanently injecting small current pulses (Amplitude: ± 10 nA, frequency: 4Hz) during approach. The resulting voltage difference between recording- and reference-electrode is

monitored via loudspeakers. Once a giga-seal has formed, the voltage increases dramatically due to the high resistance, according to Ohm's law ($V = R * I$). This results in an increased loudness of the audio-signal. The second step is to break into the cell to obtain access to the membrane-potential. This is achieved by applying light suction on the electrode. Once the membrane potential of the cell is reached, visual stimulation and electrical recording is initialized via the computer. To reduce noise, the voltage signal is low-pass filtered (cut-off frequency: 3 kHz).

Table 2 Intracellular Solution for Patch-clamp recordings

Compound	Final Concentration [mM]
K-Aspartate	140
HEPES	10
Mg-ATP	4
Na3-GTP	0.5
EGTA	1
KCl	1
Alexa Fluor 568 Hydrazide Salt	0.015
pH set to 7.26 with KOH (1M)	

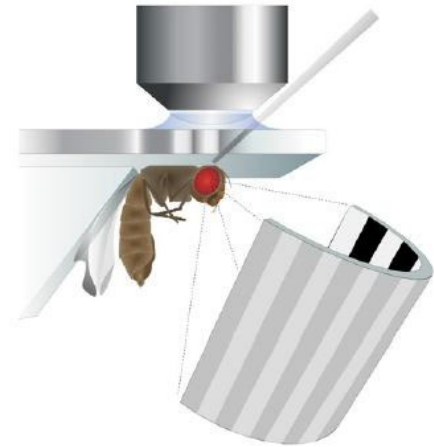


Figure 5: Scheme of experimental setup (from Schnell et al., 2010)

Data Processing

The LED-arena (Joesch et al., 2008) is controlled and initiated using Matlab (MathWorks, version R2010b). Furthermore, Matlab is used to record the voltage and the arena-trigger signal via a data acquisition card (PCI-DAS6025 Measurement-Computing) and the data acquisition toolbox in Matlab. Two generally different sets of stimuli are used for the experiments: Single bars and square wave gratings on the one hand and On- and Off-edges on the other hand. Furthermore the response to flicker (light on and light off) stimuli is tested. Table three shows an overview over both stimulation-protocols referred to as 'Stimulation' (single bars and square wave gratings) and 'Stimulation_Edges' (On- and Off-edges) respectively. Illustrations of the stimuli are depicted in Figure 6. The mean over all trials of the same stimulus is calculated and plotted using a Matlab script. These plots are shown in the results part.

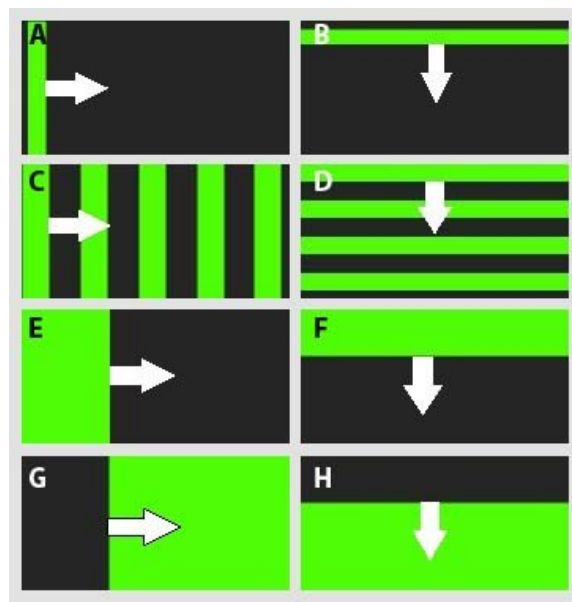


Figure 6 Illustration of Stimuli played on LED arena white arrow indicates direction of motion (also played in opposite direction); **A:** single bar moving horizontally, **B:** single bar moving vertically **C:** square wave grating moving horizontally **D:** square wave grating moving vertically **E:** On-edge moving horizontally **F:** On-edge moving vertically **G:** Off-edge moving horizontally **H:** Off-edge moving vertically

Table 3 Stimulation protocol

index	Stimulation	Stimulation_Edges
1	Flicker	Flicker
2	Single bar moving down	On Edge moving from left to right
3	Single bar moving up	On Edge moving from right to left
4	Single bar moving from left to right	Off Edge moving from left to right
5	Single bar moving from right to left	Off Edge moving from right to left
6	Square-wave-grating moving from right to left	On Edge moving down
7	Square-wave-grating moving from left to right	On Edge moving up
8	Square-wave-grating moving up	Off Edge moving down
9	Square-wave-grating moving down	Off Edge moving up

Results

Characterization of the visual response properties of lobula plate tangential cells

The main aim of this thesis is to learn the technique of whole-cell patch-clamp recordings in the visual system of *Drosophila melanogaster*. For the first trials, flies with GFP-tagged LPTC-somata were used to simplify the identification and detection of the relevant cells. By switching the filter-set in the fluorescent microscope during collagenase application, from polarized light to a filter where both red and green light can pass, the position of the cells could be controlled. Once the cell-bodies were visible under polarized light, the red-green filter was not necessary any longer. This aid simplifies the selection of the right area enormously and thereby reduces the error-rate tremendously. Figure 7 shows a maximum intensity projection of a confocal scan of an optic lobe, where the somata of LPTCs are tagged with GFP (green). To visualize the background-structures, an antibody that binds to the postsynaptic protein disc large (Dlg) was used to stain the whole optic lobe (red).

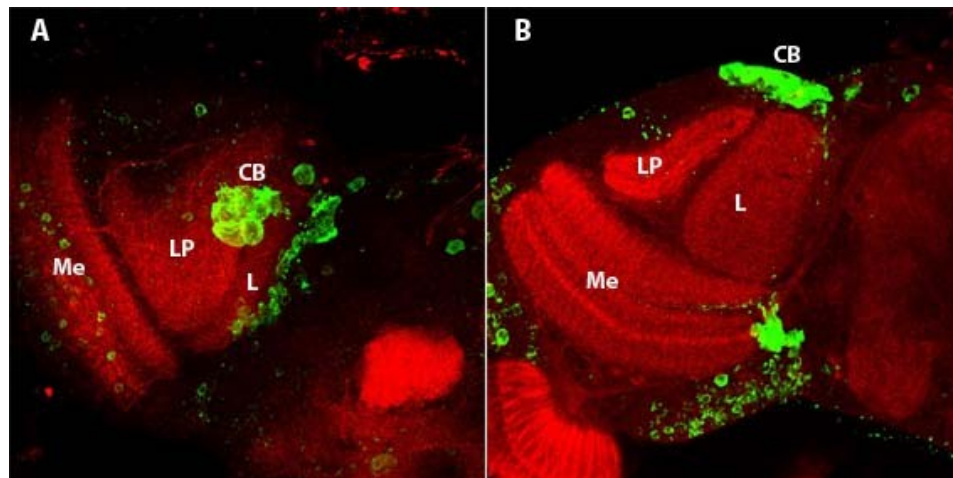


Figure 7 Maximum Intensity projection of DB331-GFP-CB fly brain; green: GFP labeled somata of LPTCs, red: background-staining (anti-Dlg Alexa 568); LP: lobula plate, L: lobula, Me: medulla, CB, LPTC cell bodies; **A**: dorsal view onto optic lobe (as during experimental procedure), **B**: lateral view on optic lobe

The physiological properties of the cells, such as motion sensitivity in a direction selective way, are considered not to be influenced by the transgenic expression of GFP. Figure 8 shows a recording from a fly that expressed GFP in the tangential cells somata (DB331-GFP-CB). The arena-controller sends a trigger signal to the computer, indicating the duration of the stimulus (green trace). The voltage recorded during the experiment is illustrated in blue. The potential measured was at -40mV. To calculate the real membrane potential of the cell, the liquid junction potential (-10mV) has to be added. Thus V_m of the cell the recording was performed is -50mV. The direction of the visual stimulation is indicated by black arrowheads. For this experiment the 'Stimulation'-protocol (see table3, first column) was used. The cell responded most strongly to vertical motion. Downward moving edges caused depolarization (PD) whereas upward moving edges elicited hyperpolarization (ND). This can be observed best during the first two stimuli in figure 8A (vertically moving single bar). For better visualization the traces for these two conditions are illustrated in a magnified way (Fig 8B). Although the cell responded primarily to vertical motion, one can also observe a slight change in membrane potential upon horizontally moving stimuli. The third stimulus (single bar moving from left to right) also causes a depolarization. These general response properties speak in favor of a VS cell. Some VS cells are known to respond partially also to horizontal motion (Joesch et al., 2008). The temporal delay of this stimulus as compared to vertically moving gratings can be explained by the properties of the visual field of the cell. The cell was recorded in the right hemisphere of the optic lobe, consequently the cells' receptive fields are also mainly on the right side of the fly. However, the single bar started in the left visual field and traveled over the whole arena to the right. When it enters the receptive field after a certain delay, the cell responds by depolarization. During stimulation with horizontal square-wave-gratings responses can be observed though again much smaller than to vertical motion. Furthermore the response saturates relatively quickly and the membrane potential slowly returns to resting potential. In order to present the data in a more significant way and to compress the information of

several trials to only one graph the average voltage across several trials for each stimulus is calculated.

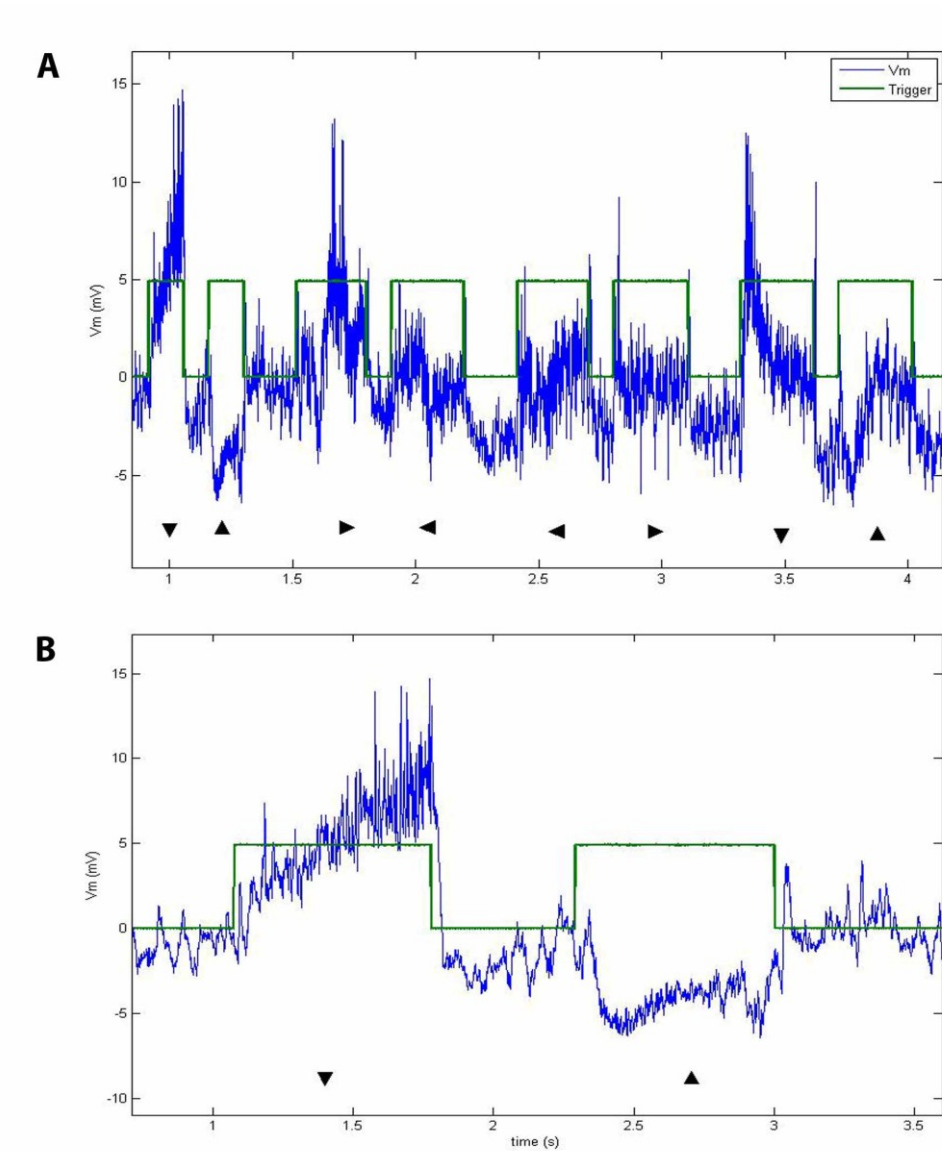


Figure 8 Recording from VS cell: unprocessed data originating from the recording of a vertical sensitive lobula plate tangential cell; green trace: trigger signal from arena, blue trace: membrane voltage (V_m); the black arrowheads at the bottom indicate direction of visual stimulation. Visual stimulation according to table3 (Stimulation), starting with downward vertical single bar stimulus. **A:** Recording of one whole repetition of the Stimulation-protocol; **B:** close up of first two stimuli (single bar PD and ND)

To this end, single trials are defined by beginning and end of the trigger signal. By setting the number of different stimuli and playing the stimuli always in the same order, one can write a program that automatically selects the same stimuli across several trials and calculates the average of all membrane potentials. Figure 9 shows the average voltage traces recorded from one VS cell in a DB331-CB fly, calculated over 5 trials. On average one can nicely see the strong depolarization upon motion in the preferred direction (5mV on average) and a little weaker hyperpolarization in null-direction (-2.5 mV on average) which is characteristic for lobula plate tangential cells. Furthermore a slightly delayed response compared to the stimulus can be observed. It takes about 30-40 ms until the cell responds to the visual stimulation. This delay can be explained by the temporal filtering properties of the presynaptic network. Another characteristic effect is the rebound of the membrane potential after termination of the stimulus. After the hyperpolarization caused by motion in null-direction (fig. 9B), the voltage does not only recover to resting potential, but has a little depolarizing overshoot of about 3 mV. As already described the effect of horizontally moving stimuli is also observed in the processed data, with the same temporal delay, caused by the size and position of the receptive field of the VS cell. By performing more precise measurements of the visual field one can also determine the type of VS cell (VS1-VS6). Another method is to identify the cell anatomically by intracellular dye filling. This technique will be used in future experiments.

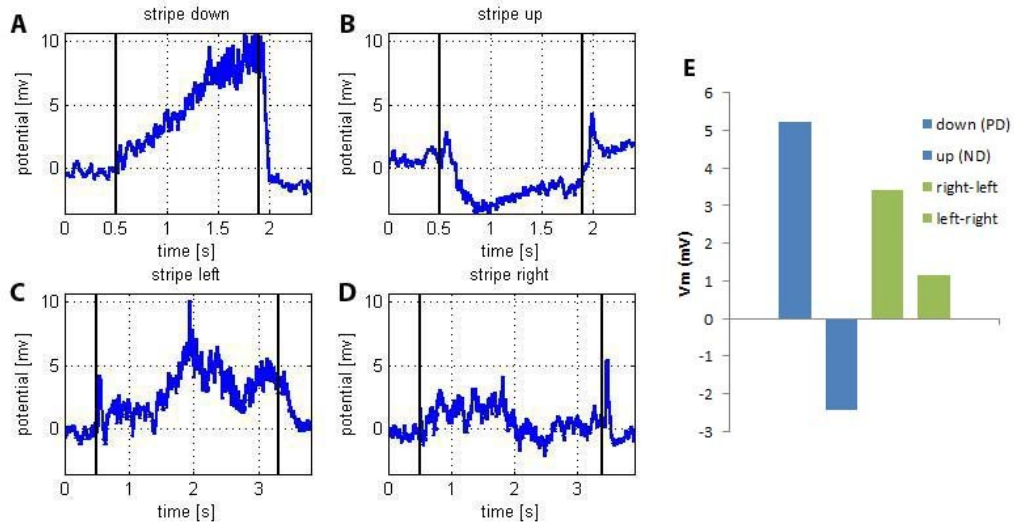


Figure 9 Recordings from VS cells, average over 5 trials, moving single bars: black vertical lines indicate beginning and end of visual stimulation, all subfigures show recordings from the same cell; **A:** membrane potential of VS cell on visual stimulation by a single bar moving downwards (PD); **B:** V_m on visual stimulation by a single bar moving upwards (ND); **C:** V_m on visual stimulation by a single bar moving from right to left; **D:** V_m on visual stimulation by a single bar moving from left to right; **E:** Quantitative illustration of the mean membrane voltage during stimulation

Besides single bars the flies are also stimulated by moving square wave gratings. The recordings from a VS cell (the same cell as used in the experiments in fig.9) in the lobula plate of a fly during visual stimulation with moving gratings are depicted in figure 10. Again strong depolarization on vertical motion in the preferred direction can be observed (fig. 10C). Differently from single bar stimuli, the cell adapts to the strong stimulation by reducing the gain of the response from about 10mV at beginning of the stimulus to 3mV towards the end. This might be due to presynaptic and intrinsic mechanisms to ensure excitability and to prevent saturation. Another difference from the single bar stimulation is the response to motion in null-direction in that the hyperpolarization for the whole field grating is weaker (fig 10D). The voltage increases 30ms after stimulus onset to about 4mV, this effect is the so called flicker response that is elicited by a rapid change in luminance. This effect can be canceled out by switching on the grating some seconds before the

stimulation starts. As the stimulus has a rather high average luminance, compared to the single bar stimulation, setting the stimulus causes such a flicker response. Comparing the mean membrane potentials during stimulation between all conditions (figure 10A,B,D) that are not thought to cause depolarization, a general shift to a higher level is presented. Given that observation one can interpret the subsequent hyperpolarization to resting potential in figure 10D as an effect caused by vertical motion in null direction. Like with stimulus onset, the flicker response is also present at stimulus offset, with a temporal delay of about 50ms.

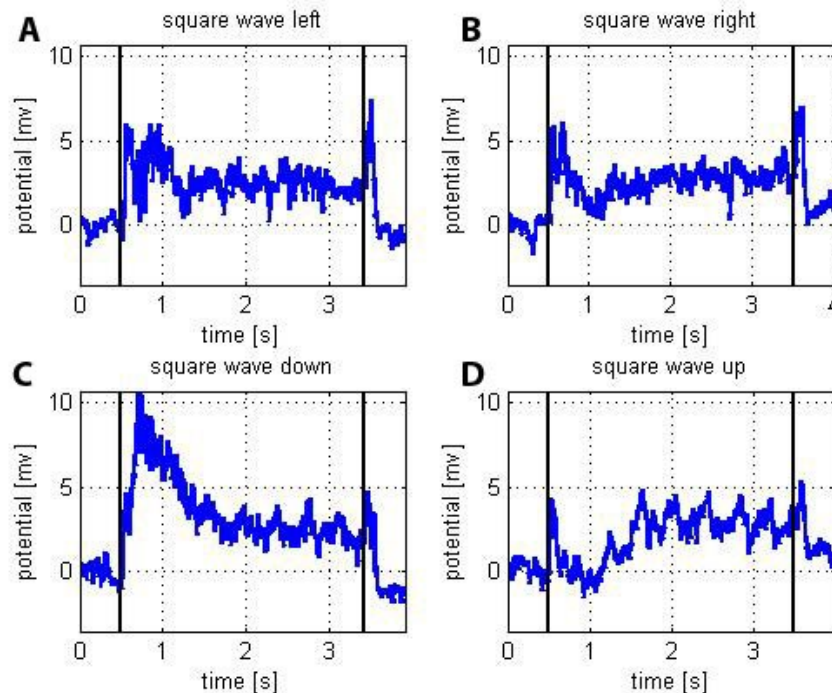


Figure 10 Recordings from VS cell, average over 5 trials, moving square wave gratings: black vertical lines indicate beginning and end of visual stimulation, all subfigures show recordings from the same cell; **A:** membrane potential of VS cell on visual stimulation by a square wave grating moving from right to left; **B:** V_m on visual stimulation by a square wave grating moving from left to right; **C:** V_m on visual stimulation by a square wave grating moving downwards (PD); **D:** V_m on visual stimulation by a square wave grating moving upwards (ND).

Genetic manipulation of the visual motion detection circuitry

Having learned the involved preparation and recording procedure on flies with GFP-tagged lobula plate tangential cell somata, the subsequent step was to perform the more difficult recordings from unlabeled cell bodies. This is necessary because during genetic manipulations of presynaptic neurons it is not possible to express GFP in the LPTCs simultaneously. It has been shown that the signal at the input channel to the Reichardt-detector is split into an On- and an Off-pathway. This finding is based on experiments in which moving On- or Off-edges were presented to flies, lacking either functional L1 or L2 to detect the dependence of the response properties of LPTCs on L1 and L2. We hypothesize that both pathways might still be separated at the output region of the motion detector. Therefore comparable stimuli were used on flies with silenced T4 and T5 cells. It has been shown that such a manipulation of both cells completely abolishes the response of tangential cells on motion indicating further the involvement of these two cell types in visual motion detection (Schnell et al., unpublished). So far there is no data about the contribution of either T4 or T5. In the following experiment T4 cells are silenced specifically by using the Split-Gal4 system in combination with visual stimulation of the same kind as Joesch and Schnell used in their experiments (moving On- and Off- edges). Therefore a fly line is used that carries an enhancer which controls gene expression of the AD part of the split Gal4 transcription factor in both T4 and T5 (42FOG-AD). Flies carrying this genetic construct are then crossed to flies that express the DNA-binding domain of Gal4 and control of a “cholinergic” enhancer (Cha-DBD). In these flies a functionally dimeric “Split-Gal4” protein will be present only in the cell that are in the expression pattern of 42FOG (T4&T5 cells) and use at the same time Acetylcholine (ACh) as a neurotransmitter. As so far only T4 is assumed to be cholinergic, the “Split-Gal4” protein should only be present in T4 cells. For the first experiment, the split-Gal4 line is crossed to UAS-shibire^{ts} expressing flies. Recordings from both, control and induced condition (i.e. incubated at 37°C) flies are performed.

Control Experiments

Figure 11 shows the recordings from two VS cells in two different control flies (no induction). The response of LPTCs to motion stimuli do not show large differences from those performed in the previous experiment. The membrane depolarizes upon motion in the preferred direction (downward, fig. 11A) and hyperpolarizes when the stimulus bar moves in the null-direction (upward, fig. 11B). Furthermore the slight depolarization in response to a left to right horizontal stimulation can be observed (fig. 11C), whereas it does not respond at all to motion in the opposite direction. Again the rebound effect is nicely visible after stimulation (fig. 11B, C, D).

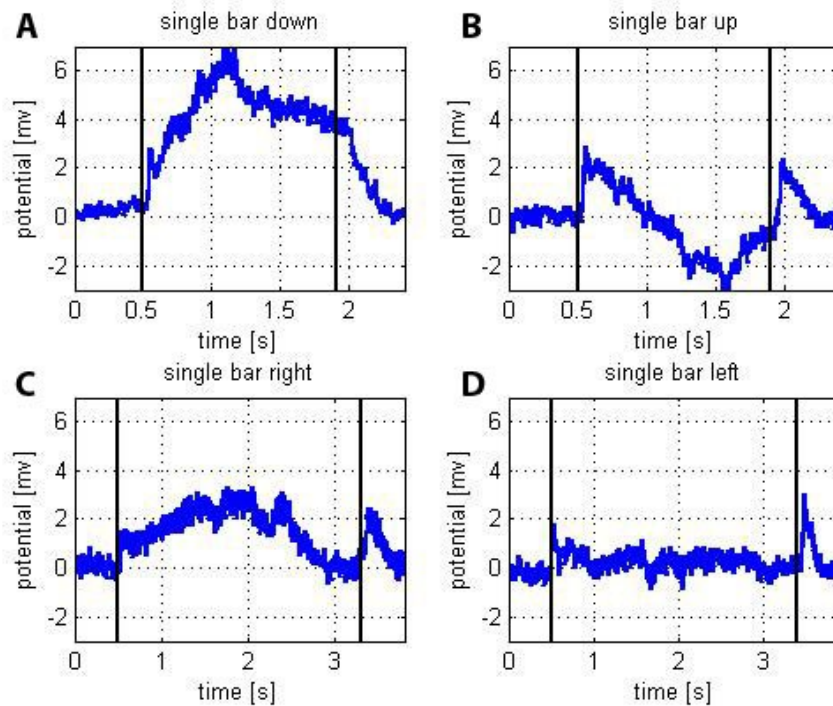


Figure 11 Single bar stimulation of control Split Gal4-UAS shibre^{ts} flies: n=2, trials per recording = 5, representation as in fig. 2&3; **A**: membrane potential of VS cell on visual stimulation by a single bar moving downwards (PD); **B**: V_m on visual stimulation by a single bar moving upwards (ND); **C**: V_m on visual stimulation by a single bar moving from left to right; **D**: V_m on visual stimulation by a single bar moving from right to left.

Besides single bar stimuli, also square wave gratings were presented to the flies during recordings. As already shown in figure 10, here again the response was lower and less clear than for single bar stimulations. Consistent with the data from single bar stimulation, the cell responded by depolarization to stimulation in the preferred direction (fig. 12 C) and by slight hyperpolarization to motion in the anti-preferred direction (fig. 12D). Also the membrane potential was higher during moving edges from left to right than in the opposite direction. The transient responses at the onset of the stimulus are very strong in all cases, due to the sudden increase in luminance. The same effect can be observed at stimulus-offset as the luminance rapidly decreases to zero. In conclusion, it appears as if the response properties of vertical sensitive lobula plate tangential cells can be considered to be normal in uninduced control flies.

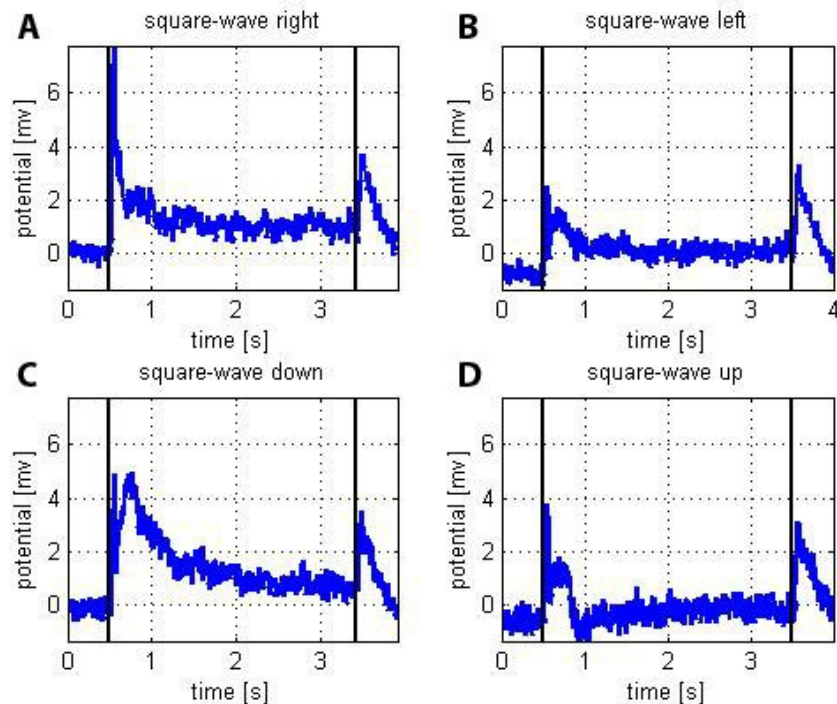


Figure 12 Moving square wave stimulation of control Split Gal4-UAS shibre^{ts} flies: n=2, trials per recording = 5, presentation as in fig. 2&3; **A**: membrane potential of VS cell on visual stimulation by a square wave grating moving from left to right; **B**: V_m on visual stimulation by a square wave grating moving from right to left **C**: V_m on visual stimulation by a square wave grating moving downwards (PD); **D**: V_m on visual stimulation by a square wave grating moving upwards (ND).

The response of VS cells on On- and Off-edge stimulation is depicted in figure 13. The depolarization of the VS cell caused by both, vertical On- and Off-edges in PD is consistent with the results presented so far. Also the hyperpolarization caused by motion in the null-direction is as expected. The slight depolarization prior to the hyperpolarization (fig. 13B) is most probably caused by a flicker response to the rapid change in luminance. The same effect is very strong for moving Off-edges, as for these conditions the arena is illuminated completely in order to present an Off-edge. As the cell is primarily sensitive to vertical motion, recordings for stimuli showing horizontal edges are not shown here.

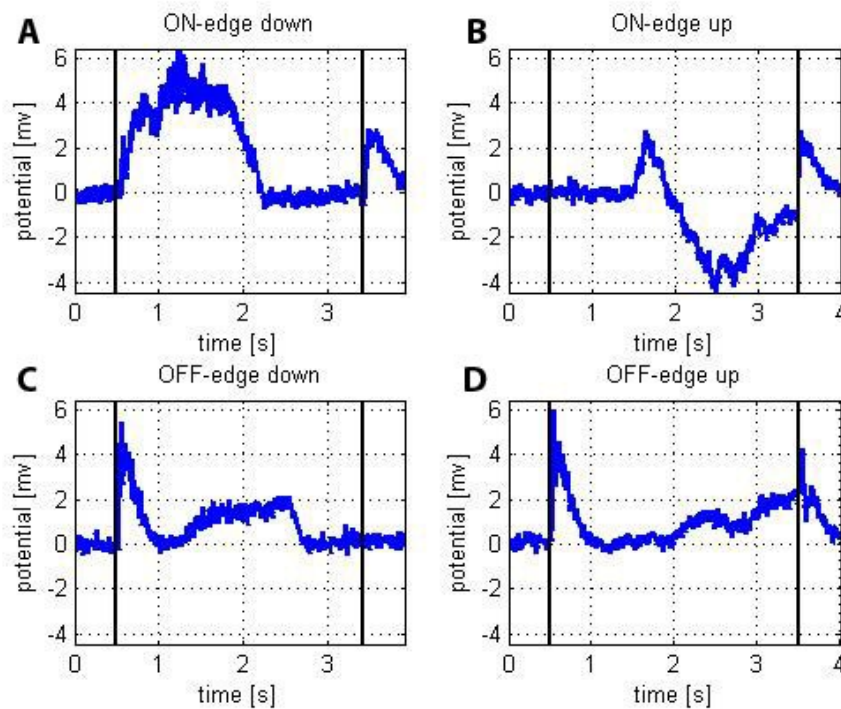


Figure 13 Moving edges stimulation of control Split Gal4-UAS shibre^{ts} flies: n=2, trials per recording = 5, presentation as in fig. 2&3; **A:** membrane potential of VS cell on visual stimulation by a square wave On-edge moving from downwards; **B:** V_m on visual stimulation by a square wave On-edge moving from upwards; **C:** V_m on visual stimulation by a square wave Off-edge moving downwards (PD); **D:** V_m on visual stimulation by a square wave Off-edge moving upwards (ND).

Induced Flies Experiments

Now that we have confirmed the regularity of the response properties of lobula plate tangential cells in not induced Split Gal4 UAS shibire^{ts} control flies, flies are used that have been kept for one hour at 37°C to inactivate dynamin and thereby suppress synaptic transmission in T4 cells. The visual stimulation protocols are as in the previous control experiments. Figure 14 shows the average response of two tangential cells from two different flies. As one can observe in all subfigures, the motion response is completely abolished. Only flicker response is still present for square wave stimulation (fig. 14 E-H). As expected, the same effect can be observed upon stimulation with On- and Off-edges (fig. 15). The response to visual motion stimuli is completely absent, only flicker response remains intact. Motion vision seems to be completely abolished. Due to the absence of any motion response, it is impossible to determine the cell type physiologically. As the injection of fluorescent dye was not successful either the exact cell-type, the response was recorded from remains unclear. During prior recordings from control flies (5 cells, 1HS, 4VS, data not shown) all cells successfully recorded from were either VS or HS cells, thus no cell was patched that did not respond to visual motion. Therefore one can assume that also the cells recorded from in induced flies are very likely lobula plate tangential cells (Either VS or HS). The results depicted in Figure 14 suggest, that either the blocking of T4 cells completely abolishes motion detection or that the Split Gal4 line used for this experiment was not entirely specific for T4 cells. To test for the second possibility, the Split Gal4 line was crossed to a UAS-mCD8-GFP line. Figure 16 shows a maximum intensity projection of a confocal image stack of the optic lob of such a fly. One can clearly identify GFP labeling in the medulla (a), and importantly also in the lobula (b). The somata are situated next to the lobula plate (c). While T5 cell dendrites are located in the lobula the dendrites of T4 cells are confined to the inner medulla layers. We may thus conclude that both cell types are contained in the Split-Gal4 expression line. This finding explains the complete abolishment of visual motion response in lobula plate tangential cells.

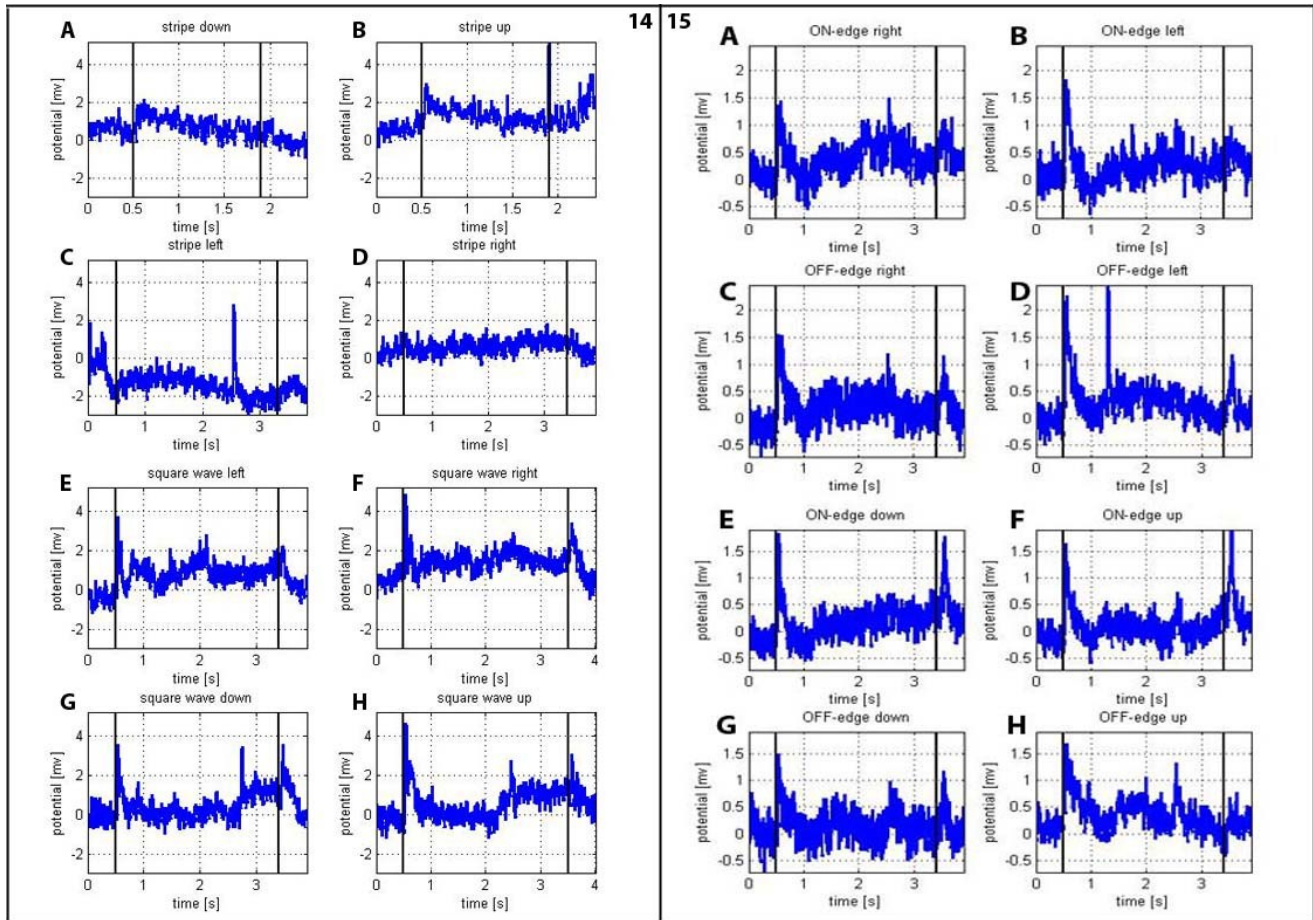


Figure 14 Induced Split Gal4-UAS shibre^{ts} flies, single bar and square wave gratings stimulation: n=2, trials per recording = 5, presentation as in fig. 2&3; **A:** V_m on visual stimulation by single bar moving downwards; **B:** V_m on visual stimulation by single bar moving upwards; **C:** V_m on visual stimulation by single bar moving right to left; **D:** V_m on visual stimulation single bar moving left to right; **E:** V_m on visual stimulation square wave grating moving from right to left; **F:** V_m on visual stimulation by square wave grating moving from left to right; **G:** V_m on visual stimulation by square wave grating moving downwards; **H:** V_m on visual stimulation by square wave grating moving upwards.

Figure 15 Induced Split Gal4-UAS shibre^{ts} flies, edge stimulation: n=2, trials per recording = 5, presentation as in fig. 2&3; **A:** V_m on visual stimulation by ON-edge moving left to right; **B:** V_m on visual stimulation by ON-edge moving right to left; **C:** V_m on visual stimulation by OFF-edge moving left to right; **D:** V_m on visual stimulation by OFF-edge moving right to left; **E:** V_m on visual stimulation by ON-edge moving downwards; **F:** V_m on visual stimulation by ON-edge moving upwards; **G:** V_m on visual stimulation by OFF-edge moving downwards; **H:** V_m on visual stimulation by OFF-edge moving upwards.

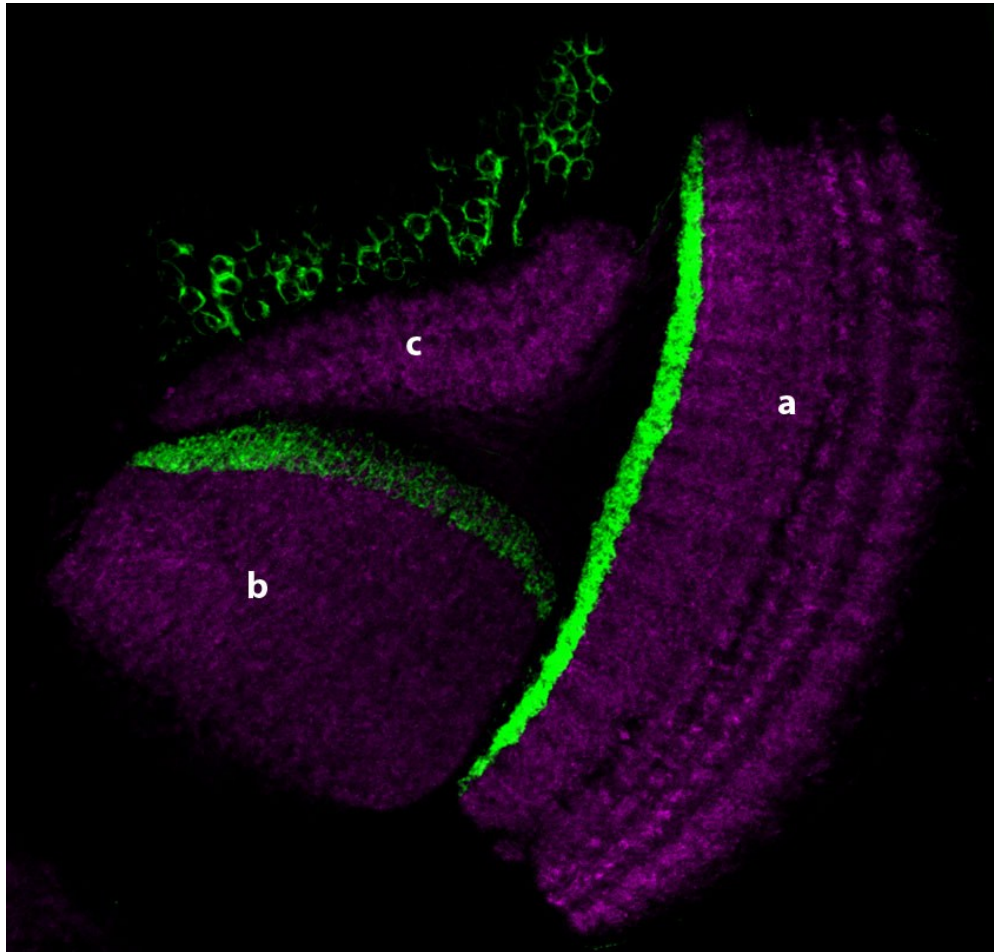


Figure 16 maximum intensity projection of Split Gal4 42FOG/Cha-DBD – UAS GFP background labeled (purple) using Dlg-antibodies; a: medulla; b: lobula; c: lobula plate; GFP expression in medulla and lobula plate indicates expression in both T4 and T5 cells

Discussion

The main aim of this thesis was to learn the general principles of whole cell patch clamp recordings in the visual system of *Drosophila melanogaster*. To this end, a recording set-up was assembled. For visual motion stimulation a LED arena was constructed. Once the basis was set, the involved preparation procedures and in vivo recordings had to be learned. While the recording from labeled somata could be established within rather short time, the detection of unlabeled cells turned out to be more challenging and time consuming. The preliminary results presented in this thesis may have interesting implications which are being discussed below. To confirm these findings, more experiments have to be performed in the future.

The method to generate fly lines with relevant genotypes used for this thesis is based on neurotransmitter specific cell types. To be able to use these lines in a meaningful way, knowledge about the transmitter specificity of certain neurons has to be available. Immunolabelings are one method to determine the neurotransmitter certain cell-types use. In this approach antibodies bind to structures that are specific for distinct neurotransmitter classes. Cholinergic neurons for instance are identified by the Choline acetyltransferase (ChAT) an enzyme that is responsible for the assembly of Acetylcholine (ACh) in the synapses. To identify the neurotransmitter-class of single neurons, pure immunohistochemical approaches are not feasible. The visual system of flies contains a large number of cholinergic neurons that are all labeled by these antibodies, thus one cannot resolve single cell types positive for ChAT. In *Drosophila* one can transgenically control the expression of fluorescent proteins in cholinergic neurons using the Cha-promoter that controls the expression of ChAT and the vesicular Acetylcholine transporter (vAChT) which are both specific for cholinergic neurons. By using a Cha-Gal4 construct and the MARCM (mosaic analysis with a repressible cell marker) method (Wu & Luo, 2007), in which the expression of a fluorescent protein is initialized by heat-shock in different stages

of development, one can create single cell clones of cells using ACh as a neurotransmitter. The analysis of *Drosophila's* visual system has shown that at least one T4 subgroup, branching in the second layer of the lobula plate, is considered to be cholinergic (Raghu et al 2011). Furthermore this study has confirmed the specificity of the Cha-Gal4 line for cholinergic neurons. The authors compared the expression pattern of the Gal4 line with immunohistochemical staining against ChAT and found 100% of the cells in the expression pattern of the Cha-promoter to be labeled by ChAT antibodies. Nevertheless only about 85% of all ChAT immuno-positive cells seem to be included in the expression pattern of the Cha-Gal4 line. The same promoter that was used for the MARCM studies has also been used to drive the DNA-binding domain (DBD) of the Split-Gal4 in the previously described experiments. In addition the promoter that drives the activation domain (AD) of the Split-Gal4 (42FOG) used in this study is thought to be active in all T4 and T5 cells. Experiments with this promoter controlling the expression of UAS-GFP have shown that the cells in this pattern ramify in all four layers of the lobula plate (data unpublished). These facts confirm that the flies used for the experiments in this thesis express in cells that are either T4 or T5 and at the same time use ACh as a neurotransmitter.

The output of the motion detector onto the large-field lobula plate tangential cells is thought to be mediated via inhibitory and excitatory inputs (Joesch et al., 2008; Borst et al., 2010). The two candidate neurons that were hypothesized to mediate these inputs are T4 and T5 cells (Strausfeld & Lee, 1991). The fact that nicotinic-acetylcholine receptors have been found on HS-cell dendrites (Raghu et al., 2009) is an indication that speaks in favor of a contribution of cholinergic neurons to motion detection. The results presented in this thesis confirm this theory and at the same time suggest new findings on the field of neurotransmitter classes for these neurons. As figure 16 shows, the Cha-Gal4 promoter drives expression not only in T4 cells as expected, but also in some T5 cells. This might be an indication that a subgroup of T5 cells also uses ACh as a neurotransmitter. Additionally the data from whole-cell patch-clamp recordings in LPTCs with silenced T4- and apparently also T5-cells support this suggestion. The response to visual motion stimulation was completely

abolished. This argues strongly for the involvement of both T4 and T5 in motion detection. Interestingly, responses to flicker stimuli are still present, indicating a different pathway for this effect. Even though more experimental flies have to be tested to confirm this hypothesis, the findings can be considered as interesting and promising results on the way to decrypt the motion detector. Moreover the described effects of silencing cholinergic neurons on motion detections are further supported by experiments performed in similar Split-Gal4 lines (unpublished data).

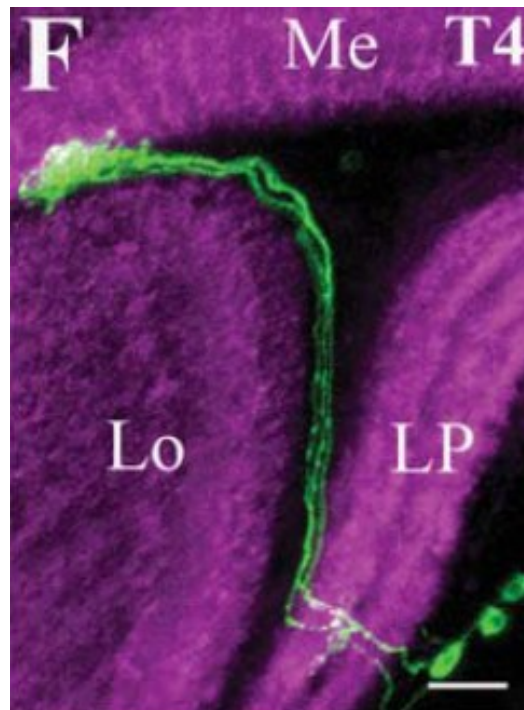


Figure 17 Cha positive T4 cell (green) in MARCM study, Me: medulla, LP: lobula plate, Lo: lobula. (From Raghu et al. 2011a)

Recent studies have brought up evidence that at least three of four T5 sub-classes are glutamatergic (Raghu et al 2011b). Glutamate is very well investigated in motor neurons and at the neuromuscular junction in insects (Usherwood & Machili, 1968; Jan & Jan, 1976), where it acts as an excitatory neurotransmitter. In other neural tissues however, it can exhibit inhibitory effects on the postsynaptic cell, for instance via glutamate gated chloride channels (Lea & Usherwood, 1973). Thus the effect of glutamate on postsynaptic neurons depends on the receptor type present at the postsynapse. It follows that to determine the role of glutamate in visual motion detection, more studies about the glutamate-receptors are necessary. Initially, the finding of glutamatergic T5 cells seems to contradict the results of the previously described experiments, but first of all not all T5 cells were found to be glutamatergic and at the same time, not all T5 cells were present in the expression pattern of the experimental Split-Gal4 line. So there might be T5 cells that are solely cholinergic. Furthermore one cell type can release more than only one neurotransmitter. There can be both, glutamatergic and cholinergic T5 and T4 cells. The fact that T5 cells were not detected in the MARCM analysis for cholinergic neurons can be explained by the difficulties of this approach per se. In the MARCM analysis the frequency of finding specific cells is rather low. For the Cha-Gal4 study for instance, approximately 3000 brains had to be analyzed to identify 33 different cholinergic cells (Raghu et al., 2011). Therefore the absence of a cell type in the MARCM analysis does not implicate that this cell type is not cholinergic. This is most probably also the reason why only one of four T4 cell-types could be found in the Cha-Gal4 expression pattern. Taken together the data presented in this thesis confirms the contribution of both T4 and T5 cells to motion detection. Furthermore evidence has been found that besides T4 also T5 cells might be cholinergic. How the at least partial usage of the same neurotransmitter types can go together with the push-pull model of separated excitatory and inhibitory inputs to tangential cells remains a matter of speculation at the moment and needs to be investigated further.

Outlook

Engineering flies that specifically express in either T4 or T5 cells has the highest priority for subsequent studies. For that purpose injections have been performed to produce flies that express a flippase under control of the ATE-promoter (provided from Bassem Hassan's lab). The flippase should thus be expressed in neuroblasts that are implicated in development of T4 and T5 cells. These flies can now be combined with neurotransmitter-specific lines, such as Cha-Gal4 and then be crossed to UAS-stop-shibire^{ts} to silence the cells. The flippase should only be active in cells that are in both expression patterns, ATE and cha-Gal4. To control the expression, flies containing the Cha-Gal4 ATE construct have to be crossed to UAS-stop-GFP flies. It will be very interesting to see whether the flies resulting from these crossings will also have labeled T5 cells, which would further confirm a possible cholinergic nature of T5. After having clarified the contribution of both T4 and T5 cells to the Reichardt detector one can go further to other columnar neurons presynaptic to the lobula plate that have been suggested by anatomical studies to be involved in motion detection.

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Name und Vorname: _____

Titel der Arbeit: _____

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Betreuer/in der Arbeit bzw. Gutachter/in bei externen Arbeiten:

Unterschrift Betreuer/in:

Datum des Beginns der Arbeit:

Datum der Abgabe der Arbeit

beim Prüfungsamt:

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