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Examination of fly motion vision by functional fluorescence techniques

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1. ABSTRACT

Over the past years, classical electrophysiological approaches to elucidate the functioning of nerve cells have been complemented by functional optical methods, in particular fluorescence imaging. This review illustrates how optical methods have proved helpful in the analysis of the neuronal principles underlying visual motion processing in the fly, a model system which allows physiological investigation under *in vivo* conditions. Many aspects of dendritic processing in large-field motion-sensitive neurons of *Calliphora* have been investigated by Ca^{2+} imaging. In addition, the function of Ca^{2+} can be addressed directly by manipulating its concentration via UV photolysis of caged Ca^{2+}. The extraction of specific motion information from visual stimuli depends on interactions between individual neurons. A powerful technique to dissect the motion-vision circuit is the photoablation of single neurons. By selective photoablation the role of individual neurons within synaptic networks has been clarified. Further advances in the disclosure of visual motion processing may in the future be achieved by imaging the activity of single neurons during the processing of natural inputs. Moreover, the combination of genetic tools with functional fluorescence approaches will help elucidate the role of classes of neurons in the visual motion pathway of the blowfly's smaller companion, the fruitfly *Drosophila*.

2. INTRODUCTION

When trying to understand how a neural system manages a specific task, the experimenter needs a technical approach that provides information on the activity of nerve cells or a method for manipulating neuronal activity. In this review we will illustrate how optical methods have been utilized to this end in a particular model system - visual motion-processing in the fly brain. This model system demonstrates in an exceptional way the usefulness of various fluorescence-based and optical techniques for studying the processes taking place in the intact brain of a living animal. Several excellent reviews have been written about optical techniques that we will address in this review, namely Ca^{2+} imaging, UV photolysis of 'caged Ca^{2+}' and laser ablation (1-4). However, we will focus on aspects of how these techniques have been applied to resolve specific questions in the context of fly motion vision.

All optical methods used so far to study fly motion vision have been built on the feasibility of introducing fluorescent substances or caged compounds selectively into single neurons or into small numbers of neurons. Such a specific targeting of cells requires a combination of optical techniques either with electrophysiological methods or with genetic tools. Before describing these optical techniques in detail, this review will briefly outline the basic principle of

electrical recording of fly visual motion-sensitive neurons. The genetic approaches that are increasingly becoming tools of choice for the study of fly motion vision in combination with fluorescence techniques are addressed later in this review.

2.1. Optic-flow processing neurons in the fly brain

During flight a fly is confronted with a rapid succession of images on its eyes, so-called optic flow. It is the task of the brain to make sense of this optic flow and to employ it for the control of a multitude of locomotor behaviours, such as targeted changes in flight direction, collision avoidance and the accurate initiation of landing manoeuvres. The velocity of flight movements – during sudden turns the blowfly *Calliphora* can reach more than 3000 degrees per second (5) – gives an impression of how challenging it is to use optic flow for the rapid control of locomotion (6). *Calliphora* has proven to be an outstanding model system for tracing activities in the brain that serve to process optic flow from the eyes (7-10), since experimental analyses can be conducted here employing a broad spectrum of methods.

Steering an animal's locomotor behaviour based on optic flow information is a complex task. It is therefore surprising that there appears to be only a small number of neurons that integrate visual motion information over a large part of the visual field. These neurons, the tangential cells in the fly's lobula plate (LPTCs), form a set of about 60 individually identifiable cells in each brain hemisphere (Figure 1A,B) (7, 10, 11).

The LPTCs integrate a large number of inputs and owe their sophisticated motion-computation capacity to complex pre-processing of visual stimuli in upstream processing layers. In the first stage of the visual system, the retina, photoreceptor cells only register the brightness in a certain part of the visual field (Figure 1A). Motion information is not explicitly given by the photoreceptor activity but has to be computed in subsequent processing stages from the pattern of brightness changes as sensed by the array of photoreceptors (12). Motion detection is thought to be implemented on a local basis by neuronal circuits in the medulla (13). LPTCs pool these local motion signals on large, retinotopically organized dendrites (14, 15). By integrating motion signals over large parts of the visual field LPTCs can extract relevant information from optic flow. This comprises information about the fly's self-motion and about the three-dimensional layout of its environment (16-18).

2.2. Recording electrical activity of fly visual motion-sensitive neurons

One crucial advantage of the fly as a model system for the study of motion vision is its accessibility to a large spectrum of physiological techniques. The position of LPTCs near the surface of the fly brain and their comparatively large size makes it possible to apply electrophysiological as well as optical methods in a nearly intact preparation. This allows an examination of the processes occurring in the motion-vision centre of the brain while the animal perceives its natural sensory inputs (9, 10). In the classic electrophysiological approach cell activity is recorded by measuring cell membrane potential with a sharp glass electrode inserted into the neuron (intracellular recording) or by placing an electrode close enough to the neuron to record its action potentials (extracellular recording).

Figure 1C illustrates the response of a VS-neuron ('Vertical System') to visual motion, registered by an intracellular electrode inserted into its axon. VS-neurons form one of the major output elements of the fly's motion-vision system (19, 20). In the lobula plate of *Calliphora*, there are ten VS-neurons per brain hemisphere. They respond predominantly to vertical motion within dorso-ventrally elongated sections of the visual field (21). The sensitivity of different VS-neurons is shifted along the azimuth, so that the entire VS ensemble covers nearly the entire visual hemifield. A prominent depolarisation is registered during downward motion in the receptive field of the VS-neuron, the cell's preferred direction (PD). In contrast, motion in the so-called null direction (ND) leads to membrane hyperpolarisation. These graded axonal voltage responses are superimposed by fast depolarising transients, in particular during PD motion and after the cessation of ND motion (22). Unlike ordinary action potentials these transients are variable in amplitude and have therefore been termed 'spikelets' by some researchers.

3. MONITORING ACTIVITY IN THE FLY MOTION-VISION SYSTEM BY CALCIUM IMAGING

3.1. Investigation of dendritic processing by Ca^{2+} imaging

Electrophysiological recordings of neuronal activity generally provide information on neuronal output signals. Unfortunately, monitoring of output activity is often insufficient to understand how neurons process incoming signals. LPTCs possess large dendrites, and these form major sites of information processing (15, 23). Electrical recordings from small dendritic branches are hardly feasible, but fluorescence microscopy provides a means to examine dendritic computation. In particular Ca^{2+} imaging can help making local dendritic activity visible.

In LPTCs, as in many other neurons, Ca^{2+} is a good marker of activity because its cytosolic concentration is largely regulated by the activity of depolarisation-activated Ca^{2+} channels (24). Different parts of the neurons may differ in the level of their depolarisation during ongoing signal processing, depending on the sites of local synaptic inputs and on the activation of voltage-dependent ion channels. Hence, imaging of cytosolic Ca^{2+} concentration changes presents a means to visualize the neuronal activation pattern (4). In order to monitor the changes in the cytosolic Ca^{2+} concentration of an individual cell, a fluorescent Ca^{2+} indicator dye has to be loaded into the neuron. In the case of LPTCs, this is accomplished by iontophoretic injection of charged dye molecules into the neuron from an intracellular electrode. Ca^{2+} dyes change their fluorescence properties upon binding of Ca^{2+}. As a consequence, the intensity of the fluorescence emitted from a dye-filled neuron upon excitation with a specific

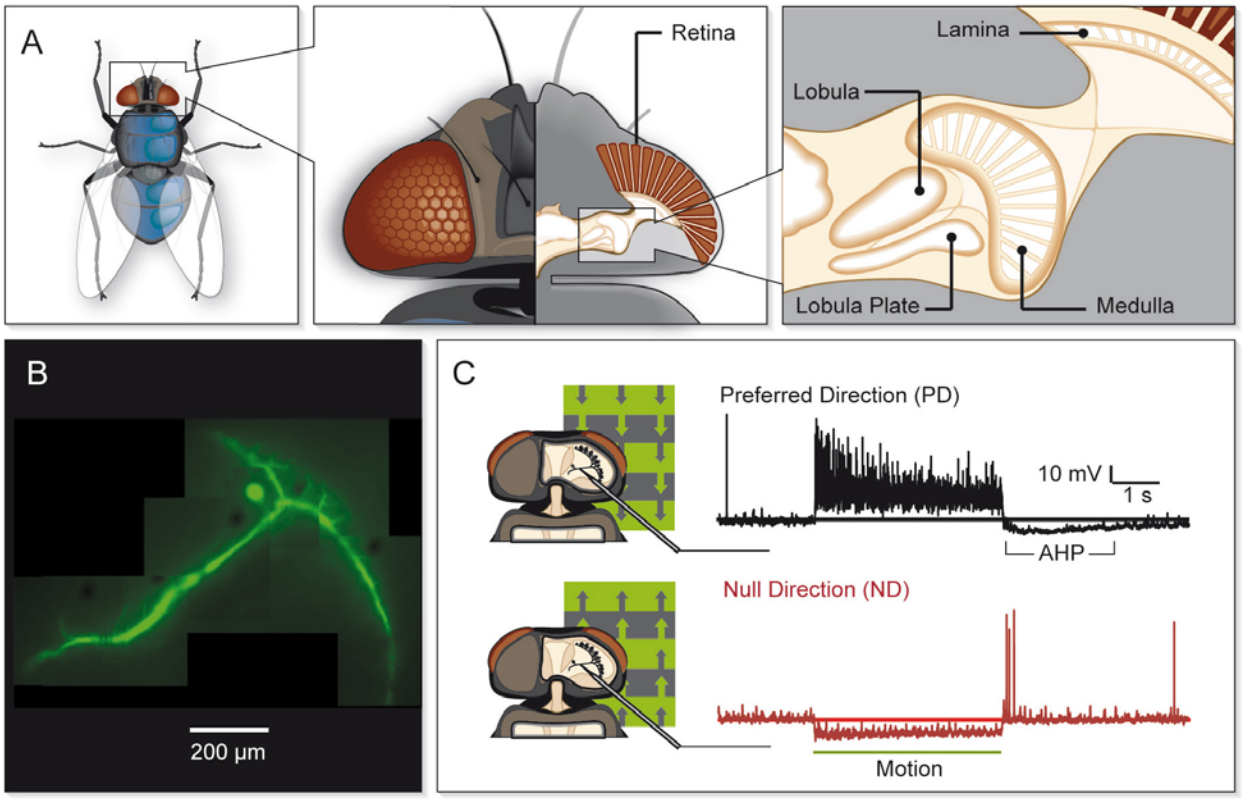

Figure 1. Neuronal processing of visual motion in the fly brain. *A*, Schematic horizontal cross-section through the fly head. Photoreceptors in the retina send their axons to the lamina. Both the lamina and the next processing stage, the medulla, are organized into columns that represent positions in the visual space in a retinotopic way. This means that in lamina and in medulla the neighbourhood relationships between image points are conserved. Neurons in the lobula complex, consisting of two structures, the lobula and the lobula plate, integrate the outputs of medulla neurons in a specific way. About 60 lobula plate tangential cells (LPTCs) are known and can be individually identified. These neurons respond in a directionally selective way to visual motion within large receptive fields, comprising in some cases almost the whole visual hemisphere. *B*, Image of a Vertical-System LPTC (VS-neuron). The cell was penetrated with a sharp glass electrode and filled with a fluorescent dye. *C*, Intracellular recording of changes in the axonal membrane potential of a VS-neuron during presentation of a moving pattern. During downward motion, the PD, a graded depolarisation is observed (top). Fast depolarizing transients of variable amplitude ('spikelets') are superimposed on the graded depolarization and are occasionally present during rest. The response to PD motion is followed by a long-lasting after-hyperpolarisation (AHP). Upward motion, the ND, causes a graded hyperpolarisation in the axon (bottom). Cessation of motion elicits a brief burst of spikelets.

wavelength is dependent on the concentration ratio of Ca^{2+}-free versus Ca^{2+}-bound dye molecules, and can thus be used to estimate the intracellular Ca^{2+} concentration. Two different classes of Ca^{2+} dyes have been used in LPTCs: 1) Fura-2 and some of its analogues, which combine a stilbene fluorophore with a Ca^{2+}-binding group (25), and 2) Calcium Green or Oregon Green 488 BAPTA, which are derivatives of fluorescein (26). Whereas the first group of dyes requires excitation in the near-UV range, the second group can be excited with visible light. In LPTCs, the first Ca^{2+} imaging experiments demonstrated that the dendrites possess a retinotopic input structure (14). This was concluded from the fact that dendritic Ca^{2+} accumulation is restricted to a certain section of the dendrite when motion is presented only in a sub area of the receptive field (Figure 2).

Axonal membrane potential responses of LPTCs have been recorded during the presentation of motion in restricted sections of the receptive field (18, 21, 27). These experiments have shown that motion sensitivity and local direction selectivity vary across the visual field in a highly ordered way. These motion sensitivity patterns, the so-called 'response fields', are specific for each of the LPTCs and were assumed to be matched to the motion patterns that are generated on the eyes during certain types of self motion. Variations in motion sensitivity and direction selectivity across the dendrite can also be monitored directly by Ca^{2+} imaging. However, the resolution of conventional wide-field microscopy is often insufficient to resolve potential differences in Ca^{2+} dynamics between small neighbouring neurites. Two-photon laser-scanning microscopy provides a higher spatial resolution than wide-field microscopy (3, 28). In wide-field microscopy image blur is caused by emission from out of focus planes within the sample. The superior spatial resolution of two-photon microscopy relative to wide-field microscopy is a consequence of the fact that the simultaneous absorption of

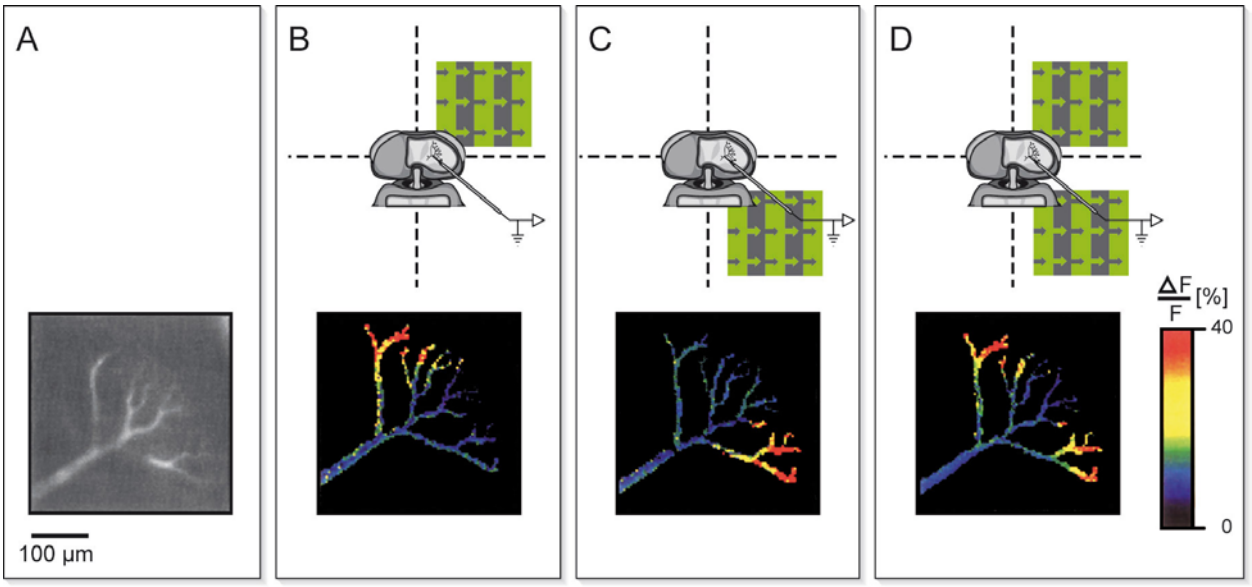

Figure 2. Retinotopic dendritic organisation of LPTCs as demonstrated by Ca^{2+} imaging. An HSE-cell ('Horizontal System Equatorial') was filled with a Ca^{2+}-sensitive dye (Ca^{2+}-Green 1) via the electrode during intracellular recording. Changes in fluorescence intensity during specific excitation of the dye were monitored by a charged-coupled device (CCD-camera) mounted on the microscope. An increase in the calculated fluorescence signal (deltaF/F) indicates a rise in the cytosolic Ca^{2+} concentration relative to its resting level. The color-coded signal is only shown in regions with high staining intensity, i.e. the major dendritic branches and the axon (see raw fluorescence image in *A*), in order to decrease noise in the calculation of deltaF/F-values. *B*, During presentation of PD motion (front-to-back) in the upper visual field Ca^{2+} rises predominantly in dorsal dendritic branches (color-coded image taken 7.5 s after motion onset). *C*, PD motion in the lower visual field leads to Ca^{2+} accumulation in the ventral dendrite. *D*, Simultaneous motion in both parts of the visual field induces Ca^{2+} accumulation in both ventral and dorsal dendritic branches. Experimental data from (75).

two photons is required to bring a fluorophore to the excited state (Figure 3A). Thus, only in the focal plane of the laser, which is used to scan the sample, is excitation intensity sufficiently high to produce fluorescence emission. One drawback of laser-scanning microscopy is its relatively slow rate of image acquisition. Frame rates are usually not higher than in wide-field microscopy, since the scan speed of the laser is limited by the inertia of moving parts. This constraint is removed, to a certain extent, by simultaneous scanning with several laser beams. In one implementation of the multifocal principle, the laser beam is split into an array of up to 64 unitary beams which can be used to create multiple excitation foci in the sample (29, 30). This excitation principle has been used to monitor Ca^{2+} signals at small dendritic branches of fly LPTCs during motion in different directions (Figure 3C).

3.2. Dendritic Ca^{2+} dynamics provide hints on the type of visual motion detection

Due to the retinotopic input structure of LPTC dendrites, local dendritic Ca^{2+} signals show characteristic temporal modulations which reflect properties of the spatial pattern (15). These local modulations are phase shifted along the direction of motion (and thus along one axis of the retinotopically organized dendrite). As a consequence, they cancel out when local dendritic activity is spatially integrated into an axonal voltage change. The dependence of the local dendritic Ca^{2+} modulations on the properties of the visual stimuli has been used to elucidate the computational principle of the local motion detectors that deliver input to LPTCs (31). If the dendrites of LPTCs receive input from correlation-type local motion detectors, local modulations synchronous with the temporal pattern frequency are expected. Implementation of an alternative processing scheme, the gradient detector, would not produce such modulations. It was suggested that the visual system might be able to shift from a correlation-type motion detection scheme at low ambient contrast to a gradient scheme at high contrast. In principle, such a shift might help the system to benefit from the specific advantages of both detection schemes. Unlike the correlation-type detector, the gradient detector provides a signal that is proportional to image velocity. On the other hand, the advantage of the correlation-type detector over the gradient detector is its lower susceptibility to noise, in particular at low contrast. Although modulations in local dendritic Ca^{2+} signals have previously been demonstrated (15), a potential contrast-dependent switch between detector schemes could not be excluded. This is due to the fact that conventional wide-field fluorescence microscopy was used, which suffers from a low effective contrast of the stimulation pattern, because the excitation light causes spurious excitation of the photoreceptors. Therefore, two-photon laser-scanning microscopy was used to resolve the question of whether different motion detection scheme are implemented at different contrast conditions. Two-photon excitation of Ca^{2+} dyes is generated by infrared light, to which the fly's photoreceptors are insensitive. Thus,

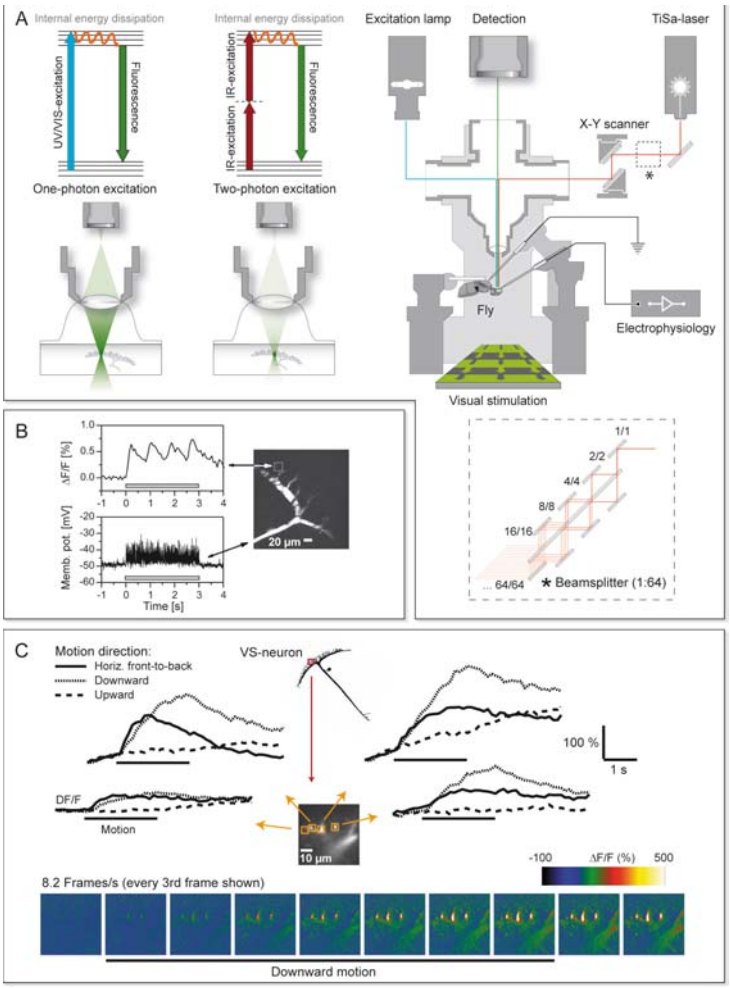

Figure 3. *A*, The principles of one-photon (left) and two-photon excitation (middle) illustrated in Jablonski diagrams (top) and sketches of the resulting spatial distributions of signal generation in one- and two-photon microscopy (bottom). Visible or UV light is used for excitation in one-photon microscopy, whereas near-infrared light is used in two-photon microscopy. A quadratic dependency of excitatition probability on laser intensity arises from the nonlinear nature of two-photon excitation. Excitation probability has a quadratic dependency on laser intensity in two-photon microscopy, since nearly simultaneous absorption of two photons is required. This confines the occurrence of excitation events to the vicinity of the laser focus and thus reduces image blur caused by out-of-focus fluorescence. Additionally, photodamage is reduced by the confinement of excitation to the focus. A schematic of a microscope set up (right) illustrates the use of two-photon microscopy in the study of fly motion vision. A fly is immobilized with bees wax, mounted on a microscope stage and stimulated with moving visual patterns. Micromanipulators holding recording and reference electrodes are installed to perform simultaneous Ca^{2+} imaging and electrophysiological recording. Wide-field fluorescence excitation with a Hg-lamp is available in addition to two-photon excitation by a pulsed Titanium-sapphire laser. In contrast to conventional two-photon microscopes, in multifocal two-photon microscopy a single laser beam is split up into several beamlets. One way to multiply the number of laser foci in the sample is the use of a mirror-optical beamsplitter (see inset indicated by asterisk), which generates an array of laser foci by repeated separation of the laser beams at a 50%-beamsplitter mirror and reflection at high-reflectivity mirrors. Multifocal laser scanning accelerates image acquisition, but necessitates the use of a camera instead of a photomultiplier tube for the detection of emission light. See (29) for a critical evaluation of multifocal two-photon microscopy and its application to Ca^{2+} imaging in fly LPTCs. *B*, Example of two-photon Ca^{2+} imaging during simultaneous intracellular recording of the axonal membrane potential. The fluorescence signal within a small area of the dendrite of a VS-neuron filled with Ca^{2+}-Green 1 shows characteristic modulations corresponding to the temporal frequency of a sinewave grating moving at constant velocity. No modulation is visible in the membrane potential recording because local dendritic signals, which are phase-shifted with respect to each other, are integrated into an axonal response. *C*, Example of Ca^{2+} imaging with multifocal two-photon microscopy at a VS-cell dendrite. A color-coded image sequence indicating changes of cytosolic Ca^{2+} concentration in a small region of the dendrite of a VS neuron is shown for downward motion (*bottom*). Ca^{2+} concentration signals obtained by excitation of Oregon-Green 1 were quantified for four different branches during motion in three different directions. The corresponding response traces hint at differences in direction selectivity between branches and differences in Ca^{2+} signal timecourse between motion directions. *B* adapted from (31).

spurious stimulation of photoreceptors was eliminated and stimulation of the fly during Ca^{2+} imaging with movement of a high-contrast grating was possible. In these experiments modulations of local dendritic Ca^{2+} signals could be demonstrated over a wide range of contrast and luminance conditions (Figure 3B). This result suggests that fly motion vision is based on a correlation-detector scheme irrespective of the signal-to-noise ratio of the visual input.

3.3. Analysis of neuronal connectivity by Ca^{2+} imaging

Ca^{2+} imaging can be applied to establish synaptic connectivity and to assess its functional properties. In this approach one neuron is intracellularly recorded and another neuron is filled with a Ca^{2+} dye. The two cells are connected by an excitatory synapse if depolarizing current injection into the first cell generates a Ca^{2+} signal in the second cell. In this way several interactions between LPTCs have been unravelled. In particular, it has been shown that bidirectional transmission by electrical synapses exists and that some specific computations are performed at dendro-dendritic synapses (32). One feature of dendro-dendritic connectivity in the fly visual motion system is the maintenance of the retinotopic organization principle. Model simulations led to the assumption that interaction by inhibitory dendritic synapses can lead to enhancement of motion contrast. This property resembles lateral inhibition in the vertebrate retina and might constitute a crucial step in figure-ground discrimination based on relative motion (33).

The use of Ca^{2+} imaging in combination with intracellular recordings of potentially coupled neurons has one advantage over dual intracellular recordings: it is not only possible to demonstrate the existence of a synaptic connection, but also to localize the site of interaction. In this way, it has been shown that VS neurons with neighboring receptive fields are synaptically coupled in their axon terminal region (34). Injection of depolarizing current into one VS neuron leads to an increase in the concentration of Ca^{2+} in an adjacent VS neuron. Unlike the injected neuron, which shows a Ca^{2+} increase both in its dendrite and in its axonal region, the increase in the adjacent neuron was strongest in the axon terminal region (Figure 4A).

4. ANALYSIS OF SYNAPTIC CONNECTIVITY BY SINGLE-CELL PHOTOABLATION

In the fly visual system it is feasible to fill a single neuron with a fluorescent dye that is toxic upon intense illumination, e.g. Carboxy-fluorescein. It is then possible to selectively ablate the dye-filled neuron by directing a laser beam on the preparation. All other neurons that are not filled with the dye are left intact. Single-cell photoablation, also termed the 'fill-and-kill technique', is an elegant technique to assess the function of a neuron by recording neurons at postsynaptic processing stages before and after the ablation (35). Due to its specificity, laser ablation is superior to any other lesion technique. Microsurgical brain lesions, for instance, usually affect fibre tracts containing axon projections of several neurons (36). Lesions of neuronal precursors in larval flies can also be performed by laser ablation. Unlike single-cell laser ablation with phototoxic dyes in adult animals, this technique results in elimination of classes of neurons or entire brain regions (37, 38). Certain classes of neurons can also be targeted by the expression of neurotoxins in transgenic *Drosophila* (39, 40) (see also section 6). However, single-neuron specificity has not yet been reached in genetics-based lesion approaches.

4.1. Dendritic and axonal interactions elucidated by photoablation

In the first study to use single-cell photoablation in the fly brain, it was established that a particular LPTC - a so-called Figure-Detection neuron (FD1) - is inhibited by a GABAergic Ventral Centrifugal Horizontal neuron (VCH) (41). This inhibition leads to an important feature of the FD1 neuron, so-called 'small-field tuning': FD1 responds only weakly when a motion stimulus covers its entire receptive field. In contrast, when pattern motion is restricted to a small part of the receptive field FD1 shows a strong response (42). This 'small-field tuning', which is thought to mediate visual-motion based figure-ground discrimination, was abolished after ablation of VCH. This circuit was further analyzed by photoablation of neurons that are presynaptic to the CH-cells. The dorsal and ventral CH-cells (DCH and VCH) receive their main ipsilateral input not from retinotopic local motion-detecting elements, as is the case for other LPTCs, but via dendro-dendritic synapses from HS cells. The response of CH-cells to ipsilateral motion was abolished after ablation of HS neurons (43).

Interactions in the ensemble of LPTCs sensitive to vertical motion have also been unravelled by single-cell photoablation. The ten VS-neurons form one major output element of the lobula plate (see sections 3,4). Since the dendrites of VS-cells have been shown to receive retinotopically arranged input, one would expect that their receptive fields correspond to the spread of their dendrites in the lobula plate. This is not the case however, since the VS-neurons respond to vertical motion in a much larger section of the visual space than estimated from the extent of their dendrites (20). This discrepancy was resolved by a photoablation study: the receptive field of a VS-neuron was compared before and after photoablation of a neighbouring VS-neuron (44). Photoablation led to narrowing of the receptive field of the recorded VS-neuron on the side of the lesioned neighbouring cell (Figure 4B). The form of synaptic interaction between neighbouring VS-neurons was resolved by current injections during dual intracellular recordings: VS-cells are electrically coupled, having stronger connections to their immediate neighbours than to the VS-cells with more distant receptive fields (45). This suggests a chain-like coupling of the VS-neurons. As described above (see section 3.3 and Figure 4A), by Ca^{2+} imaging the axonal regions were identified as the sites of electrical coupling (34). Interestingly, a very similar role of electrical synapses in enlarging receptive fields was recently demonstrated in vertebrate retina. The receptive fields of individual horizontal cells in the mouse retina were found to extend beyond their particular dendritic arbors. However, deletion of connexin57 (Cx57), a

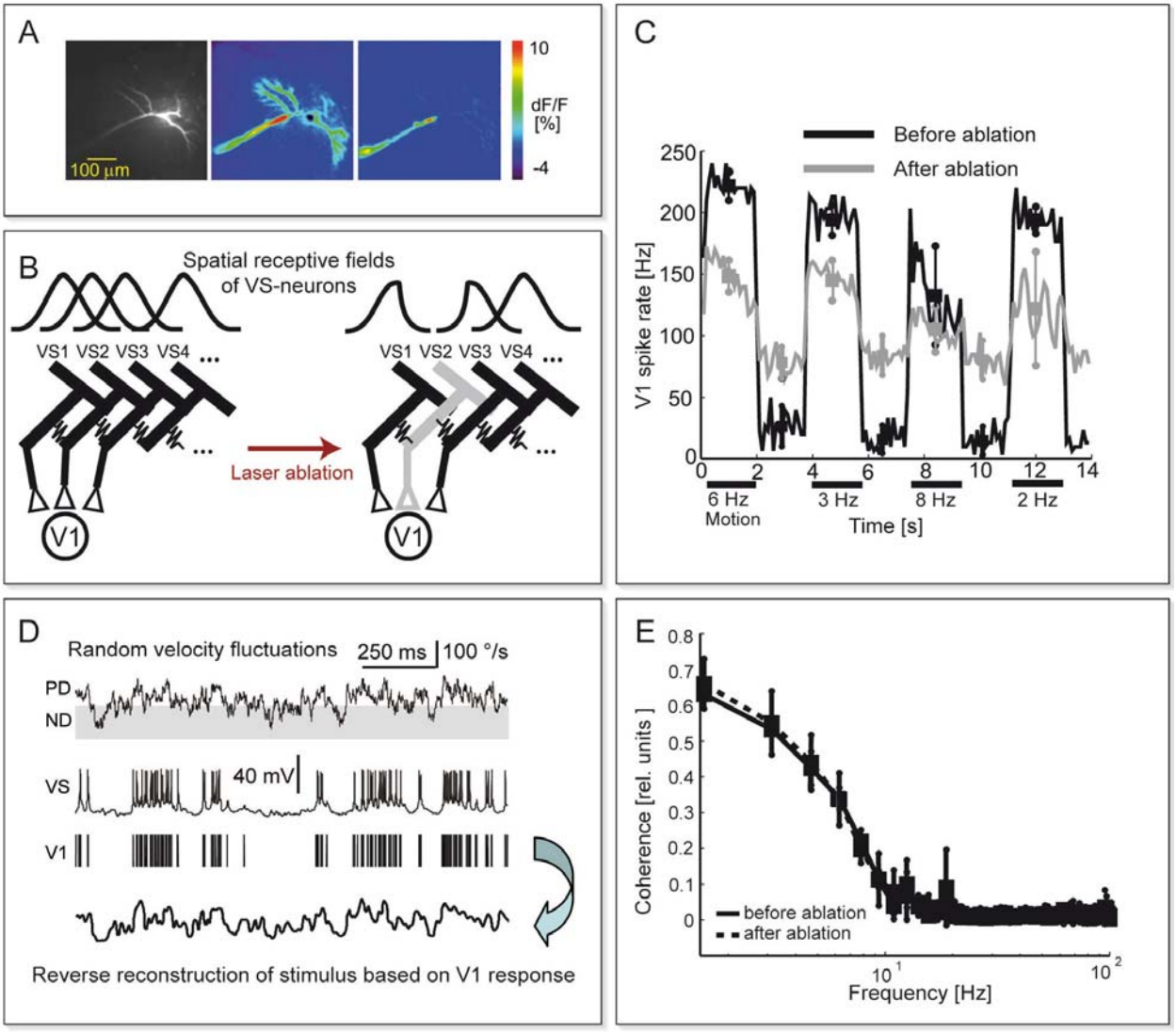

Figure 4. Examination of the VS-V1 network in the fly brain by Ca2+ imaging and single-cell laser ablation. *A*, VS neurons are coupled at their axon terminal region: fluorescence Ca2+ concentration signals were monitored in a VS1-neuron (left, raw fluorescence image) during depolarizing current injection into the neuron itself (middle) and into the neighboring VS2-neuron (right). The response during current injection into the neighboring cell was strongest in the axon terminal region, suggesting this region to be the site of synaptic contacts. *B*, Scheme illustrating network connections and receptive fields. VS neurons are thought to be connected by electrical synapses with their neighbours in a chain-like manner (45, 76). The V1-neuron receives strong synaptic input from VS1 – VS3. VS-neurons possess dorsoventrally elongated stripe-like spatial receptive fields. In the chain of VS neurons the centres of maximum sensitivity shift from frontal to lateral, with considerable spatial overlap in the receptive fields of neighbouring VS-neurons. Selective laser ablation of one VS-neuron leads to narrowing of the spatial receptive fields of its neighbours on the side of the ablated neuron. See (44) for details. *C*, In the postsynaptic V1-neuron the ablation of one of the presynaptic VS-neurons leads to a decrease in motion-induced responses and to an increase in baseline firing rate. The averaged timecourse of V1's spike responses to a square-wave grating moving with different temporal frequencies is shown before (*black*) and after VS-ablation (*grey*). *D*, Example responses of VS and V1 to dynamic motion stimulation. The coherence is a measure of how well the stimulus can be reconstructed from the response (in this case the response of V1) by convolution with a linear filter (reconstructed stimulus trace shown schematically). See (49) for details. *E*, The coherence between V1 and the dynamic motion stimulus is plotted for different frequencies of velocity fluctuations. The coherence when V1 receives input from the intact VS-cell ensemble is not different from the state in which one VS-neuron is ablated. *A* adapted from (34). Experimental data in *C* and *E* from (50).

constituent of vertebrate gap junctions, significantly reduced horizontal cell receptive field size (46).

4.2. Investigation of synaptic integration by photoablation of inputs

One neuron that reads out the ensemble activity of VS-neurons is the V1-neuron (47). V1 receives strong input from VS-cells with frontal to slightly lateral receptive fields (VS1 – VS3) and transforms the resulting postsynaptic potential into spike activity (Figure 4B). This integrated motion signal is conveyed to the contralateral lobula plate, where V1 forms a large output arborisation. One postsynaptic target of V1 is the VCH-neuron, which gains sensitivity to contralateral vertical motion by this interaction (48).

During flight, a fly is rarely confronted with visual motion of uniform velocity. As a somewhat more naturalistic stimulus, the response of fly LPTCs was investigated using moving patterns that continually modulate their velocity. This approach tested how well the graded-potential VS-neurons and the spiking V1-neuron can encode different motion velocities. Both the presynaptic VS-neurons and the postsynaptic V1-neuron were shown to encode PD velocity fluctuations linearly and reliably over a frequency range of up to about 10 Hz (49). The overall synaptic transfer between VS and V1 was shown to operate almost linearly over a range of presynaptic voltage fluctuations that covers this frequency range. But what is the contribution of individual VS-neurons to motion velocity coding in V1? Is the convergence of several VS-neurons onto a single V1-neuron essential to guarantee reliable transfer of velocity information? To address these questions the visual motion response of V1 before and after eliminating individual VS-neurons by laser ablation was registered (50). Ablation of single VS-neurons had two effects on the postsynaptic V1-neuron. First, motion-induced responses of V1 decreased and baseline spike rate increased after lesioning a VS-neuron (Figure 4C). The most parsimonious explanation of the latter effect is an increase in leak conductance in VS-neurons that are nearest neighbours of the lesioned VS-cell. Such effects have been reported to accompany the ablation of electrically coupled counterparts in neuronal networks (51). Increased leak conductance in VS would lead to depolarization and, consequently, to increased graded synaptic input to the V1-neuron. The second effect of ablation of single VS-neurons on the postsynaptic V1-neuron was that coding of fluctuations in motion velocity by V1 was quantified by information measures before and after ablation of a VS-neuron. Surprisingly, in most cases there was no pronounced effect on motion velocity coding by V1 when a presynaptic VS-neuron was ablated (Figure 4D,E). This indicates the presence of redundancies in the synaptic integration of motion information by the V1-neuron. Otherwise, elimination of a presynaptic element would strongly degrade V1's capacity to code motion velocity. Redundancies in synaptic wiring patterns might help establish robust representations of neuronal information, particularly when sensory information is sparse or fragmentary.

5. MANIPULATION OF CALCIUM SIGNALLING IN SINGLE NEURONS BY UV PHOTOLYSIS OF CAGED CALCIUM

As detailed in section 3, Ca^{2+} signals in neuronal dendrites present a good marker of local activity. But what is the functional role of Ca^{2+} in the dendrite? Prompted by correlations between Ca^{2+} accumulation in the dendrites of LPTCs and membrane after-hyperpolarisation (AHP) following motion in PD (Figure 1C), a Ca^{2+}-dependent inhibitory conductance, such as Ca^{2+}-activated K^+-channels, has been proposed as a physiological basis of direction-selective adaptation (52). The AHP following PD motion is an interesting effect for two reasons. First, it modulates neuronal sensitivity in a specific way. Second, it can explain a peculiar behavioural after-effect observed in *Drosophila:* after the cessation of rotatory visual motion deviations in the resumption of a straight walking course were observed. This after-effect is reminiscent of the well-known 'waterfall illusion' in human psychophysics, in which the observer experiences the impression of movement in opposite direction after viewing a pattern moving in one direction for several seconds (53-55). In fly LPTCs, Ca^{2+} appeared to be a plausible mediator of direction-selective adaptation for two reasons: first, as outlined in section 3.1, Ca^{2+} accumulation stays local within the dendritic tree (14, 56). Likewise, adaptation is stronger if adapting motion and test motion are presented at the same location within the receptive field of the LPTC (57, 58). Second, the time courses of the build up and the recovery from adaptation resemble a temporally low-pass-filtered version of the membrane potential (52). These adaptation time courses are similar to the dynamics of cytosolic Ca^{2+} signals (52, 56, 59). In this sense, Ca^{2+} could act as a kind of memory signal for stimulus history.

Although Ca^{2+} imaging is helpful to assess correlations between the cytosolic Ca^{2+} dynamics and neuronal adaptation, this correlation alone does not necessarily imply a causal relationship. To test the involvement of Ca^{2+} in the control of adaptation in LPTCs, the cytosolic Ca^{2+} concentration has to be manipulated in the absence of visual stimulation (60). Through this approach, the effect of Ca^{2+} can be dissociated from other activity-dependent mechanisms. One way to quickly and selectively manipulate the cytosolic Ca^{2+} concentration in a single neuron is through the release of Ca^{2+} from a UV-sensitive caged compound (1). NP-EGTA is a chelator of divalent ions, which exhibits a high selectivity and affinity for Ca^{2+} (61). UV illumination causes cleavage of NP-EGTA, leading to a several thousand fold decrease in its affinity for Ca^{2+}. Thus, NP-EGTA, which can be selectively introduced into a cell by a iontophoretic current through a recording electrode, can be used to switch single neurons rapidly from a low- Ca^{2+} to a high- Ca^{2+} state (Figure 5A). Surprisingly, release of Ca^{2+} by UV photolysis of NP-EGTA in LPTCs neither elicited an AHP nor a rise in conductance (Figure 5B,C). Both effects were expected if Ca^{2+} would activate an inhibitory conductance. Nevertheless, a rise in conductance was present after adaptation with motion in PD. It is therefore plausible that direction-selective adaptation is caused by an activity-

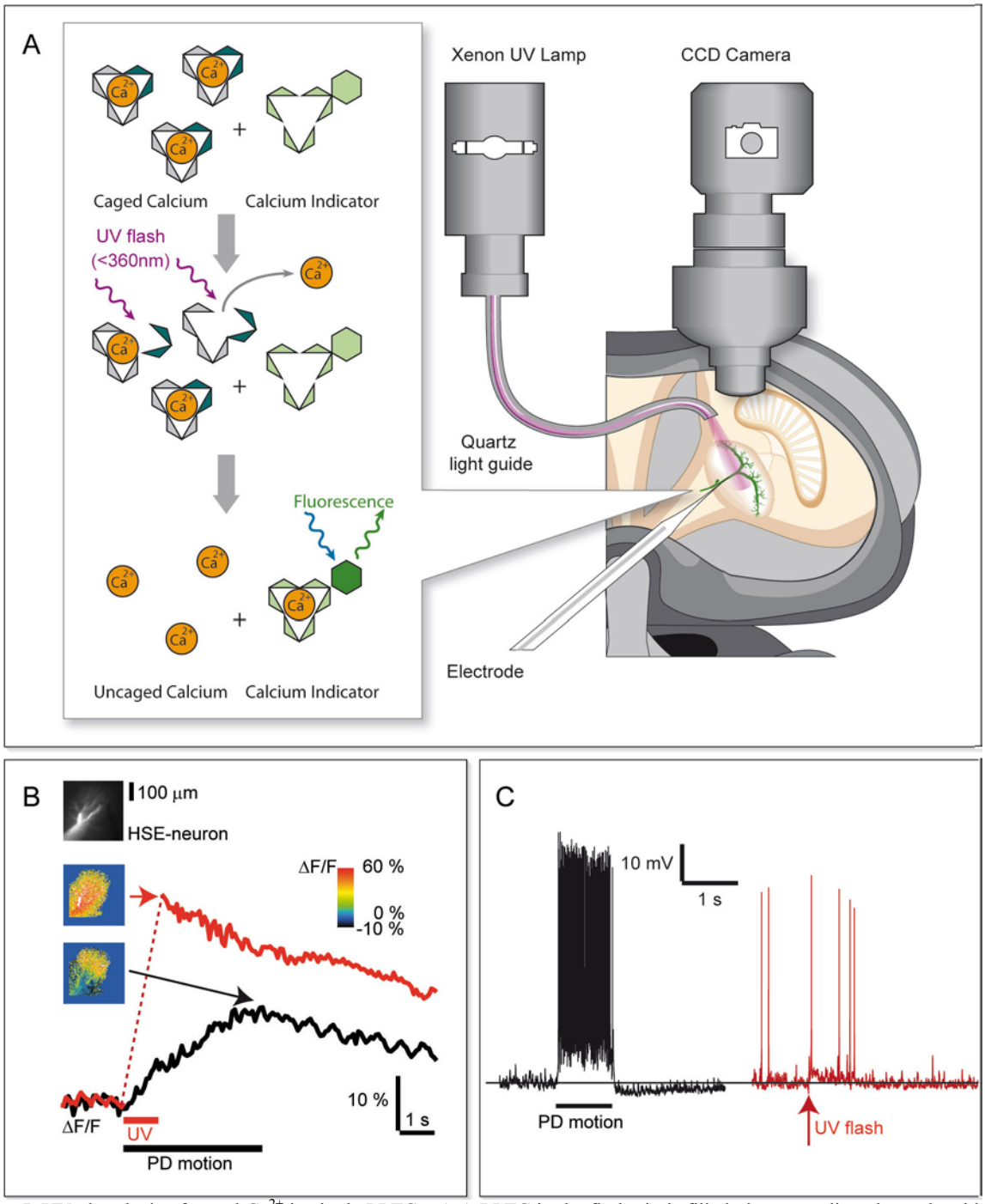

Figure 5. UV photolysis of caged Ca^{2+} in single LPTCs. *A*, A LPTC in the fly brain is filled via a recording electrode with Ca^{2+} indicator dye and NP-EGTA, so-called 'caged Ca^{2+}', which releases Ca^{2+} upon exposure to UV light. Filtered UV light, either from a Xenon flash lamp or from a continuous UV light source, is delivered to the sample by a quartz light guide to induce release of Ca^{2+} into the cytosol during membrane potential recording and fluorescence Ca^{2+} imaging. *B*, Example traces of dendritic Ca^{2+} elevation in an HSE-neuron by UV photolysis of caged Ca^{2+} (*red*) and during visual stimulation with PD motion (*black*). The color-coded images show the fluorescence signals at the end of UV illumination and of visual stimulation, respectively. *C*, Membrane potential traces of an HSE-neuron during visual stimulation with PD motion (*black*) and during UV photolysis of caged Ca^{2+} (*red*). Whereas an AHP follows PD motion (see also Figure 1C), no such response is present after artificial elevation of cytosolic Ca^{2+} by UV photolysis of caged Ca^{2+}. In contrast, a slight depolarisation, presumably caused by excitation of photoreceptors by the UV flash, is observed. This speaks against an induction of AHP by Ca^{2+}, and thus against Ca^{2+}-dependent regulation of adaptation in LPTCs (see main text for details). Experimental data from (60).

egulated inhibitory ion conductance that is independent of Ca^{2+} (60). One potential candidate is a Na^{+}-regulated conductance, which appears to exist in fly LPTCs (62). Alternatively, direction-selective adaptation might be generated by a voltage-gated inhibitory ion conductance with very slow activation and deactivation characteristics, such as members of the KCNQ family of K^{+}-channels, which have been shown to exist in *Drosophila* (63). In vertebrates, activation of KCNQ-type channels generates an AHP, suggesting a role in neuronal adaptation (64-66).

6. COMBINATION OF OPTICAL METHODS WITH GENETIC APPROACHES

Penetrations with intracellular electrodes are used to fill single neurons with fluorescent substances, e.g. calcium-sensitive probes or phototoxic dyes. However, intracellular recordings from LPTCs in the fly brain are a difficult and time-consuming procedure. By using genetic approaches in *Drosophila* it is, in principle, possible to circumvent this problem by expressing proteins in particular groups of neurons. For example, it is already possible to address the function of classes of neurons in the fly visual system by the targeted expression of toxins that block synaptic transmission (39, 40). These methods have not yet reached specificity for single neurons. Nonetheless, the ability to test large numbers of flies with identical defects will make these genetic approaches good complements to existing methods with single-cell specificity such as photoablation. A prerequisite to draw parallels between *Drosophila* and *Calliphora* is, of course, the close anatomical similarity of their visual systems. Importantly, both fly species possess the same classes of lobula plate visual motion-sensitive neurons, LPTCs, although the number of neurons within a class and cell shape of individual neurons may differ (67, 68). One major drawback of *Drosophila* in functional neurophysiological studies is its tiny body size. Whereas behavioural tests, such as monitoring of optomotor responses, are well applicable in *Drosophila*, it is still difficult to assess neuronal responses directly. Therefore, *Calliphora* still presents the best model both for electrophysiological recording and for functional imaging. With respect to the latter, approaches to express genetically encoded functional fluorescence dyes in classes of neurons in *Drosophila* are promising, given the enhanced sensitivity of recently developed genetically encoded Ca^{2+} dyes (69, 70). By the MARCM method (71), i.e. by mosaic analysis with a repressible cell marker, it has recently become possible to express specific proteins in small numbers of LPTCs derived from a common progenitor or even in single LPTCs (72). It is, however, not feasible with this method to generate large numbers of individuals carrying exactly the same cellular expression pattern, which is the prerequisite to perform a systematic functional analysis of individual neurons.

7. PERSPECTIVE

Fluorescence imaging in living animals offers a wide range of possibilities to investigate neuronal activity in real time and at increasing spatial resolution, complementing classical electrophysiological methods. Technical advances in the field of fluorescent indicators and caged compounds provide not only the possibility to monitor various ionic concentrations *in vivo*, but also to influence these concentrations and other cellular processes on fast timescales.

The high spatial resolution of confocal and two-photon-imaging opens up a new experimental approach to small animals like *Drosophila*, whose small neurons previously made *in vivo* approaches difficult. The introduction of transgenic fluorescent probes and functional compounds, especially in *Drosophila*, might some day solve the problem of selectively marking single cells which are hard to access with intracellular dye injection techniques. Until then the exquisite amenability of larger flies, in particular *Calliphora*, to the simultaneous application of functional optical and electrophysiological techniques will help elucidating how the brain manages sensori-motor control tasks by specific wiring and synaptic processing in small neuronal circuits. With *Calliphora* it is possible to monitor by sophisticated techniques body movements during flight in order to reconstruct the visual stimuli seen in a natural situation for replay when recording neuronal activity (17, 73, 74). Such approaches will help clarify how behaviourally relevant visual information is processed in the insect brain.

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Abbreviations: AHP: after-hyperpolarisation, CCD: charged-coupled device, CH: centrifugal horizontal, DCH:

dorsal centrifugal horizontal, FD: figure detection, HSE: horizontal system equatorial, ND: null direction, PD: preferred direction, LPTC: lobula plate tangential cell, UV: ultraviolet, VCH: ventral centrifugal horizontal, VS: vertical system

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