Differentiation of Dendrites and the Analysis of Spine- Like Structures on Lobula Plate Tangential Cells in *Drosophila melanogaster*

Dissertation

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To My Parents

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Abbreviations

AcCh	Acetyl choline
ADF	Actin depolymerizing factor
ADP	Adenosine diphosphate
AMPA	Amino-3-hydroxy-5-methylisoxazole-4-propionic acid
APF	After puparium formation
ATP	Adenosine triphosphate
BDNF	Brain derived nerve growth factor
Brp	Bruchpilot
BSA	Bovine Serum Albumin
CA	Constitutively Active
CaMKII	Calcium/ calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
CNS	Central Nervous System
d	Day
DAB	3, 3' Diaminobenzidine
da	Dendrite arborization
dfmr1	*Drosophila* fragile X mental retardation
DN	Dominant Negative
DNC	Descending Neuron Cluster
DNA	Deoxyribonucleic acid
EM	Electron Microscopy
F-actin	Filamentous actin
FMR	Fragile X Mental Retardation Syndrome
g	Gram
G-actin	Globular actin
GABA	γ-amino butyric acid
GEF	Guanine Nucleotide Exchange Factor
GFP	Green Fluorescent Protein
GOF	Gain of function

GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
h	Hour
Ham	Hamlet
HRP	Horse Radish Peroxidase
HS	Horizontal System
HSN	Horizontal System North
HSE	Horizontal System Equatorial
HSS	Horizontal System South
KC	Kenyon Cells
L2	Monopolar Laminar Cell 2
LOF	Loss of function
LPTC	Lobula Plate Tangential Cell
MAP1	Microtubule associated protein 1
MAP2	Microtubule associated protein 2
MARCM	Mosaic Analysis with a Repressible Cell Marker
MB	Mushroom Body
md	Multiple dendrite
MKLP1	Mitotic kinesin-like protein-1
µl	Microliter
µm	Micrometer
ml	Mililiter
mRNA	Messenger ribonucleic acid
NGF	Nerve Growth Factor
nm	Nanometer
NMDA	N-methyl-D-aspartic acid
NMJ	Neuromuscular Junction
NT-3	Neurotrophin 3
OE	Overexpression
omb	Optomotor blind
P1	Pupal Stage 1
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction

PFA	Paraformaldehyde
pH	Potential of Hydrogen
PNS	Peripheral Nervous System
PSD	Postsynaptic Density
RGC	Retinal Ganglion Cells
RT	Room Temperature
SER	Smooth Endoplastic Reticulum
SIs	Spine- like Structures
Syt	Synaptotagmin
Ss	Spineless
UAS	Upstream Activation Sequence
VS	Vertical System
VS1	Vertical Cell One
WASP	Wiscott- Aldrich Syndrome Protein
wt	Wild type

Summary

The development of dendrites leads to the establishment of cell-type specific morphology of dendritic trees that eventually determines the way in which synaptic information is processed within the nervous system.

The aim of this study was to investigate dendritogenesis of *Drosophila* motion-sensitive Lobula Plate Tangential Cells (LPTCs) and to understand the role of cytoskeletal molecules in these developmental processes. I employed genetic techniques to obtain fluorescent labeling exclusively in the neurons of interest. In order to visualize the LPTCs confocal imaging was applied.

Time point analysis allowed me to follow and describe the phases of LPTC differentiation in the intact *Drosophila* brain starting from the third instar larva throughout the pupal stages until adulthood. I determined the time when the initial growth of LPTC dendrites starts and showed it to be directional from the beginning. Additionally, I demonstrated that the phase of extensive dendritic growth and branching precedes reorganization processes that lead to establishment of the final architecture of LPTC dendritic trees. In parallel, I attempted to analyze the contribution of actin and tubulin in the shaping of the neurons. In these experiments actin-GFP localized to dendritic termini whereas tubulin-GFP was mainly observed in the primary dendritic branches. These data showed clear similarities between the cytoskeletal organization of LPTCs dendrites and vertebrate neurons.

The discovery of the actin enrichment in dendritic termini made me conduct a set of experiments to test if these protrusions are the counterparts of vertebrate spines. I performed a thorough quantitative analysis of spine- like protrusions present on LPTC dendrites. Morphological features like the density and shape of the LPTC spine– like protrusions appeared to be comparable to hippocampal spines. Using immunohistochemical methods I demonstrated that LPTC spine-like protrusions are sites of

synaptic contacts. The ultrastructural analysis supported the immunohistochemical data and showed that synaptic transmission takes place at the LPTC spine-like protrusions.

Next, I tried to genetically modify these structures by generating LPTC mutant for genes which have vertebrate homologues known to alter spine morphology. I showed that dRac1 can modulate significantly the LPTC spine-like structure density. Finally, I tried to check if *Drosophila* LPTC spine-like structures are motile.

To conclude, I showed an initial description of LPTC dendritogenesis and the subcellular localization of actin and tubulin in these neurons. The actin enriched spine-like structures detected on the LPTC dendrites are sites of synaptic contacts, thus resemble vertebrate spines.

1 Introduction

Dendrite morphology

Dendrites are at least as important as axons in establishing synaptic connections, and forming the neuronal circuitry. They are designed to integrate multiple inputs that the neuron receives. The cell- type specific dendritic morphology determines the way that the synaptic or sensory information is presented to a given neuron and processed within the nervous system (Grueber and Jan, 2004). One can observe a correlation between the specific function of the dendritic tree and its morphological appearance, e.g. the functional autonomy of individual dendritic branches of a retinal starburst amacrine cells (Taylor and Vaney, 2003) (Figure 1.1, D) or olfactory bulb mitral cells (Yokoi et al., 1995) (Figure 1.1, C) that exhibit dynamic odorant responses based on experience. The other prominent example are the motion sensitive interneurons in the visual system of Diptera that possess very complex dendritic trees designed to receive the input from the multiple upstream columnar partners (Figure 1.1 B) (Borst and Haag, 2002).

Studying the mechanisms that lead to shaping of characteristic dendritic forms is necessary for understanding their ultimate function. Mechanisms used in the initial steps of dendritic development may also allow remodeling and plasticity in the mature nervous system (Grueber and Jan, 2004).

Factors involved in Dendritogenesis

Dendrites differ from axons molecularly, morphologically and functionally. They contain mRNA, ribosomes, endoplasmic reticulum and Golgi which makes local protein synthesis possible (Huber et al., 2000). The polarization of neurons takes place before they elaborate their dendrites and axon. There is evidence that both extrinsic and intrinsic factors contribute to the initial polarization of the neuron (Horton and Ehlers, 2003). As shown in cultured hippocampal neurons, local actin dynamics plays a crucial role in the initial neurite selection (Bradke and Dotti, 1999).

Components that are involved in diverse aspects of dendrite shaping and maintenance can be divided into two groups of intrinsic and extrinsic cues that act throughout the development. Some examples of those cues are listed below.

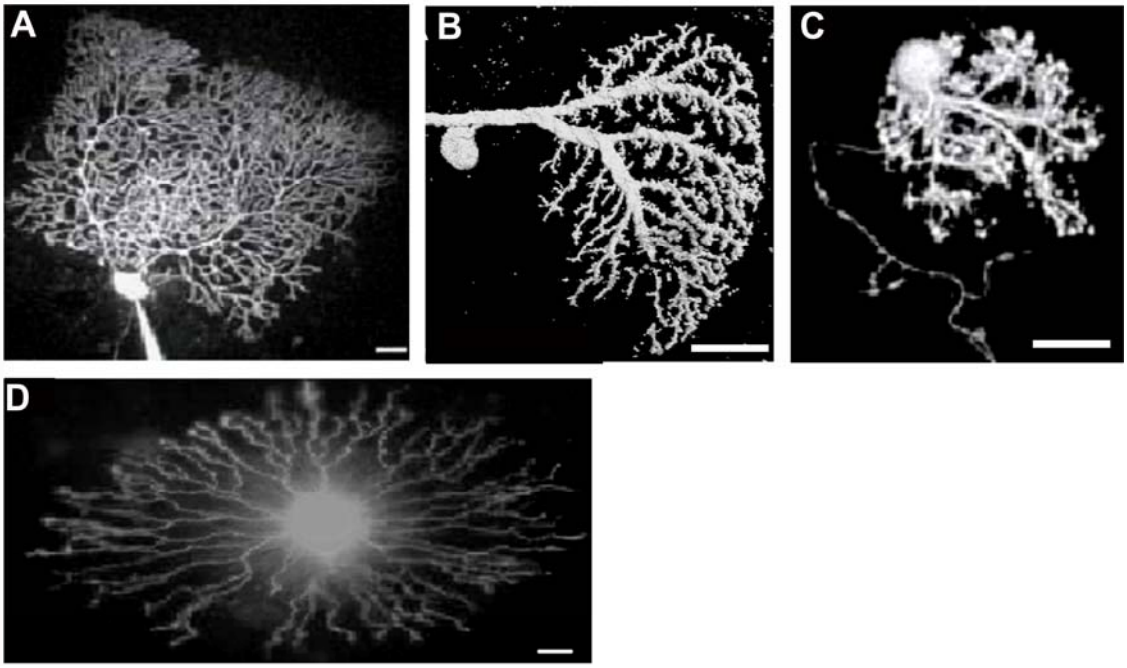

Figure 1. 1: Dendrite morphologies of single neurons. A: Cerebellar Purkinje cell adapted from Wang *et al* (2000). B: Arborization of a single horizontal system neuron of the adult *Drosophila*. Clone expressing mCD8GFP by use of the MARCM technique (current study). C: Mitral cell from the zebrafish olfactory bulb adapted from Friedrich *et al* (2001). D: Retinal starbust cell labeled with enhanced GFP using a gene gun adapted from Masland *et al* (1999).

Intrinsic Factors

Members of the family of small Rho GTPases act as cytoskeletal regulators and integrators of many environmental cues (Redmond and Ghosh, 2001; Van Aelst and Cline, 2004) and play a role in neuronal dendritic development (Luo, 2000; Redmond and Ghosh, 2001). These proteins serve as molecular switches, transducing signals when in their active GTP-bound state, but not when in their inactive GDP- bound state. They were reported to be involved in actin polymerization (Cdc42, Rac), and regulation of actin depolymerization (Rac) or myosin activity (Rho) (reviewed in (Redmond and Ghosh, 2001). Among the transcription factors, *Cut* has been shown to regulate branching patterns of *Drosophila* multidendritic (md) neurons based on its level of expression (Grueber et al., 2003). A study on another transcription factor, *hamlet* (*ham*) (Moore et al., 2002) has

demonstrated that Ham is a binary genetic switch between dendritic outgrowth and branching. A recent study conducted in the sensory neurons of the *Drosophila* peripheral nervous system (PNS) has shown that the *Drosophila spineless* (Ss) gene, a transcription factor, serves diversification of dendrite morphology. It executes the simple dendritic morphologies of class I and class II dendritic arborization (da) neurons, whereas it enables class III and class IV neurons to elaborate complex dendrites (Kim et al., 2006).

Extrinsic Factors

Neurotrophic factors play a role in dendritic tree establishment either by inhibiting or promoting dendritic outgrowth. The best studied are interactions between brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and nerve growth factor (NGF). For example, the antagonistic actions of BDNF and NT-3 on cortical layer 4 and 6 neurons dynamically regulate dendritic growth and retraction (McAllister, 2000).

Transmembrane proteins like cadherins contribute to the stabilization of branches both in invertebrate (Ye and Jan, 2005) as well as in vertebrate (Shima et al., 2004) dendrites.

Interestingly, some molecules important in axon guidance, exhibit a different or even opposite function during dendrite outgrowth. Semaphorin 3A for instance, acts as a repulsive guidance cue in axons and as an attractive cue in cortical dendrite outgrowth (Polleux et al., 2000). Lately, the knowledge about dendrite morphogenesis has increased, for example due to employing *Drosophila* PNS as a genetically amenable system where intact nervous tissue can be analyzed (reviewed in (Jan and Jan, 2001). Furthermore, technical advances like *in vivo* time lapse imaging performed in invertebrates as well as in vertebrates enables visualization of dendrite differentiation in real time (Brown et al., 2006; Knott et al., 2006) offering an insightful contribution to understanding how dendrites are constructed. Nevertheless, dendritic development still remains a field with many open questions.

Building a Dendritic Tree

The processes that shape dendritic trees are very complex and diverse. Generally, they can be separated into several essential phases (Scott and Luo, 2001) (Figure 1.2).

Dendrites extend in a defined direction and increase in diameter. The branches become elaborate; many also generate small specialized protrusions called spines. The emergence of spines involves the same initial stages of development as the formation of branches (Scott and Luo, 2001).

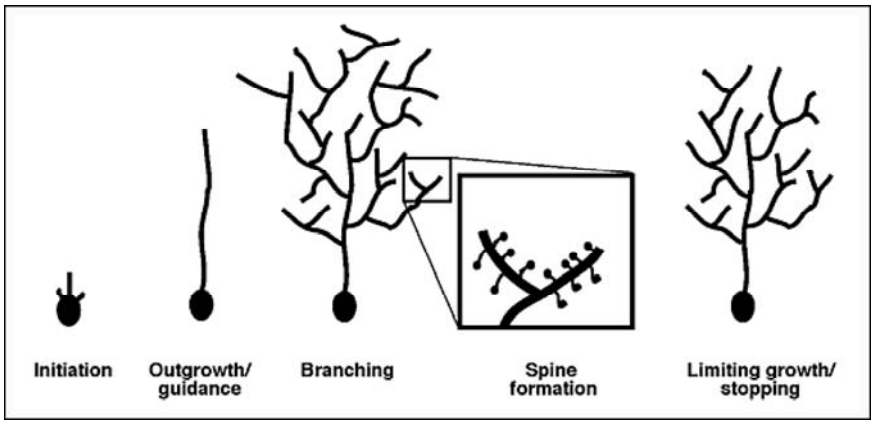

Figure 1.2: Scheme of dendritic development. Adapted from Scott *et al.* (2001).

Both structures begin as transient filopodia, which can then retract and disappear, extend to form a branch, or stabilize and become a spine (Dailey and Smith, 1996). For simplification, I presented dendritogenesis as a set of subsequent steps, but normally the processes of growth, branching, steering and retracting strongly overlap (Scott and Luo, 2001).

Limiting Dendrite Growth

Dendrites stop growing at defined borders, giving rise to the mature overall shape. The molecular mechanisms underlying limitation of growth in dendrites are not yet fully understood. However, one of the identified players is RhoA, a member of small GTPases mentioned previously. Studies of constitutively active RhoA in hippocampal neurons (Nakayama et al., 2000) and loss of function analysis in *Drosophila* mushroom bodies showed its role in restricting dendrite growth (Lee et al., 2000).

The dendrites of some neurons with the same physiological function stop growing once they have completely but not redundantly covered their receptive field. This so called 'tiling' phenomenon is well described in the mammalian retina (Devries and Baylor, 1997;

Wassle, 2004). In the *Drosophila* larval md neurons laser ablations and genetic manipulation have also demonstrated the existence of tiling and selective repulsion between dendrites of the same neuron (Gao et al., 2000). Motion-sensitive neurons of *Drosophila* horizontal system (HS), avoid direct contact with each other (Heisenberg et al., 1978).

Elimination of Branches

Regressive events that refine already existing connections are of great importance for neuronal development (Cowan et al., 1984). Pruning meant as a neuronal degeneration, has been shown to be critical for establishment of motoraxon projections at neuromascular junctions (Keller-Peck et al., 2001). Defective pruning has been blamed for increased spine density seen in Fragile-X-syndrome (FMRX) (Irwin et al., 2001).

In insects that undergo complete metamorphosis, pruning is particularly prominent. Many of the factors that pilot these regressive processes in insect axons and dendrites have been recently identified. Pruning can be regulated hormonally by ecdysone signaling (Schubiger et al., 2003), by the ubiquitin proteasome system (Watts et al., 2003) and by local caspase activity (Williams et al., 2006). Additionaly, phagocytic blood cells and the epidermis were also shown to be actively involved in dendrite elimination in *Drosophila* (Williams and Truman, 2005) . Instead, for some mammalian neurons the glia cells were recently shown to mediate pruning (reviewed in Freeman, 2006).

Insect metamorphosis provides promising grounds for exploring developmental regulation of programmed cell-death and structural and functional modifications of neurons (Levine et al., 1995).

Dendritic Cytoskeleton

Microtubules, actin filaments and neurofilaments set up and maintain dendrite morphology. Each of these cytoskeletal components has its unique properties and makes specific contributions to the overall function of the neuron. They provide the substrates upon which regulators of dendritic development act.

Regulation of Actin Dynamics

Actin filaments provide the main foundation for dendritic and synaptic shape, motility and stability (Matus, 2000; Luo, 2002).

Those filaments have two ends: a fast growing barbed end and a slow growing pointed end. The monomers (G-actin) are added to the 'barbed' end of an existing actin filament (F-actin) and are lost from the filament's 'pointed' end (Figure 1.3) (Calabrese et al., 2006).

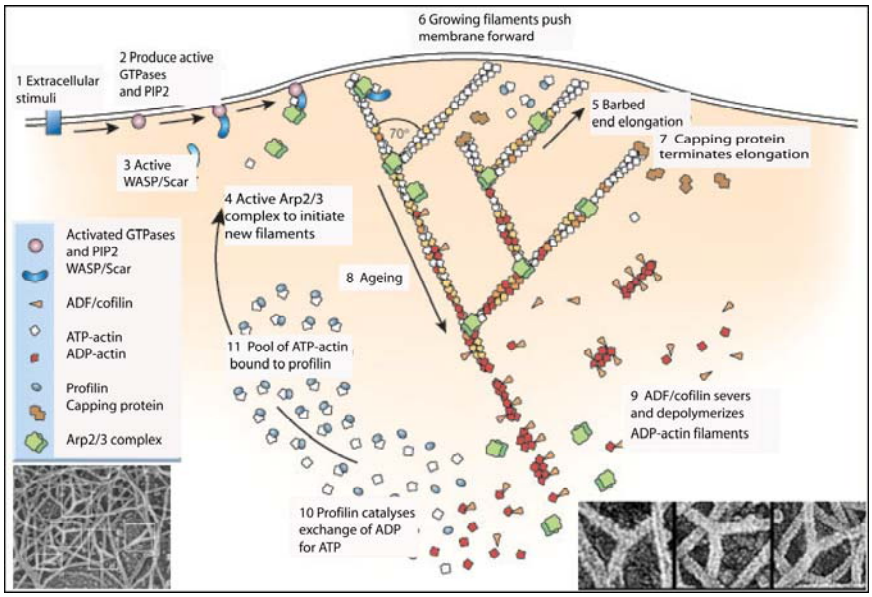

Figure 1.3: Organization and regulation of actin. For the description see text. Inset on the left: an electronmicrograph of actin filaments. Inset on the right: Close up of forked actin filament from the left inset. Figure adapted from Pollard *et al* (2003). Inset images adapted from Svitkina *et al* (2003).

A variety of actin- binding and regulatory molecules determines the degree of polymerization and thus the equilibrium between G- actin and F- actin. Actin regulatory pathways activated by transmembrane signals (Figure 1.3, 1) lead for instance to the activation of GTPases (Figure 1.3, 2), and thus to the activation of Wiskott- Aldrich syndrome protein (WASP) (Figure 1.3, 3) which subsequently leads to initiation of a new filament by Arp2/3 complex as a side branch of an existing filament (Figure 1.3, 4). Each new filament grows rapidly (Figure1.3, 5), fed by a high concentration of profilin- bound actin stored in the cytoplasm, and this pushes the plasma membrane forward (Figure 1.3, 6). A capping protein binds to the growing end, terminating elongation (Figure 1.3, 7). Actin-depolymerizing factor (ADF)/cofilin then severs and depolymerizes the ADP filaments, mainly in the older regions of the filaments (Figure 1.3, 8, 9). Profilin re- enters

the cycle at this point, promoting dissociation of adenosine diphosphate (ADP) and binding of adenosine triphosphate (ATP) to dissociated subunits (Figure 1.3, 10). ATP– actin binds to profilin, refilling the pool of subunits available for assembly (Figure 1.3, 11) (Pollard and Borisy, 2003).

F- actin is distributed at the cortex of the dendrites, but is highly enriched in dendritic spines that are devoid of microtubules. Via a network of protein interactions, actin filaments indirectly link up with the neurotransmitter receptors and other transmembrane proteins that regulate spine shape, development, and function, including Eph receptors, cadherins and neuroligins (Calabrese et al., 2006).

Microtubule Organization in Dendrites

Bundles of microtubules fill the interior of the dendrites and provide their structural integrity (Figure 1.4, A) (Matus, 2000). In dendrites, microtubules have mixed polarity, in contrast to axons in which they have unidirectional plus- end distal polarity. Populations of plus- end and minus- end distal microtubules have been reported in vertebrate neurons (Craig et al., 1992) as well as in *Drosophila* cells (Sharp et al., 1997).

The microtubule- associated motor protein CHO1belonging to a subfamily of the mitotic kinesin- like proteins (MKLP1), establishes this non-uniform microtubule polarity in dendrites by transporting microtubules from the cell body into the developing dendrite with the minus ends leading. Bidirectional orientation of microtubules may be important for dendrite establishment, since it has been demonstrated that in hippocampal neurons inhibition of MKLP1 suppresses dendritic differentiation (Sharp et al., 1997). The dendrite-specific microtubule associated protein (MAP2) regulates dendritic size and stability. MAP2 deficient mice show a reduction in microtubule density in dendrites leading to a reduction in dendrite length (Harada et al., 2002). Cultured neurons treated with an antisense oligo nucleotide of MAP2, failed to form dendrites (Caceres et al., 1992). Disruption of fly homolog of MAP1B-Futsch was shown to induce changes in the neuronal cytoskeleton and progressive neurodegeneration (Bettencourt da Cruz et al., 2005).

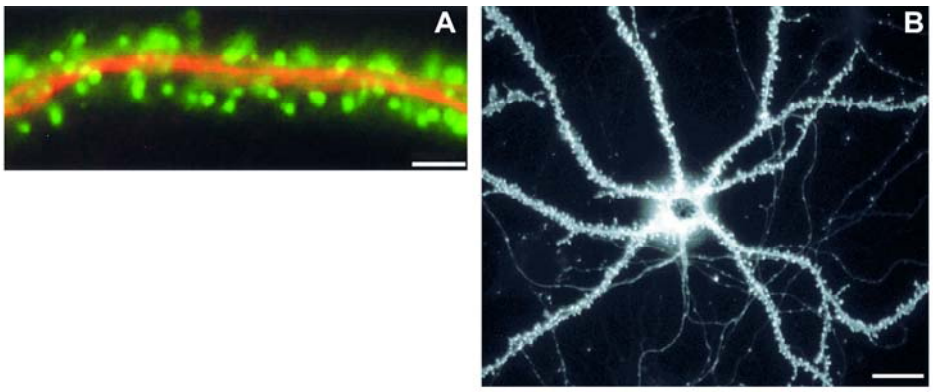

Figure 1.4: Cytoskeletal organization of dendrites. (A) Part of a dendrite from a GFP- actin- expressing hippocampal cell that was fixed and then stained with antibodies against the dendrite- specific microtubule protein MAP2. Red MAP2 labeling shows microtubules concentrated in the shaft of the dendrite compared to green actin- GFP labeling of actin present in dendritic spine heads. Scale bar= 5 µm. B: Living hippocampal neuron in cell culture expressing γ- cytoplasmic actin tagged with GFP- actin. The numerous fluorescent dots on the dendrites are spine heads where actin accumulates. Scale bar= 15 µm. Adapted from Matus (2000).

The studies to date suggest a crosstalk between many cytoskeletal molecules and signaling pathways in dendritic development. However, there is still a need to decipher new players to complete the picture of dendritogenesis.

Dendritic Spines

Vertebrate spines are discrete membrane protrusions (Figure 1.5) where the large majority of excitatory synapses are located (Sala, 2002). Typically, mature spines have a single excitatory synapse located at the head, but the same spine may also have an inhibitory input (Knott et al., 2002). Spines are characteristic for mammalian neurons where inputs from diverse sources converge; prominent examples are pyramidal cortical cells and cerebellar Purkinje neurons (Figure 1.1 A) (Calabrese et al., 2006).

Spine Functions

The presence of spines increases the surface of dendrites. Spines reach out to the axons and allow for more synaptic connections providing the postsynaptic component of the synapses (Swindale, 1981).

Spines constitute separate morphological compartments where Ca^{2+} and other signaling cues are segregated (Nimchinsky et al., 2002). Due to the presence of the thin neck, which allows the chemical and electrical isolation (Araya et al., 2006) from the rest of the cell, so called 'compartmentalization' occurs (Nimchinsky et al., 2002). Biochemical signals rise and fall without spreading to the neighboring synapses along the parent dendrite, thus allowing the isolation and amplification of incoming signals. Similarly, a spine compartment may help confine membrane trafficking to a localized region. Such restriction of molecular signals to one spine may contribute to the phenomenon of 'input specificity', assenting to a given set of terminals to induce changes only at those synapses that are specific to their postsynaptic contacts and not at other synapses on the same neuron that are driven by different axons (Malenka and Nicoll, 1999).

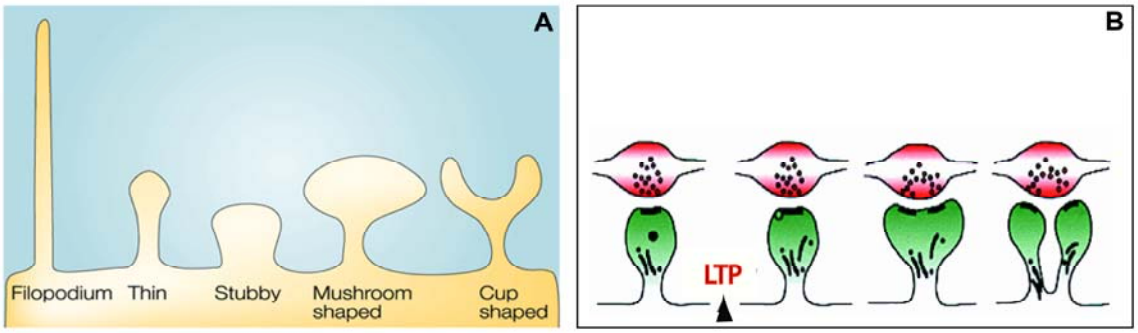

Figure 1.5: Spines are dynamic structures. A: Morphological classification of dendritic spines. B: A model of changes in spine and PSD morphology after LTP. LTP may induce activity- dependent metamorphosis from continuous PSDs in small spines to enlarged segmented PSDs in bigger spines and bifurcation of spines contacting the same presynaptic terminal. Figure A adapted from Hering *et al* (2001). Figure B modified from Luscher *et al* (2000).

Results of experiments conducted *in vitro* (Maletic-Savatic et al., 1999; Nagerl et al., 2004) but also *in vivo* (Lendvai et al., 2000) showed that the number or morphology of spines is increased in response to long- term potentiation (LTP) induction, an experimental paradigm for learning and memory (Figure 1. 5, B). LTP is associated with a shift of actin equilibrium towards F-actin that results in spine head enlargement. In contrast, long-term depression (LTD) shifts the equilibrium towards actin depolymerization, spine retraction and loss (Nagerl et al., 2004).

Correspondingly, it has been showed that animals exposed to enriched environments have an increased number of spines on cortical neurons in comparison to animals grown in non-

stimulating environments (Greenough and Volkmar, 1973; Rampon et al., 2000). Since it is widely assumed that the formation of long-term memory requires activity-dependent long-lasting morphological alterations in plastic neuronal networks, spines are suggested to be the cellular effectors of such processes as learning and memory (Yuste and Bonhoeffer, 2001).

Furthermore, since spine morphology is linked to synaptic function, altered spines in disease states are likely to have diverse functional effects (Calabrese et al., 2006). Several neurological and psychiatric disorders like epilepsy, Down's syndrome, Fragile X Mental Retardation, stroke and schizophrenia exhibit spine abnormalities as does normal aging (reviewed in Fiala et al., 2002).

Spine Components

Spine cytoplasm is denser than the dendrite cytoplasm. In case of bigger spines it usually contains smooth endoplastic reticulum that in a subset of Pyramidal neurons is laminated into a spine apparatus that plays a role in Ca^{2+} handling (Westrum et al., 1980). Poliribosomes serve protein synthesis within the spine. The presence of mitochondria at the base of the spine has been demonstrated to be essential for spine formation and function in hippocampal neurons (Li et al., 2004).

As mentioned before, each spine is supported by a dynamic actin cytoskeleton that responds to internal and external cues to allow spine development, elongation, retraction and movement (Johnson and Ouimet, 2006). Spines are nearly devoid of intermediate filaments and microtubules that are abundant in the dendritic shaft (Husi et al., 2000). They are very dynamic structures thus their morphologies are transient and very diverse (Figure 1.5, A). Spines contain a dense collection of hundreds of transmembrane and scaffolding molecules (Figure 1.6) accumulating at the spine head matrix, precisely– at the postsynaptic density (PSD), a dense thickening of the membrane. It is a site where glutamate receptors (NMDA, AMPA and metabotropic glutamate receptors), adapter proteins, adhesion molecules and other signaling molecules can be found. They are involved in a number of signaling pathways controlling synaptic plasticity.

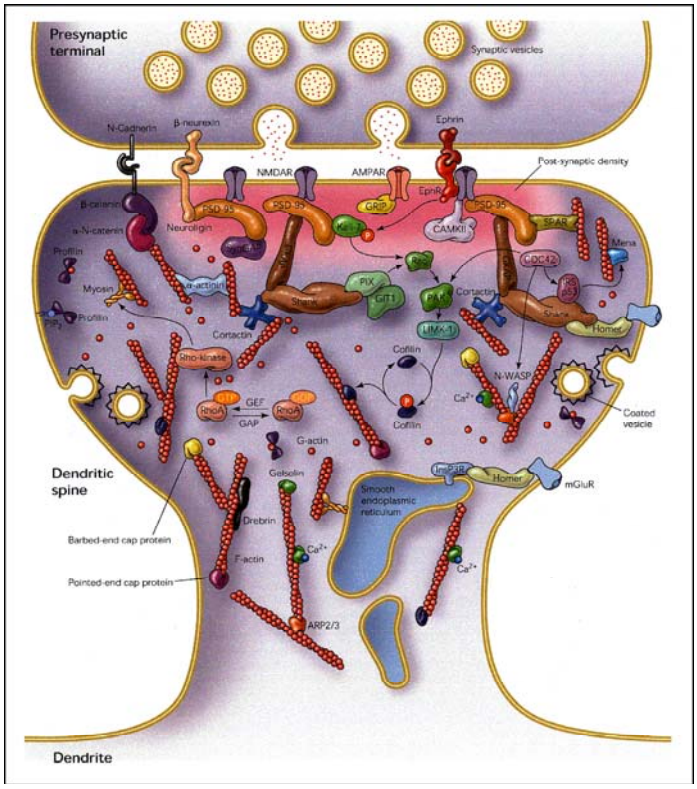

Figure 1.6: Some important components of dendritic spines. Spines use the excitatory neurotransmitter glutamate, which is released from the synaptic vesicles clustered in the presynaptic terminal. Receptors connect to scaffolding molecules such as PSD-95 which recruit signaling complexes (e.g. regulators of Rho GTPases or protein kinases). Actin- regulatory molecules such as profilin, drebrin, gelsolin and cofilin control the extend and rate of actin polymerization (PSD; pink) Figure adapted from Calabrese *et al* (2006).

Spine Morphologies

Typically, spines consist of a bulbous head that is connected to the shaft by a narrower neck. On the basis of detailed ultrastructural studies in fixed brain tissue, dendritic spines have been classified by their shape as thin, stubby, mushroom shaped and cup shaped (Harris et al., 1992) (Figure 1.5, A). This diversity of forms may reflect the level of maturation and activity or simply represent different functions. Mushroom shaped spines are postulated to be the mature ones indicating the presence of a machinery capable of forming active synapses (Fiala et al., 2002; Noguchi et al., 2005). Additionally, in younger animals the percentage of filopodia is higher than in adults, indicating a potential role of filopodia in synapse formation (Yuste and Bonhoeffer, 2004).

Spine Motility

It is believed that an extensive turnover of spines is a normal part of brain physiology (Calabrese et al., 2006). Two major types of spine motility driven by actin dynamics can be distinguished. So called 'morphing' describes continual changes of spine shape and size on the scale of seconds (Fischer et al., 1998). It is likely that this kind of rapid change of the biochemical compartmentalization allows for adjustment of signaling properties of the synapse (Bloodgood and Sabatini, 2005). It might be involved as well in the diffusion of molecules through the plasma membrane into the spine and fast delivery of receptors into the synapse, a process that is likely to be accelerated during the acquisition of a memory (Yuste and Bonhoeffer, 2001; Nagerl et al., 2004).

The second type is the long term change in spine size and number over days and months revealed using multiphoton microscopy (Trachtenberg et al., 2002). Changes in spine density have also been observed *in vivo*, correlating with environmental factors that affect brain activity, such as visual deprivation (Globus and Scheibel, 1967), visual stimulation (Parnavelas et al., 1973) or hibernation (Popov and Bocharova, 1992). This kind of motility indicates that the adult brain retains the capacity to form synapses and remodel the circuitry throughout its life. The dynamic nature of spines could offer a morphological substrate for neurons to adjust constantly the number of axospinous synapses, allowing them to maintain excitatory homeostasis (Holtmaat et al., 2005).

Spine- like Protrusions in Flies

Up to now, there has been no thorough analysis of spines in the *Drosophila*. The presence of processes with spine morphology was reported along tangential neurons of *Musca* and *Calliphora* (Pierantoni, 1976; Hausen et al., 1980). Their presence was further suggested by several recent studies, including the identification of spine- like processes in motion sensitive Lobula Plate Tangential Cells (Scott et al., 2003a, b), and synaptic contacts onto small-spine like protrusions in lateral horn neurons receiving input from the mushroom body Kenyon cells (Yasuyama et al., 2003). However, none of these studies demonstrated that the observed structures possess essential structural and functional spine features.

Dendritic Filopodia

Spines are not the only appendages that protrude from dendrites. During development, dendrites are first decorated by filopodia (Morest, 1969; Jontes and Smith, 2000). Due to similarities between filopodia and spines, information on filopodia has been secondary to the robust knowledge about spines (Ziv and Smith, 1996).

Filopodia are highly motile (rapidly extending and retracting their entire length) in an actin- dependent manner. Their actin matrix is denser then the spine matrix. Their longish shape (>3μm) is due to the longitudinal actin bundles organized similarly to that in the spine neck. Actin polarizes around the tip surface of the filopodium. This electrodense thickening of the membrane is more transient than the spine PSD but serves the same function- it is a place for signaling (Rao and Craig, 2000).

Several types of filopodia have been observed in developing dendrites, both in the vertebrate Purkinje neurons (Laxson and King, 1983) and in the invertebrate motorneurons (Evers et al., 2006).

Function of Filopodia

The elongated shape of filopodia suggests an exploratory function in the extracellular space. Another potential role of filopodia is in guiding the growth of dendrites (Portera Cailliau and Yuste, 2001), a function analogous to that of axonal growth cone filopodia.

However, the major controversy exists as to whether filopodia are precursors of spines (Ziv and Smith, 1996) and which role they play in synaptogenesis. Indeed, according to Vaughn's synaptotropic hypothesis filopodia seek out synaptic partners, and synapses are first formed on the filopodia before being incorporated into dendritic shaft (Vaughn et al., 1974). The fact that they can form synaptic contacts has been demonstrated in the *Manduca* developing motorneurons (Evers et al., 2006).

Spines versus Filopodia

One could conclude that spines and filopodia might not be different structures, but could be a part of a continuum of morphologically plastic structures. On the other hand, stable spines have been shown to emerge directly from shaft synapses (Marrs et al., 2001). Overall, these data suggest that it is probably incorrect to assume that all spines go through the same stages (beginning as filopodia, proceeding to thin or stubby spines, and

ending as mushroom shaped spines). Filopodia and spines could still represent two completely different processes with different functions (Calabrese et al., 2006).

Synaptic Contacts

The most important structure for cell-to-cell communication within the nervous system is the synapse, where neurons meet for the relay of chemical messages. Fast chemical synaptic transmission is mediated by neurotransmitter-containing synaptic vesicles that rapidly fuse with the presynaptic membrane in response to an influx of Ca^{2+}. It not only requires very close apposition of presynaptic and postsynaptic partners but also necessitates a precise structural alignment of cellular components on both sides of the synaptic cleft to facilitate effective synaptic transmission (Atwood, 2006).

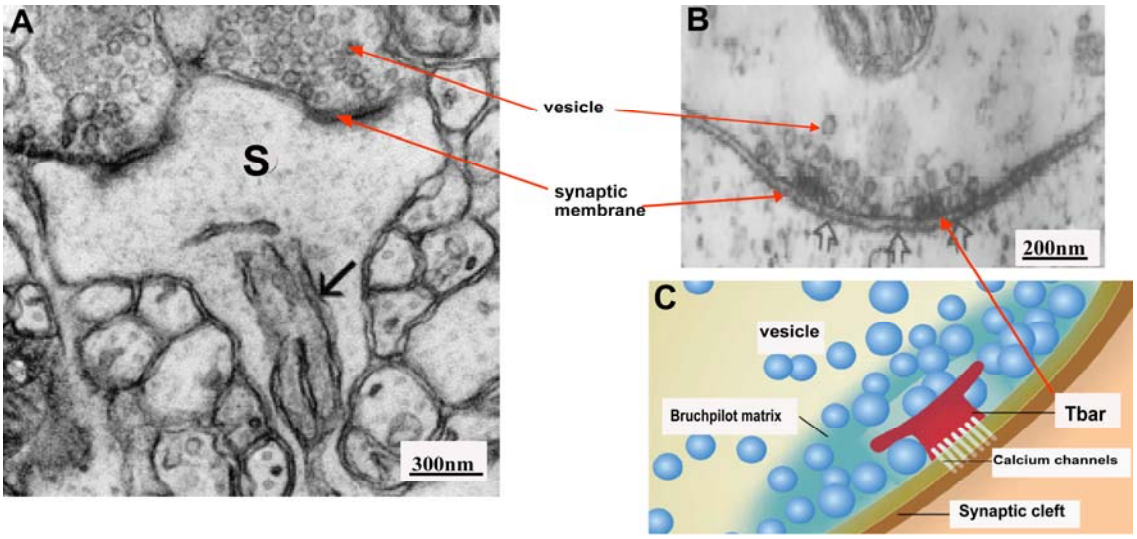

Figure 1.7: *Drosophila* synapses versus vertebrate synapses. A: Hippocampal spine synapse with membrane and docked vesicles. S: spine. Spine apparatus marked with a black arrow. B: *Drosophila* synapse at the neuromuscular junction. Presence of a characteristic T- bar structure. C: Schematics of a *Drosophila* NMJ synapse with T- bar structure -docking site for the vesicles and a Bruchpilot matrix. Figure A modified from Fiala *et al* (2002). Figure B modified from Prokop *et al* (2005). Figure C modified from Atwood *et al* (2006).

Mammalian central nervous system (CNS) synapses share several basic features. Post-synaptic sites are formed in dendritic shafts or spines (Sala, 2002). The electron dense postsynaptic membrane of spines consists of neurotransmitter receptors, usually glutamate receptors, and scaffolding molecules like postsynaptic density (PSD-95). Active zones are

highly specialized sites for release of neurotransmitter from presynaptic nerve terminals. A typical active zone in the central nervous system consists of the plasma membrane juxtaposed to the PSD where synaptic vesicle fusion occurs and cytomatrix where the synaptic vesicles dock (Figure 1.7 A)(Zhai and Bellen, 2004).

T bar Structures at the *Drosophila* Synapses

Drosophila synapses, as reported for the neuromuscular junction as well as the laminar connection in the visual system (Prokop and Meinertzhagen, 2006) are equipped with a characteristic presynaptic density structure called T- bar (Figure 1. 7 B and C). T- bars are surrounded by a structural protein called Bruchpilot (Kittel et al., 2006). Matrix formed by this protein is required for normal localization of docked vesicles and possibly also for clustering of calcium channels (Atwood, 2006).

Detection of synaptic contacts

Synaptic contacts can be detected by immunocytochemistry using antibodies recognizing molecular components of synapses (Figure 1.7). In the vertebrate system both post- (anti-PSD95 (Shiraishi et al., 2003)) and presynaptic (anti- synapsin (Zagrebelsky et al., 2005)) molecules can be identified simultaneously, which allows almost unambiguously to estimate the presence of synaptic contacts.

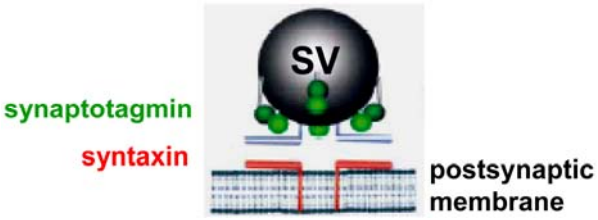

Figure 1.8: Scheme of a synaptic vesicle docked at the membrane. Adapted from Littleton *et al* (2002).

In the *Drosophila* central nervous system this detection method is limited to the analysis of presynaptic components (Figure1. 8) by staining the tissue with antibodies raised against e.g. vesicle associated molecules (Littleton et al., 1993). Dlg (the closest homolog of PSD-95, membrane associated guanylate kinase, originally discovered as tumor suppressor and encoded by *disc-large-1*) a postsynaptic marker allowed obtaining reliable results exclusively at the neuromascular junctions (NMJ).

This limitation in accurate detection of synaptic contacts can be partially overcome by using 3D software (Image J) for the analysis. However, in order to rule out false positive synaptic connections, analysis at the ultrastructural level is necessary.
Even though there are at least several theories on synaptogenesis (reviewed in Yuste and Bonhoeffer, 2004), and recent data provided insights into synapse formation in the visual neocortex *in vivo* (Knott et al., 2006), there are still many gaps remaining.

Anatomy of Lobula Plate Tangential Cells

The lobula plate contains approximately 60 motion sensitive tangential cells (LPTCs) that are a substantial part of the flight control system. They have been extensively examined for their anatomy and physiology in *Musca*, *Phaecinia*, and *Calliphora* (Eckert and Bishop, 1975; Hausen et al., 1980; Hengstenberg, 1982). Studies done in *Drosophila* focused on the behavioral and anatomical analysis of the *optomotor blind* (omb^{H31}) mutant (Buchner et al., 1984; Bausenwein et al., 1986) which misses horizontal and vertical system cells and as a consequence has impaired flight control.
Lobula plate tangential cells can be grouped on the basis on their anatomy and response characteristics. When the LPTCs are grouped according to a preferred orientation one can find two groups: horizontally and vertically sensitive cells (Borst and Haag, 2002). These two groups dominate the lobula plate, with their dendrites fanning over each other and covering almost the entire area of that neuropile. The gigantic size of VS and HS neurons (φ of the cell body =10-12μm, compared to cell body of other LPTC cell bodies φ = 3-7μm) and localization makes them relatively well accessible for physiological recordings and microscopic studies.
Ultrastructural analysis performed in *Musca* and *Calliphora* to determine the location of synaptic structures on VS and HS cells revealed that both classes of giant cells are purely postsynaptic in their dendritic regions and that synaptic sites are located on dendritic branches, and, in highest density on the fine higher order profiles. The terminal axonal arborizations of both classes of giant neurons show pre- and postsynaptic specializations, indicating that they are not simply output regions but also receive inputs from other cells (Pierantoni, 1976; Hausen et al., 1980).

Vertical and horizontal systems can be further divided as shown in (Figure 1. 9):

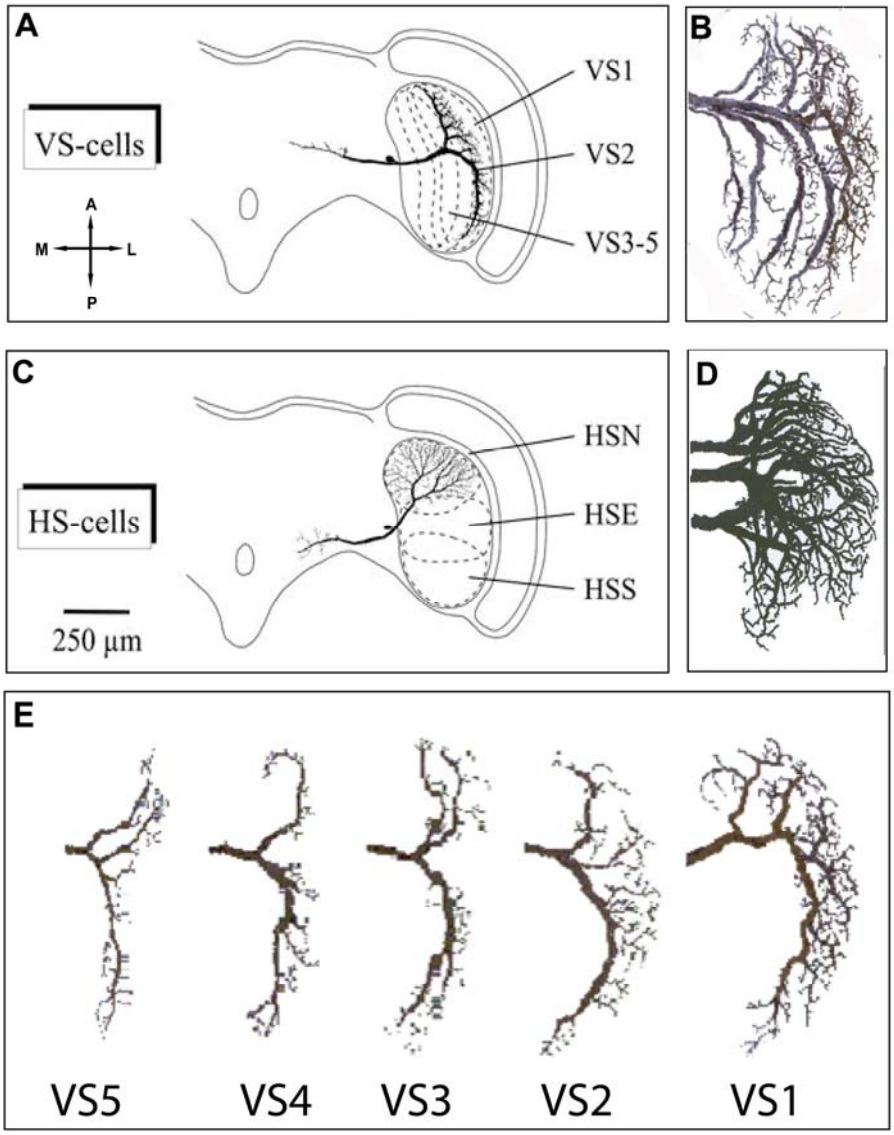

Figure 1.9: Anatomy of the Lobula Plate Tangential Cells. A and C: Frontal view of a *Calliphora* brain highlighting the position of different LPTCs within the lobula plate- the vertical and horizontal system respectively. The dotted lines indicate the dendritic extent of the different LPTCs. B, D and E: Camera lucida reconstructions of *Drosophila* neurons. B: Vertical system dendrites grouped. D: Horizontal system cells grouped. E: Dendritic extensions of VS1-VS5 cells represented separately. Figure A and C adapted from Borst and Haag (1996). Figures B, D and E modified from Shamprasad (2003).

Vertical system (VS) Cells

In *Drosophila*, VS cells are a group of at least 6 giant output cells projecting into the perioesophageal region of the brain. One could hypothesize that the higher number of VS cells in *Musca* (11) may be due to the bigger size of their compound eyes.

Vertical system cell main dendrites are arranged one after the other at the posterior surface of the lobula plate. They have been shown in *Drosophila* to share common lineage (Scott et al., 2002).

In Diptera they respond maximally to downward motion, and are suited to perceive wide field motions that occur when the fly rotates (Hengstenberg, 1982). The main shaft of the *Drosophila* outermost cell, VS1 (Figure 1.9, E), produces branches that combine to cover the most lateral band of the lobula plate. In comparison to its counterpart in the *Calliphora* it lacks a dorsal component. The VS1 neuron in *Drosophila* as well as other members of the VS and HS system is highly stereotyped and can be individually identifiable.

Horizontal System (HS) Cells

The organization of the horizontal system seems to be a conserved feature among Diptera. This class consists of three giant output cells called north, equatorial and south horizontal cell (HSN, HSE, and HSS) according to the position of their dendrites in the lobula plate. They have been identified in *Drosophila* by Heisenberg *et al* (1978) using semi-thin serial sections stained by toluidine blue. HS cells occupy two anterior layers of the lobula plate in *Drosophila* (Fischbach and Dittrich, 1989). As demonstrated by Scott *et al*. (2002) all three of them develop from one neuroblast. In Diptera, HSN and HSS respond to ipsilateral progressive movements and HSE to both ipsilateral progressive movements.

Visual System of a Fly

Meinertzhagen (2006) states: "As anyone knows who tried to catch one, flies see extremely well". Already much earlier Cajal (1937) claimed, perhaps exaggeratedly, that "the insect optic lobes are infinitely more complex that their vertebrate counterparts, comprising an exotic variety of uniquely identifiable neurons". In flies, visual processing

starts with the detection of intensity variation by light sensitive omatidia (Figure 1.11, B). Compound eyes are capable of sensing colors, polarized light and recognizing patterns. Each omatidium is a functional unit and has its own lens consisting of inert cone and pseudocone that focuses light onto a group of eight photoreceptors, R1-R8. The photoreceptors send their axons to a set of brain structures called neuropiles that are devoted to image processing. The photoreceptors R1-R6 project to the lamina while photoreceptors R7 and R8 project to the medulla (Nicol and Meinertzhagen, 1982). These two optic neuropiles are interconnected by interneurons and the medulla is connected with the lobula complex consisting of lobula and lobula plate (Figure 1.10) (Borst and Haag, 2002).

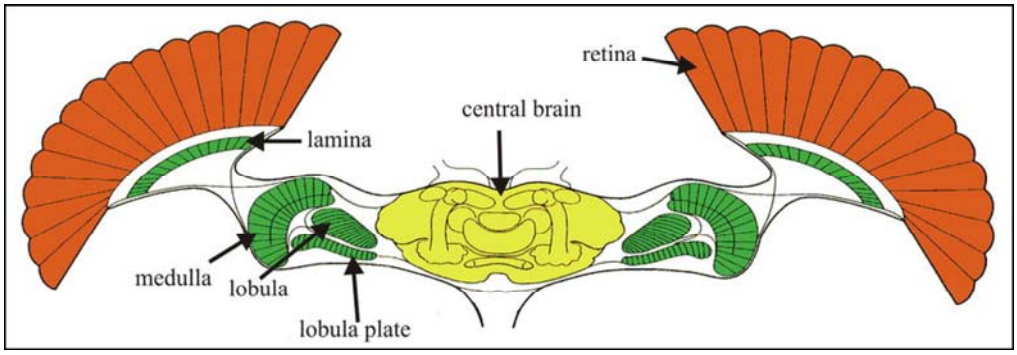

Figure 1. 10: Scheme of a fly visual system. Horizontal section through the fly head. Note: visual ganglia labeled with green. Figure adapted from Borst and Haag (2002).

For each point in the visual world there is one column comprising five monopolar laminar cells (L1-L5). The retina, lamina, and medulla are organized into columns that retinotopically represent positions in the visual world (Figure 1.11, A).

Plasticity in the visual system

Neuronal plasticity is an important feature of the brain and refers to any biochemical, morphological or physiological change in the adult and developing nervous system (Luscher et al., 2000).

The visual system of mammals as well as frogs and fish is capable of plastic changes (Cline, 1991; Tian and Copenhagen, 2003; Karmarkar and Dan, 2006). In contrast, studies carried out on the photoreceptor synapses mutants in *Drosophila* suggest cell

autonomous control of synapse numbers as part of the developmental program of activity-independent steps that leads to a hard- wired visual map in the fly brain (Hiesinger et al., 2006). High resolution experiments done on the VS cells indicate that neither visual experience nor spontaneous activity from the photoreceptors play a role in the development of complex dendritic trees of VS neurons (Scott et al., 2003b). Based on the current data one could state that LPTCs, similarly to other elements of visual map are hardwired, however additional experiments would need to be carried out to verify if they are indeed incapable of plastic morphological changes. It is likely that direct upstream partners of LPTCs, T4 and T5 neurons (Strausfeld and Lee, 1991), will need to be silenced to completely stop synaptic input to LPTCs and highlight possible morphological change (Mizrahi and Libersat, 2002) (see Discussion).

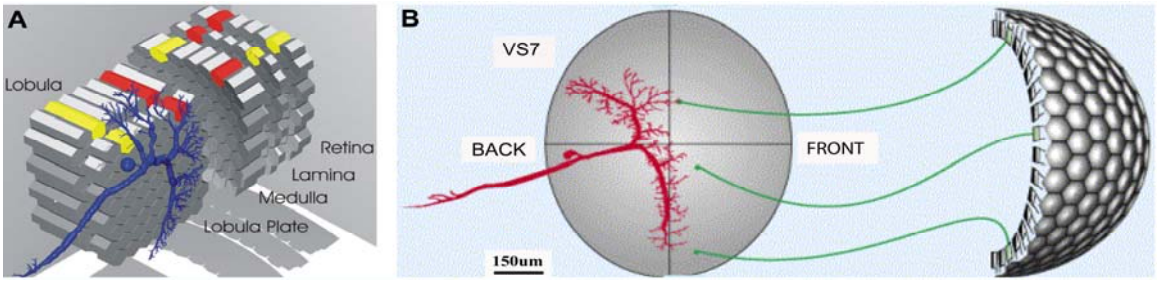

Figure1. 11: Retinotopic pathway in the fly visual system. A: Visual information from the eyes is processed in the subsequent layers called the lamina, the medulla and the lobula complex. Each layer is divided into series of columns. The columns in each layer represent the facets of the retina in a one-to-one fashion giving a retinotopic projection of the visual input onto the dendrites of LPTCs. B: Inputs from an array of photoreceptors project onto the lobula plate to form a spatial map of the visual field. Figure A adapted from Borst and Haag (2002). Figure B adapted from Laughlin *et al* (1999).

LPTCs as a Part of Neuronal Circuitry

Lobula Plate Tangential Cells are motion- sensitive high order processing centers responsible for guiding optomotor responses of the fly and in consequence determine the survival of these insects.

Upstream partners of LPTCs

LPTCs' upstream partners include bushy columnar neurons, namely T4 and T5 (Figure 1. 12, A) situated in the inner layer of the medulla. Sections across the lobula plate's depth

show these levels occupied by T4 and T5 endings as demonstrated in *Musca*, *Calliphora*, *Sarcophaga* and *Drosophila* (Strausfeld, 1984; Fischbach and Dittrich, 1989; Strausfeld and Lee, 1991).

Downstream partners of LPTCs

Among the downstream partners of the LPTCs are descending neurons (DNs) whose dendritic trees are grouped into clusters. Each of the clusters localized in the dorsal part of the deutocerebrum is visited by wide- field motion- sensitive neurons and by small field retinotopic elements (Figure 1.12, B). Typically, wide field neurons establish presynaptic sites onto the trunks or major branches of DNs (Gronenberg and Strausfeld, 1990).

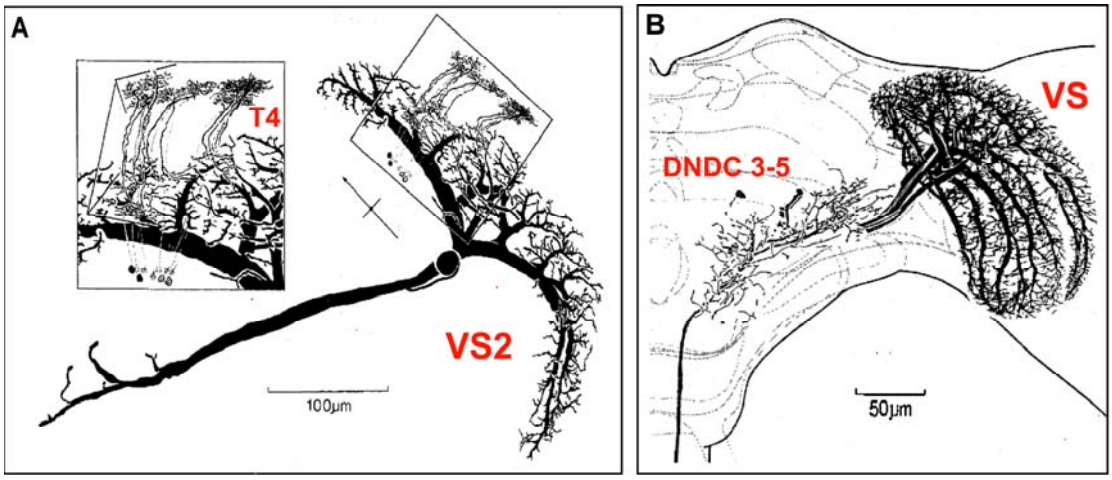

Figure 1.12: Vertical system cells as part of a neuronal circuit. A: Golgi impregnated VS2 neuron visited by T4 neurons from the medulla. Inset illustrates linear order of columnar neurons. B: Reconstruction of a descending neuron DNDC3-5 visited by the vertical VS cells. Figure A adapted from Strausfeld (1991), figure B adapted from Gronenberg (1990).

Dorsal DNs project to thoracic motor neurons of the neck and flight motor. It is suggested that in Diptera, dorsal descending neurons may separately be involved in velocity, stabilization and steering manoeuvres (Strausfeld and Gronenberg, 1990).

Development of the Fly CNS

Unlike many other larval organs, the central nervous system persists into the adult stage. The optic lobe starts as a small vesicle attached to the basal surface of the early larval

brain (Figure 1.13, A). Then it proliferates and gives rise to the outer and inner optic anlagen. The outer optic anlage forms the lamina and part of the medulla; the inner optic anlage gives rise to the remaining part of the medulla, the lobula and the lobula plate.

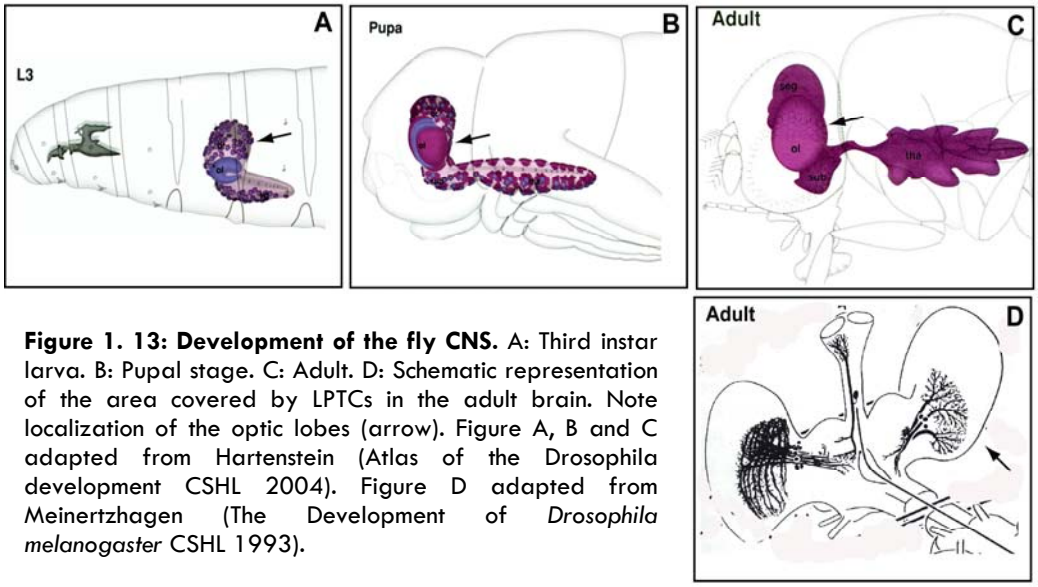

Figure 1. 13: Development of the fly CNS. A: Third instar larva. B: Pupal stage. C: Adult. D: Schematic representation of the area covered by LPTCs in the adult brain. Note localization of the optic lobes (arrow). Figure A, B and C adapted from Hartenstein (Atlas of the Drosophila development CSHL 2004). Figure D adapted from Meinertzhagen (The Development of *Drosophila melanogaster* CSHL 1993).

The anatomy of *Drosophila* CNS changes remarkably in late postembryonic development. The larval brain hemispheres, to which the optic lobes are attached, become the supraesophageal ganglion of the adult brain (Figure 1.13, C) (Hartenstein, 2004).

The postembryonic remodeling in insects evokes issues that are reminiscent of vertebrate CNS development, including neurogenesis, programmed cell death, neuron- glia interactions, and the structural and functional modifications of neurons during the transition to adulthood (Levine et al., 1995). Given these similarities, cellular and molecular studies on the insect nervous system could shed light on mechanisms underlying diverse phenomena like synaptic plasticity.

2 Materials and Methods

Fly stocks

Name/ Genotype	Source/Donor
Gal4 DB331	Alexander Borst/ Reinhardt Stocker (Germany)
Gal4 3A	Alexander Borst / Martin Heisenberg (Germany)
w; UAS-actinGFP 2#2 (II)	Hiroki Oda (Japan)
w;P[w^+UASpGFPS65Cα-tub84B]/TM3Sb#56	Nicole Grieder (Switzerland)
w; P[w^+UASpGFPS65Cα-tub84B]/SM 6a#57	Nicole Grieder (Switzerland)
UAS-GMA (I)	Daniel Kierhart (USA)
UAS-GMA (II)	Daniel Kierhart (USA)
Elp/CKG; scrb e FRT80/TKG	Juh Nung Jan (USA)
y^1 w^*; P{tubP-GAL80}LL9 P{neoFRT}80B	Juh Nung Jan (USA)
elavGal4,UASmCD8GFPHsFLP/ywFM6B;Pin/Cyo	Juh Nung Jan (USA)
UAS-CD2-HRP/CyO	Jean Paul Vincent (UK)
UAS sraRNAi	Christian Klaembt (Germany)
Cyo/Sp; UAS-sra myn/TM2 #5	Christian Klaembt (Germany)
Cyo/Sp; UASsraΔC$^{Myc\#5}$/TM2	Christian Klaembt (Germany)
UAS-CaMKII.T287A	Leslie C. Griffith (USA)
UAS-CaMKII.T287AD1	Leslie C. Griffith (USA)
UAS-CaMKII.T287AD	Leslie C. Griffith (USA)
9-9Gal4 (L3 cells driver)	Larry Zipursky (USA)
9-9Gal4 mCD8GF	Larry Zipursky (USA)
Gal4 21D	Thomas Raabe (Germany)
UAS-mRed/Cyo (O)	Generated in the Lab
w; UAS-mCD8GFP/Cyo	Bloomington Stock Center
P{UAS-mCD8::GFP.L}LL4, y^1 w^*; Pin^{Yt}/CyO	Bloomington Stock Center (BL#5136)

w^{1118}; P{UAS-myr-mRFP}2/TM6B, Tb^1	Bloomington Stock Center (BL#7119)
w^{1118}; P{UAS-myr-mRFP}1	Bloomington Stock Center (BL#7118)
w^{1118}; P{UAS-Fmr1.Z}3	Bloomington Stock Center (BL# 6931)
w^{1118}; $Fmr1^{\Delta 50M}$/TM6B, Tb^1	Bloomington Stock Center (BL#6930)
w^{1118}; $Fmr1^{\Delta 113M}$/TM6B, Tb^1	Bloomington Stock Center (BL#6929)
P{UAS-Rac1.L89}6	Bloomington Stock Center (BL#6290)
P{UAS-Rac1.N17}	Bloomington Stock Center (BL# 6292)
P{GawB}OK307	Bloomington Stock Center (BL#6488)
y w HsFLP; FRT 42B UAS mCD8GFP ^{LL5}	Bloomington Stock Center (BL# 5131)
w DB331 UASmCD8GFP; Pin/Cyo (#30)	Generated in the lab
yw; HsFLP; tub Gal80 FRT80	Generated in the lab
yw; TM3Sb/TM6Tb	Bloomington Stock Center
y w HsFLP;; FRT 82B / TM3 ry Sb	Bloomington Stock Center

Antibodies

Name	**Source/Donor**
Anti-synaptotagmin DSYT2 (rabbit 1:25)	Hugo Bellen (USA)
Anti-synaptotagmin 3H2 2D7(mouse 1:3)	Hybridoma Bank (USA)
Anti-syntaxin 8C3 (mouse 1:3)	Hybridoma Bank (USA)
Anti-mouse conjugated with Rhodamine Red X	Jackson Laboratories (USA)
Anti-rabbit conjugated with Rhodamine Red X	Jackson Laboratories (USA)
Alexa Fluor 488 (goat 1:100)	Invitrogen (Germany)
Alexa Fluor 633	Invitrogen (Germany)
Alexa Fluor 680	Invitrogen (Germany)
Alexa Fluor 350	Invitrogen (Germany)
Alexa Fluor 405	Invitrogen (Germany)
Anti-mouse-Cy3	Jackson Laboratories (USA)
Anti-rabbit-Cy5	Jackson Laboratories (USA)

Anti-mouse-Cy5	Jackson Laboratories (USA)
Anti-CD8 (rat 1 :100)	Caltag Laboratories (USA)
Anti-GFP (rabbit)	Invitrogen (USA)
Anti-Dlg	Hybridoma Bank (USA)
Anti-dFMR1 5A11	Hybridoma Bank (USA)
Anti- HRP (rabbit)	ICN Biomedical Cappel (USA)

Instruments

Name	Source
Leica SP2 Confocal Microscope	Leica GmbH, Heidelberg (Germany)
Leica SP2 UV Confocal Microscope	Leica GmbH, Heidelberg (Germany)
Leica MZ16 Fluorescent Dissectoscope	Leica GmbH, Heidelberg (Germany)
Zeiss Stemi 2000-C Dissectoscope	Zeiss GmbH, Oberkochen (Germany)
Schott KL 1500 LCD Light Source	Schott, Germany

Consumables

Name	Source
Forceps DuMont Nr.5	FST, Germany
Fly food vials	Greiner Bioone, Germany
Fly food plugs	Kunststoffteile Klühspies, Germany
Microscope slides 76mm x 26mm	Menzel Gläser, Germany
Microscope cover glasses 24mm x 40mm	Menzel Gläser, Germany
Small petri dishes	Mat Tek Corporation, USA
Staining Cups	Lymphbecken, Germany
Immersion Oil	Leica GmbH, Heidelberg (Germany)
Granulated Yeast	Fermipan, Holland

Solutions and Media

Name	Recipe
Blocking buffer for Antibody Staining	10% serum in1xPBT
PBS (10x)	100mM Na_2HPO_4, pH7.4 20mM KH_2PO_4 1.37 M NaCl 27mM KCl
PBT (1x)	0.05% Triton X-100 in 1x PBS
PFA (4%)	4% Paraformaldehyde in 1x PBS, pH 7.4
Vectastain ABC Elite Kit	Vector Laboratories (USA)
Grace Medium	Invitrogen (Germany)
Shields and Sang M3 Insect Medium	Sigma (USA)

Fly Maintenance

Drosophila melanogaster flies were raised at 25^0 C, 70% relative humidity to achieve the fastest generation time (app. 10 days from egg to eclosed adult). Standard fly media was used:

Fly food (1L)	Yeast	15.0g
	Agar	11.7g
	Molasses	80.0g
	Corn flour	60.0g
	Methylparaben	2.4g
	Propionic Acid	6.3ml

Yeast paste (yeast granules and fly water) was added to the bottles in order to enhance egg lying.

Fly water	0.8% CH_3 COOH in dd H_2O

Staging of the Animals

To examine how the LPTC dendrites grow, I designed a protocol that allowed me to analyze the consecutive stages of their development. Eggs were collected in bottles over the time course of 2h. Then, they were left to develop for 3-4 days and when the animals reached 2nd instar stage I selected the females. Chosen animals were put in vials for further aging. I precisely marked the moment when they stopped crawling and turned into white prepupae. From that time point (puparium formation) I counted the hours (APF= After Puparium Formation), collected and dissected the fly at the desired time.

Dissection of Adult Fly Brains

Female *Drosophila melanogaster* flies were briefly anesthetized with CO_2 and transferred with the forceps to a watchmaker dish with 70% EtOH for 30s in order to remove the wax that covers the cuticles of the flies. Then they were sunk in the PBS solution and dissected under the dissectoscope. First, the thorax was cut away from the head and the gut was pulled out. Second, the proboscis was removed. To access the brain, the head capsule was cut in the middle starting from the hole created after detaching the proboscis and pulled simultaneously from both sides. Next, trachea and the air sacs that normally cover the brain were removed. Generally, if not stated differently, brains from 4-8 day old flies were analyzed.

Dissection of Larval and Pupal Fly Brains

For the dendritic differentiation studies I dissected larval, prepupal and all pupal (P1-P10) stages of *Drosophila melanogaster* flies. To dissect out the larval brains, I put the

animals into PBS and using the forceps I opened the body pulling from both sides. Internal organs and the surrounding lipid tissue were discarded.

In case of pupal stages, the animals first needed to be released from their pupal cases and then transferred directly into PBS. The following steps were similar to the dissection of the brains from the adult animals. Since the pupal brains are far more fragile then the adult ones, special care was exerted in order to avoid damaging their nervous tissue.

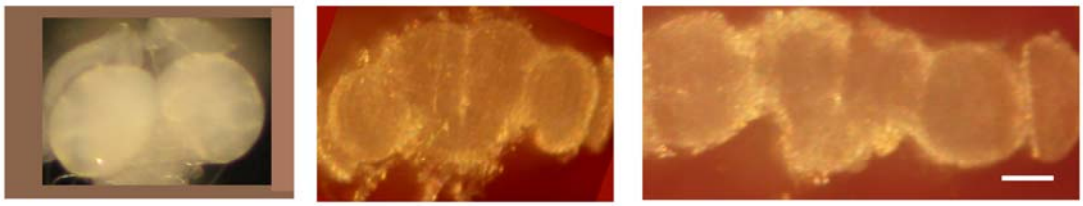

Figure 2.1: Stages of *Drosophila melanogaster* brain development. A: Third instar larva brain. B: Pupal brain 65hAPF. C: Adult brain. Scale bar= 100μm.

Sample Preparation

Dissected brains of all stages were fixed for 50min in 4% PFA. Then they were washed 3x 15 min in PBT (0.1% Triton X-100 in PBS) at RT and transferred with a pipette tip onto the microscope slide. In the central part of a slide I prepared a rectangular frame from stripes of double sided TESA Film (Beiersdorf, Germany) to obtain a proper spacing. The brains were placed on a droplet of anti- fading Vectashield medium (Vector Laboratories). I oriented them dorsally to improve visualization quality. Samples were stored at 4^0C.

Gal4- UAS System

For fluorescent labeling of the LPTCs I used the Gal4-UAS system (Brand and Perrimon, 1993). This genetic tool allows visualization of fluorescently tagged molecules in a desired subset of cells, in which the marker expression is restricted by a Gal4 driver enhancer trap line.

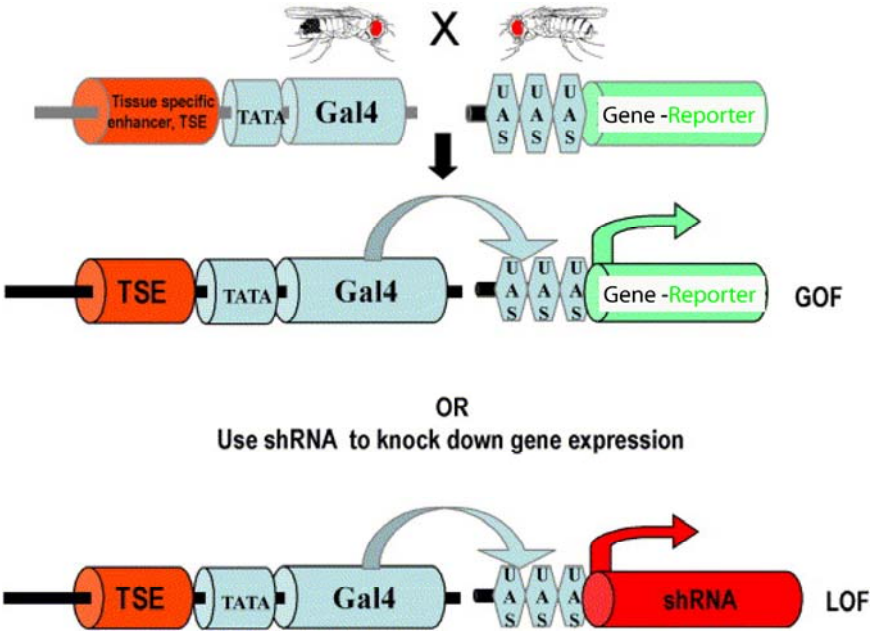

Figure 2.2: Gal4 UAS system. Gal4 expression is driven by a tissue- specific genomic enhancer (TSE). Sequence of Gal4-dependent reporter gene is subcloned 3' of Gal4 binding sites. In the progeny of flies carrying the target (UAS-*Gene* X-Reporter) crossed to flies expressing Gal4 (Enhancer Trap Gal4) the target gene is activated in a tissue-specific pattern. GOF= Gain of function, LOF= Loss of function. Figure adapted from Marsh *et al* (2006).

In my studies I used two drivers that are relatively specific for LPTCs and several GFP fused reporters (Table 2.1). Gal4-UAS system was used to study the effect of the overexpression (Figure 2.2- GOF) as well as the loss of function (Figure 2.2- LOF), of the genes of interest.

Gal4 DRIVERS	ESSENTIAL REPORTERS
DB331 (Stocker R.)	UAS- mCD8GFP (Luo L.)
3A (Heisenberg M.)	UAS- actin GFP (Oda H.)
	UAS- tubulin GFP (Grieder N.)

Table 2.1: List of Gal4 drivers and UAS- reporters used in the study.

1. DB331 - drives expression throughout the developmental stages in VS and HS cells as well as in the columnar elements of the medulla (Figure 2.2 A).
2. 3A - drives expression notably in the VS cells starting from the late developmental stages (from P8 on). Background GFP signal is detected (Figure 2.2.B).

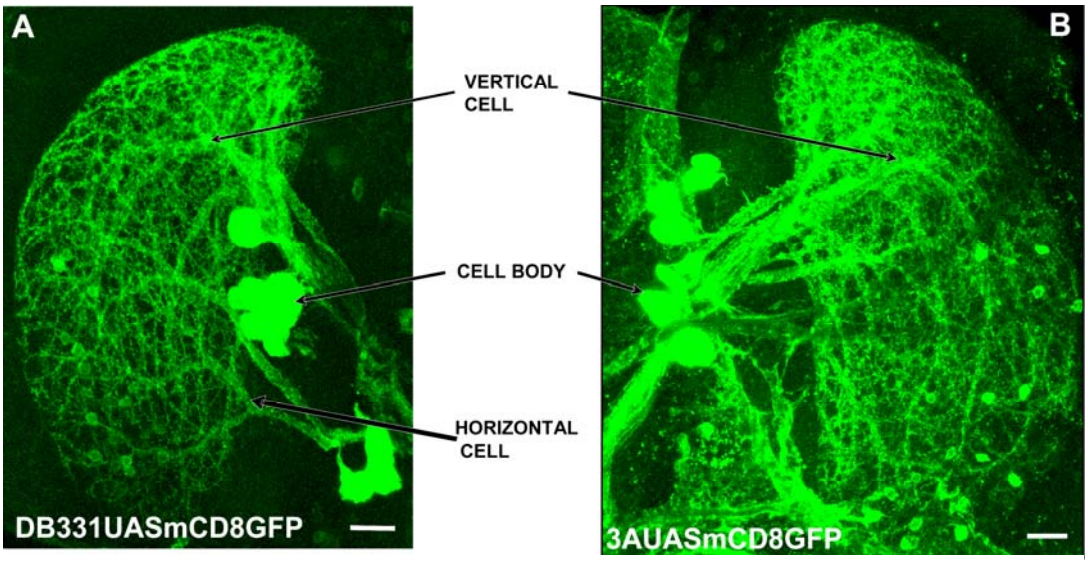

Figure 2.3: Expression patterns of the Gal4 drivers in the adult brain. A: DB331 driver. Note expression in the HS cells in contrast to B: expression pattern of 3A driver. Scale bar=20μm.

The expression pattern of the reporter in the dendrites of VS and HS cells is complex. The dendrites fan over each other and this was an obstacle to obtaining high resolution and reproducible data. To solve this issue I aimed to restrict the expression pattern of the DB331 driver. I undertook several approaches:

1. I mapped the DB331Gal4 driver, meaning I found the place in the genome where the PGawB element was inserted. To map the driver I used the plasmid rescue method (O'Kane, 1998). DB331 maps to the region 12 DE on the X chromosome. The neighboring gene is CG 32611, a transcription factor (data not shown). The potential role of this gene in the LPTCs formation needs to be verified. Then, the gene could be modified in a way that it would result in a more specific expression

pattern. Molecular refinement of the enhancer could be performed in order to make the driver even more specific or inserting a reporter eg. mCD8-GFP downstream of DB331.

2. I generated single cell clones using the MARCM technique (Figure 2.7)
3. I developed an imaging protocol that allowed me to take images of the same neuron from animal to animal. I focused on the middle region of the dorsal VS1 neuron branch (Figure 2.3).

VS1 is the most distal of the vertical neurons and there is very little overlap with neighboring vertical neurons distal of the primary dendrite. Dendritic branchlets from VS1, which are highly stereotyped, can be distinguished from horizontal cell dendrites by a small gap between vertical (more anterior) and horizontal neurons (more posterior).

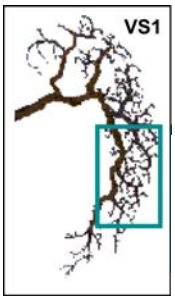

Figure 2.4: Schematic representation of a VS1 neuron. Boxed is a representative middle region of a ventral branch of VS1 used for quantifications. Modified from Shamprasad (2003).

Confocal Image Acquisition

Optical section of z stacks were taken from fixed brains using a Leica SP2 (Heidelberg, Germany) confocal microscope. Z step was 0.15μm- 0.5μm.

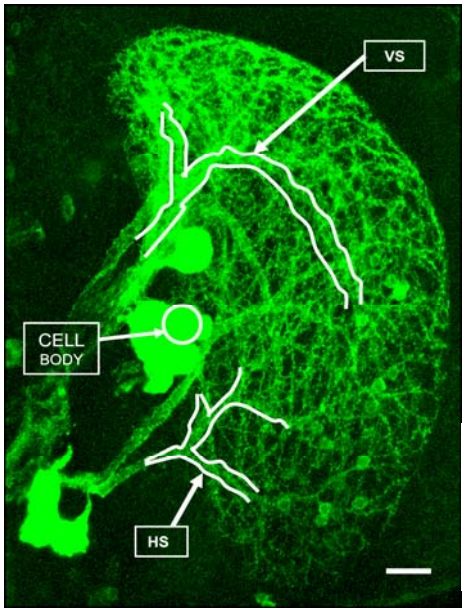

Figure 2.5: Representative confocal image of the entire LPTCs tree. Note the marking of VS, HS primary dendrites and gigantic cell body. HS= horizontal cell, VS=vertical cell. Scale bar=20μm.

Overall thickness of a stack was 2μm- 30μm for the middle fragment of VS1 or entire dendritic tree respectively.

MARCM Technique

To examine the morphology of LPTCs dendrites and spines with a single cell resolution and to look at the homozygous mutant neurons in phenotypically wild type brains I used Mosaic Analysis with a Repressible Cell Marker (MARCM) (Lee and Luo, 1999).
This technique enables one to obtain positively marked single cell clones in an otherwise unlabeled background.

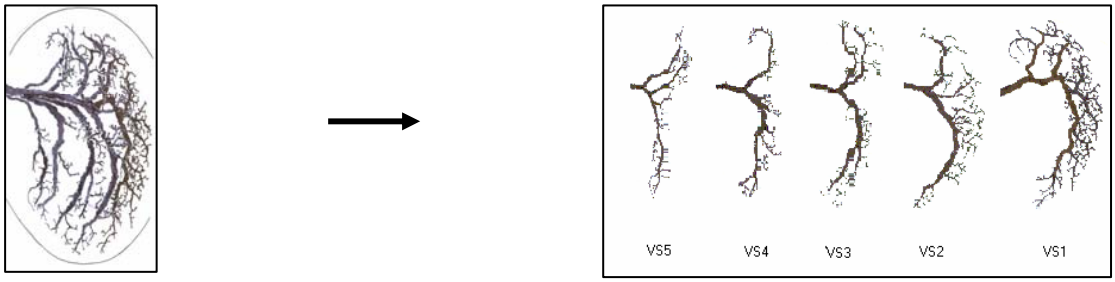

Figure 2.6: A principle of MARCM technique. Restricting the Gal4 expression pattern driven in all VS cells to single cell MARCM clones. Modified from Rajashekhar and Shamprasad (2003).

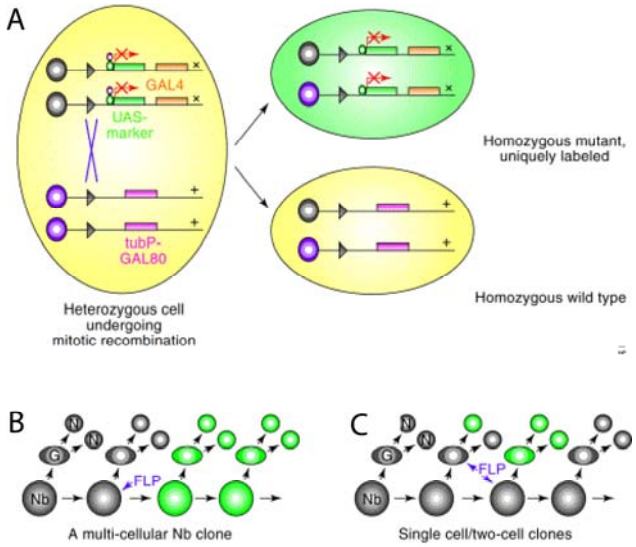

Figure 2.7: Genetic basis of MARCM technique. A: After site-specific mitotic recombination, a heterozygous mother cell can give rise to two daughter cells in which the chromosome arms distal to the recombination site become homozygous. Driven by the *tubulin 1* α promoter, Gal80 is ubiquitously expressed and efficiently suppresses Gal4-dependent expression of a *UAS-marker* gene (i.e. the Gal80 pink rectangle binds to the Gal4 orange box at the UAS site, preventing transcription). If *tubP-Gal80*, but not *Gal4* or *UAS-marker*, is inserted on the chromosome arm carrying the wild-type (+) gene of interest, the daughter cell homozygous for the mutant gene (x) no longer contains *tubP-Gal80* (pink rectangle). Therefore, the marker gene can be specifically turned on by GAL4 (orange box) in homozygous mutant cells. B and C: Schematic diagram showing a typical CNS neuroblast division pattern in *Drosophila*. Nb, neuroblast; G, ganglion mother cell; N, neuron. Green circles represent those that will be marked as members of the clone lacking Gal80. B: If a Nb becomes Gal80-negative ($Gal80^-$) after FLP- mediated mitotic recombination, all neurons derived from this $Gal80^-$ Nb are specifically labeled and appear as a multicellular Nb clone. C: If a G loses Gal80 after mitotic recombination, two neurons derived from the $Gal80^-$ G are labeled and become a two-cell clone. By contrast, if mitotic recombination occurs in a dividing GMC, only one of the two post-mitotic neurons will be labeled. Adapted from Lee and Luo (2001).

To obtain labeled single cells, Gal80 (ubiquitously expressed repressor of Gal4), has to be eliminated from a cell (Figure 2.7), thus allowing the expression of a UAS-driven marker gene. Exclusion of Gal80 is possible by the inclusion of Flp recombinase target (FRT) proximal to Gal80 on the chromosome being used for recombination and on the homologous chromosome. The *flp* gene is included and driven by a heat shock promoter, thus allowing for heat- shock- induced mitotic recombination at the FRT sites. The resulting elimination of Gal80 in this cell allows the driver (Gal4-DB331) for expression of the marker.

I obtained multiple, double and single cell clones achieved via mitotic recombination induced via heat shock. My results confirmed previous observations (Scott et al., 2002) that LPTCs are born in a rapid succession with no consistent birth order. Since those neurons are born in too short time window to establish precise time points for inducing recombination I heat shocked the animals 2 or 3 times in the I st, IInd and IIIrd instar larvae. The heat shock was done for 30 min in 37-38^{0}C.

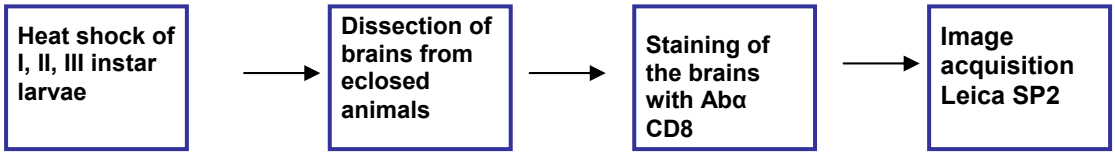

Figure 2.8: MARCM data analysis.

Afterwards the GFP signal was enhanced by the α mCD8 staining and the brains were scanned under the confocal microscope (Figure 2.8).

Analysis of the Images

Qualitative analysis of the confocal images was performed with the Leica SP2 software (Heidelberg, Germany), Adobe Photoshop and Adobe Illustrator (Adobe Inc., Illinois, USA). Quantitative assessment of synaptic contacts was done using Image J software (Synchro Plug in)(NIH, USA). Juxtaposition of a presynaptic partner to a spine was traced synchronously for both channels in projections of a z stack and confirmed in single confocal

slices. The number of synaptic contacts was verified by rotating 3D reconstructions of the stack between 0^0 and 180^0 using AMIRA software (Mercury/TGS, Berlin, Germany). Only the contacts that were confirmed at every rotation angle were counted in the statistics. Volume rendered images and movies adding information about the actual 3D structure of the dendritic trees were produced from confocal stacks using AMIRA software.
Measurements on the electron microscopy data were done using FIVE Software (Soft Imaging System, USA).

Quantitative Morphological Analysis of Spine- Like Structures

Quantification of the spine density and length was done for the following genotypes: *DB331-GAL4 UAS-mCD8GFP/+; UAS-myr-mRFP/+*, *DB331-GAL4/+; UAS-actinGFP/+; UAS-myr-mRFP/+*, *DB331-GAL4/+; UAS-GMA/+; UAS-myr-mRFP/+, DB331-GAL4/+; UAS-GMA/+; UAS-myr-mRFP* or *UAS-rac1.L* or *UAS-rac1.N17/+*. Flies were between 4 and 8 days old. Image stacks of small dendritic branchlets of VS1 were taken with a Leica SP2 confocal microscope. Branchlet length and spine length were measured with ImageJ on projections of confocal stacks. Number of spine– like structures were counted on 3D reconstructions generated in AMIRA. All protrusions between 0.2 and 3 µm length were considered as spine- like structures. Density and length of spine– like structures were calculated for individual flies. Five animals were analyzed and averaged per data point. Images of 5-10 dendritic branchlets were quantified for each animal. Data for the branchlets or individual spine– like structures was averaged to obtain the value per animal for spine– like structure density and length.
Analysis of spine– like structure morphology classes was also done on the middle region of VS1 neurons of *DB331-GAL4/+; UAS-GMA/+* flies. All animals were 5- 8 days old. Projections from z stacks of confocal images were processed in ImageJ. First, the total number of spine– like structures present on a restricted fragment of VS1 was counted and labeled. Then, spine- like structures were assigned to one out of four classes following the criteria described in the text. The morphology of more than 100 spine- like structures per fly was analyzed. The data obtained from 5 animals were averaged per data point. The

original data were processed by volume rendering (AMIRA, Mercury/TGS, Berlin, Germany) for presentation purposes.

Immunohistochemical Methods

Anti-presynaptic Marker Staining

Fly heads were fixed in 4%PFA in PBT for 3h on ice and subsequently rinsed with PBT (0.1% Triton X-100 in PBS) three times. Dissection was performed in PBT to remove the cuticle and connective tissues. Samples were incubated in 10% fetal calf serum for 4h at RT and then incubated overnight with primary antibodies: pre-absorbed anti-synaptotagmin raised in rabbit (1:25, kindly provided by H.Bellen) or mouse (1:3) or mouse anti-syntaxin (1:3) (both from the Developmental Studies Hybridoma Bank). Samples were washed three times for 10min with PBST before incubation for 2h with secondary antibodies: anti-rabbit conjugated with Rhodamine Red X (1:100) or anti-mouse-Cy3 (1:100; Jackson Laboratories) and anti-mouse Cy5 (1:50; Jackson Laboratories). After three 10min rinses brains were mounted in Vectashield (Vector Labs) using TesaFilm as spacers. Image stacks were taken with Leica SP2 confocal microscope.

Anti- mCD8 Staining

Fly brains were fixed in 4% PFA for 30min at RT. Then, they were washed 3 times for 15min in PBT (with 0.1%Triton X). Then, they were incubated in PBT with 5% fetal calf serum for 30min and stained with primary anti- mCD8 α chain antibody (1:100, Caltag Laboratories, Burlingame, CA) over night at 4^0. The brains were washed 3 times for 15min in PBT and then incubated with secondary goat anti– rat -Alexa 488 antibody (1:500, Invitrogen, Germany) in PBT. After three 10min washing the brains were mounted in Vecta Shield (Vector Labs) anti- fading medium.

Electron Microscopy

Ultrastructural analysis was done on brains of DB331 UAS-CD2 HRP/CyO and eventually 3A UASmCD8GFP/+ adult females. Brains of 4 to 8 day- old flies were dissected in 0.1M PBS- BSA 0.1% buffer and fixed for 30min in 2% paraforaldehyde. The samples were rinsed and stained with an anti- mCD8 antibody (1:50 Calteg) and biotinylated secondary antibody, treated with Vectastain ABC Elite Kit (Vector Laboratories) and developed in DAB solution. The brains were then transferred for post- fixation to 2% glutaraldehyde for 45min, then to 1% osmium tetroxide O_2S_4 and to Dalton solution. Brains were further dehydrated and embedded in Araldit. Slices of thin sections (50nm) were obtained with an Ultracut Ultramicrotome (Reichert-Jung). Spine- like structures were re- identified in serial sections on the basis of image landmarks. To examine the size and dimensions of spine- like structures, as well as the T-bar structures, images with a magnification of 12 700 x were taken.

Culturing Fly Brains

Brains from the staged flies (pupal stages for the differentiation studies and late pupae P10 and adult for the adult for the motility studies) DB331UAS-mCD8-GFP and DB331 UAS-GMA were dissected and a single brain put in a droplet of medium in a small Petri dish with a cover slip at the bottom allowing the imaging. To enable the oxygen exchange the medium was surrounded by a layer of modeling clay (OkoNORM, Germany) that served also as the top cover slip holder. Immobilization of the brain was hampered due to its globular shape.

First, the brain was put onto a droplet of medium, and then the medium was sucked out with a thin tip so that the brain attached to the bottom surface of the dish. Afterwards, the medium was gradually added back to cover the brain. To improve the adhesion of the brains Poly- Lysine and chicken plasma were used.

Among several approaches to achieve the optimal imaging conditions the most promising was to take a single slice with Δ T= 5-10min intervals. This kind of imaging reduces the probability of bleaching of the sample in every round of imaging as well as it allows obtaining images from one sample for a longer period of time.

3 Results

The steps that are essential during the vertebrate CNS development like neurogenesis, programmed cell death, neuron- glia interactions, and the structural and functional modifications of neurons during the transition to adulthood are also essential for *Drosophila* postembryonic remodeling. Given these similarities, cellular and molecular studies on the *Drosophila* nervous system promise to shed light on mechanisms underlying diverse phenomena for instance synaptic plasticity. Below is presented a set of experiments where first I describe the differentiation of LPTC dendrites in the intact brain tissue and then analyze their subcellular cytoskeletal organization. The second set of experiments shows the novel finding that LPTCs bear spine- like structures that share many similarities with vertebrate spines.

Developmental Time Points

To study dendritic development I chose a subset of *Drosophila* Lobula Plate Tangential Cells (LPTCs), system consisting of at least 6 vertical and 3 horizontal neurons. The prominent size, together with their highly stereotyped structure (Scott et al., 2002; Rajashekhar and Shamprasad, 2004) made them appropriate for my experiments.

Since developmental processes are extremely dynamic, the optimal and straightforward way to investigate them would be to perform time lapse experiments. Imaging from cultured brains *in toto* could provide an insight into the real timing of the differentiation phases of the LPTCs. However, since one could not exclude that the time lapse imaging conditions affect the developmental processes, single time points from intact control flies throughout development (mCD8GFP membrane marker) were obtained to serve as a reference for the real time imaging.

I designed an experiment that allowed me to precisely determine the sequence of events in the life line of LPTC dendrites (Figure 3.1).

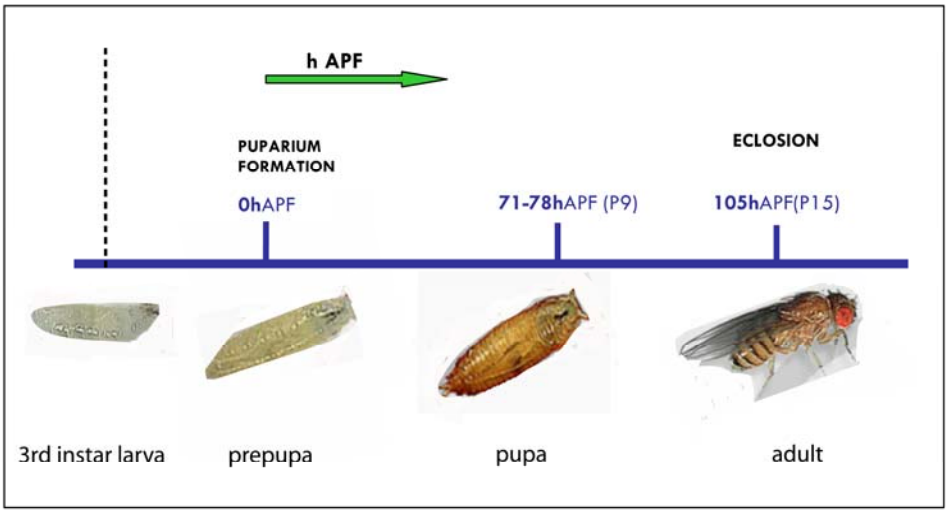

Figure 3.1: Life line of the pupal developmental stages of *Drosophila* (blue line). Dashed line indicates the starting point of the experiment. Green arrow indicates starting point for the counting hours after puparium formation (APF). Images modified from http://flymove.uni-muenster.de/.

Flies starting from the 3rd instar larvae through the pupal stages to the eclosed animals were staged and female brains were dissected with time intervals of 10-20h. The development of flies from white prepupae until adulthood for animals raised in 25^0 C lasts 100-110h (Figure 3.1).

To visualize neurons, I used the mCD8GFP marker (Lee and Luo, 1999) which as a transmembrane protein labels the cell surface. Due to the high surface to volume ratio mCD8 is highly concentrated in neuronal processes. Among the available drivers I used DB331, since it showed a reliable expression pattern in the early developmental stages. A combination of these tools enabled me to follow the differentiation of dendritic branchlets and to define the differentiation phases of tangential interneurons.

At each time point I took images of 5 animals to ascertain the extent to which extend the LPTC development is stereotyped. I performed confocal imaging, producing z- projection stacks of 100-130 serial optical sections through the complete region of arborization of

the LPTCs. The sections were 0.15- 0.35 μm thick depending on the size of the area imaged. All of the images show a frontal view of the dorsal side of the brain. Comparison of the measurements on dendritic tree width and hight (data not shown), from equally aged animals, showed that the pace of sculpturing of the dendrites is highly stereotyped, confirming that the system is appropriate for the current studies.

Late Larval Stage

Based on clonal analysis (Scott et al., 2002) LPTCs are known to arise in the third instar larvae. Indeed, I could observe fluorescently labeled cells with short diversely oriented extensions, already at that stage (Figure 3.2).

Based on localization (corresponding to the area of the lobula plate location within the larval optic lobe), and cell body diameter I could identify the LPTCs even at early stages. Studies where LPTCs were laser ablated in the larval stages (Geiger and Nassel, 1981) are in agreement with the localization of LPTC cell bodies that I observed. To prove the specificity of the early expression pattern this could be compared to the expression pattern of the *omb* Gal4 driver, which is known to drive expression within the lobula plate exclusively in LPTCs. This driver was on the other hand not appropriate for wider use in my experiments since it induces a broad reporter pattern, also in neighboring neuropiles, which compromises imaging quality.

Early Pupal Stages

The images of LPTCs obtained for the first 20 hours of pupal development provided insight into the initial dendritic outgrowth. In spite of evident obstacles disturbing the achievement of sharp images (such as poor tissue preservation, weak GFP signal, and high level of autofluorescent background staining) I managed to show that around 12hAPF faint dendritic branchlets are already present (Figure 3.3. A, B, arrows).

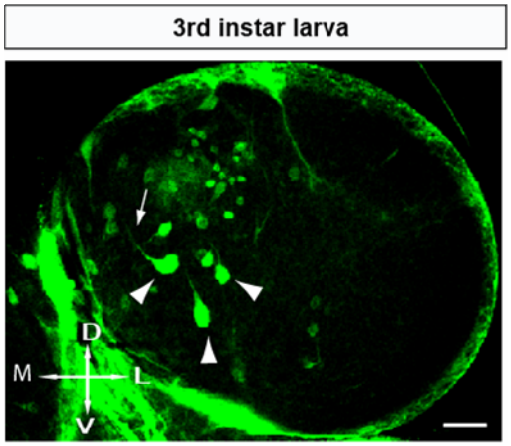

Figure 3.2: Optic lobe overview- LPTCs in the third instar larval. Note the giant cell bodies (arrowheads) with short multidirectional extensions (arrow). L, lateral; M, medial; D, dorsal; V, ventral. Scale bar= 20μm.

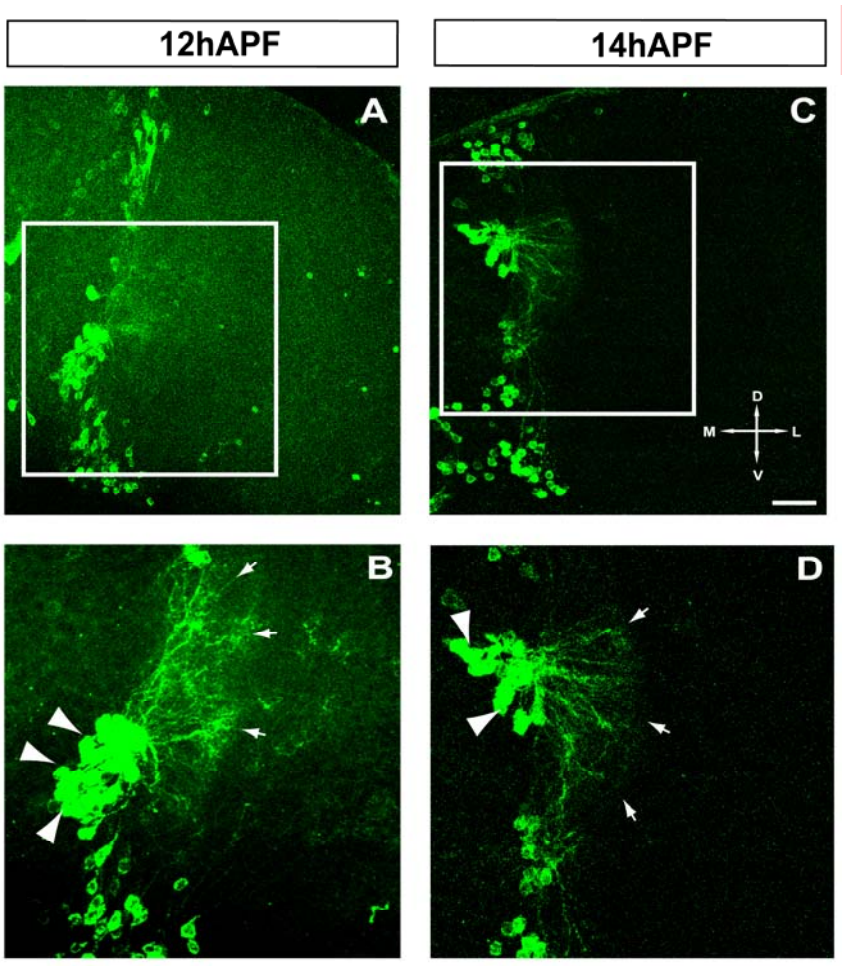

Figure 3.3: View of LPTCs in the early pupal stages. A and C: 12hAPF and 14hAPF respectively; localization of LPTC within optic lobe. The boxes show areas magnified in B and D. B and D: Close-ups of the LPTC dendrites (arrows) and a central cluster of the cell bodies (arrowheads). L, lateral; M, medial; D, dorsal; V, ventral. Scale bar= 20μm.

It is clear that the dendrite outgrowth is widespread, though directional. The tiny extensions steer laterally towards the neighboring medullar neuropile. The central cluster consists of a high number of cell bodies (app. 20) bearing comparable morphological parameters. I hypothesized that during the later stages more than half of them undergo degradation and only 9 neurons remain (6VS and 3HS), although it is possible that at this stage DB331 could drive expression in more than VS and HS neurons.

In addition to the initial LPTC dendritic tree main trunks, some dorsal and ventral extensions were observed. At least a subset of them seemed to arise from dorsal and ventral side cell clusters. The contribution of these neurons in the formation of the final LPTC tree or a surrounding medullar ring cannot be excluded.

Intermediate Pupal Stages

By 30hAPF I could visualize a bundle of axonal extensions directed towards the protocerebrum (Figure 3.4 A). However, it is possible that like *Drosophila* PNS sensory neurons or zebrafish motorneurons (Westerfield et al., 1986) in *in vivo* conditions, as well as cultured hippocampal neurons (Dotti and Simons, 1990), axon growth precedes the outgrowth of dendrites. Since I focused my imaging on the layer where dendrites are located it is possible that I missed the initial steps of axonal expansion in the previous phases.

At the presented example the dendritic tree at 30hAPF (Figure 3.4 A) the number of cell bodies in the central cluster was reduced. Second order branches appeared, however the overall dendritic tree was relatively sparse. The latter is due to the fact that this z stack contains fewer slices, in another two examples of brains at 30hAPF, the dendritic tree was denser. Over the next hours, (until 40hAPF) (Figure3.4 B, arrows) rapid branching occurred.

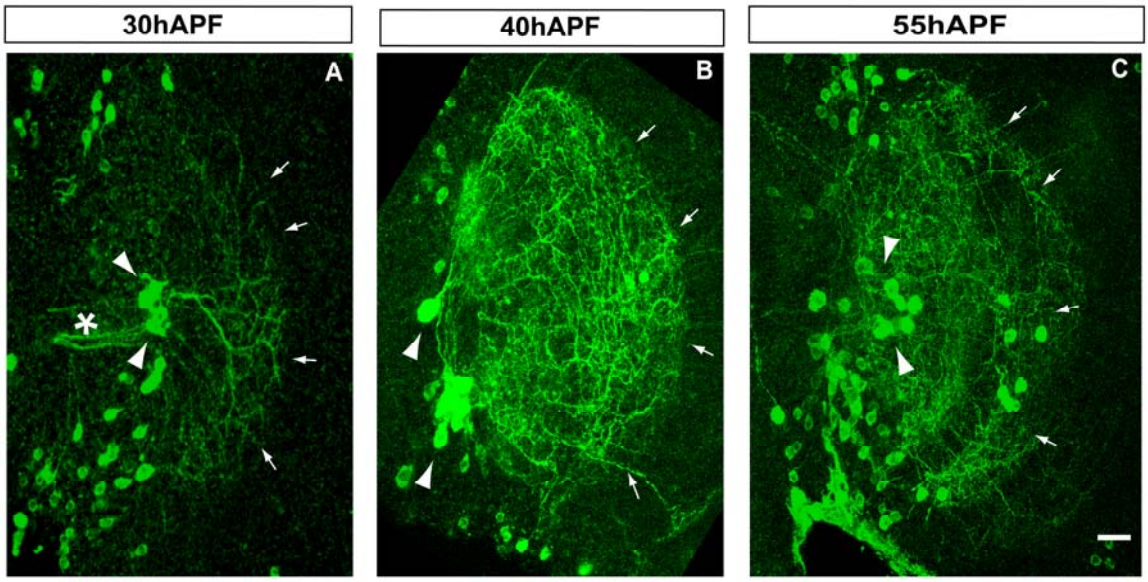

Figure 3.4: LPTCs during the Intermediate pupal stages. A: 30hAPF, B: 40hAPF, C: 55hAPF. Cell bodies indicated by arrowheads, position of LPTC dendrites by arrows, axonal extensions by white star. L, lateral; M, medial; D, dorsal; V, ventral. Scale bar= 20μm.

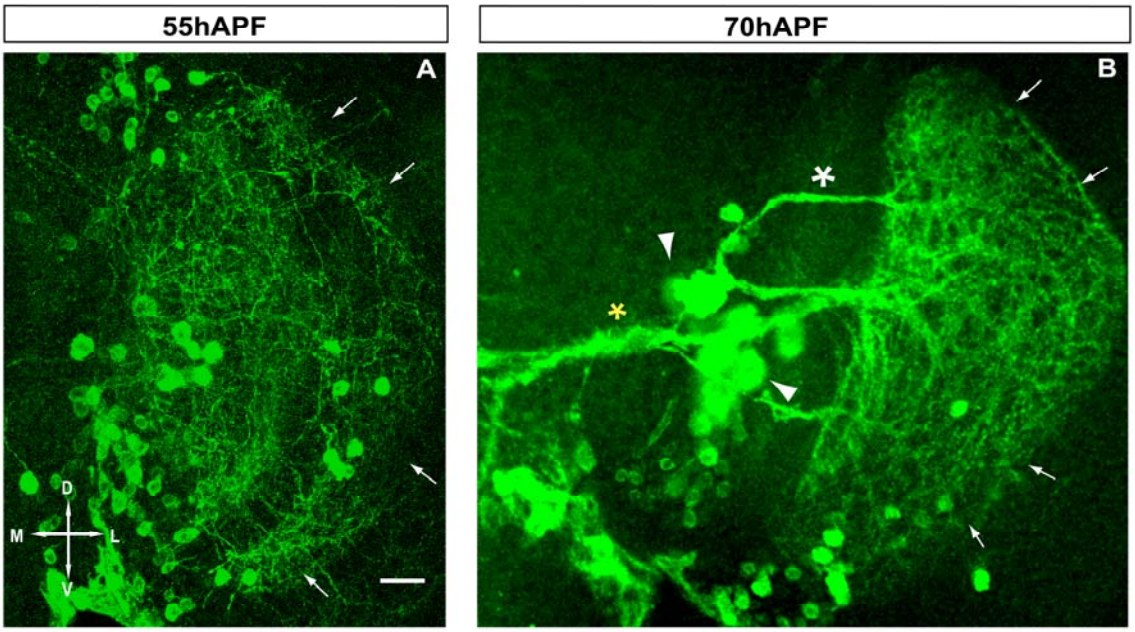

Figure 3.5: Comparison between 55hAPF and 70hAPF LPTC dendritic patterns. A: 55hAPF, in B: 70hAPF. Cell bodies indicated by arrowheads, position of LPTC dendrites by arrows, proximal segment of dendrites by white star, and axonal extension by yellow star. L, lateral; M, medial; D, dorsal; V, ventral. Scale bar= 20μm.

The dendritic pattern became significantly more complex with numerous tiny protrusions emanating from the cell bodies. Higher order branches could be identified but the diameter did not differ among the bulk of extensions. The branches seemed to be undirected and intermingled with each other. Thus they were still devoid of defined targets. The contribution of extensions from the columnar neurons to that network is rather likely, especially, since a 3D reconstruction of the z stack at that time point showed the existence of perpendicular connections between two layers of the branching clusters (data not shown).

Taken together, the picture from the differentiation time point of 55hAPF (Figure 3.4 C) remained similar with the branches fanning compactly over each other. The outgrowth of the branches resulted in more extensive coverage of the lobula plate. The formation of an outer ring of branching could be easily observed. It probably represents medullar cells that are upstream partners of LPTCs. In addition to the cells bodies localized in the central cluster of somata, further cell bodies were scattered all over the lobula plate.

Late Pupal Stages

The rapid outgrowth phase is followed by a stage of very dynamic reorganization accompanied by retraction of a notable number of branches (Figure 3.5A, arrows). Between 55hAPF (Figure 3.5, A) and 70hAPF (Figure 3.5, B) the tree sculpted and started to be reminiscent of the final LPTC dendritic pattern. The primary branches could be identified (Figure 3.5 B, white asterix) and their proximal parts were devoid of any extensions. Detailed measurements could reveal whether this was due to pruning of the higher order branchlets or the dendritic network was pushed laterally by the growing primary branches. At 70hAPF I could view for the first time some of the cell bodies located on a pedunculus- a short extension sticking out from the branch and marking the border between dendritic and axonal parts of the neuron. This type of spacing between the main neuron structure and the somata is characteristic for LPTCs in the adult animal (3D data not shown, and Figure 3.7 B. labeled HS cell body).

The bundles of axonal extensions heading towards the central brain were clearly visible. Also, the typical terminal forking of the axons could be observed.

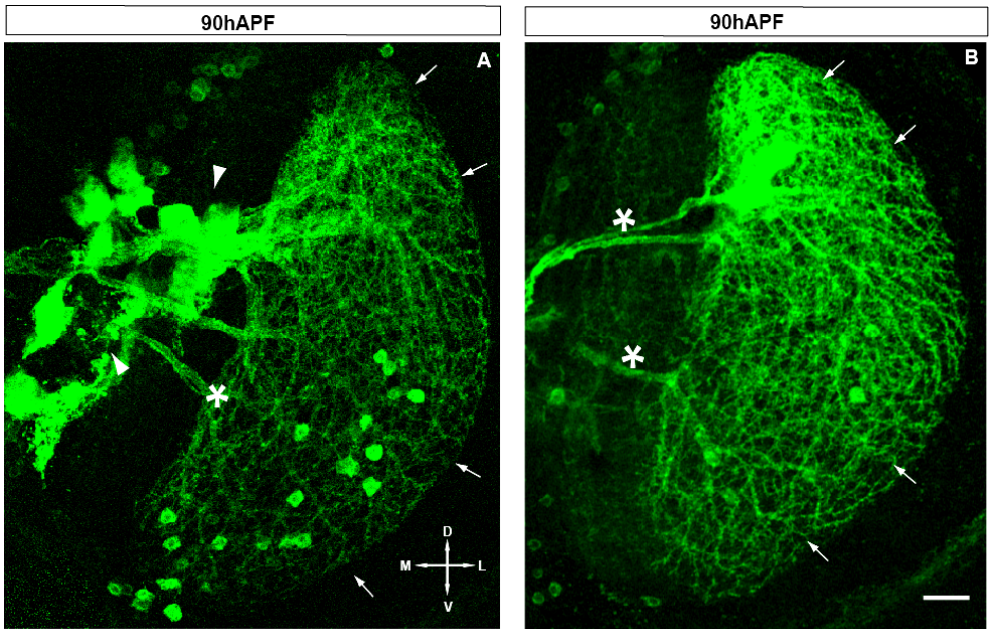

Figure 3.6: Stereotypy of the dendritic tree in the two different 90hAPF animals. A and B: View at 90hAPF animals. Cell bodies are indicated by arrowheads, position of LPTCs dendrites by arrows, and proximal segment of dendrites by white star. L, lateral; M, medial; D, dorsal; V, ventral. Scale bar= 20μm.

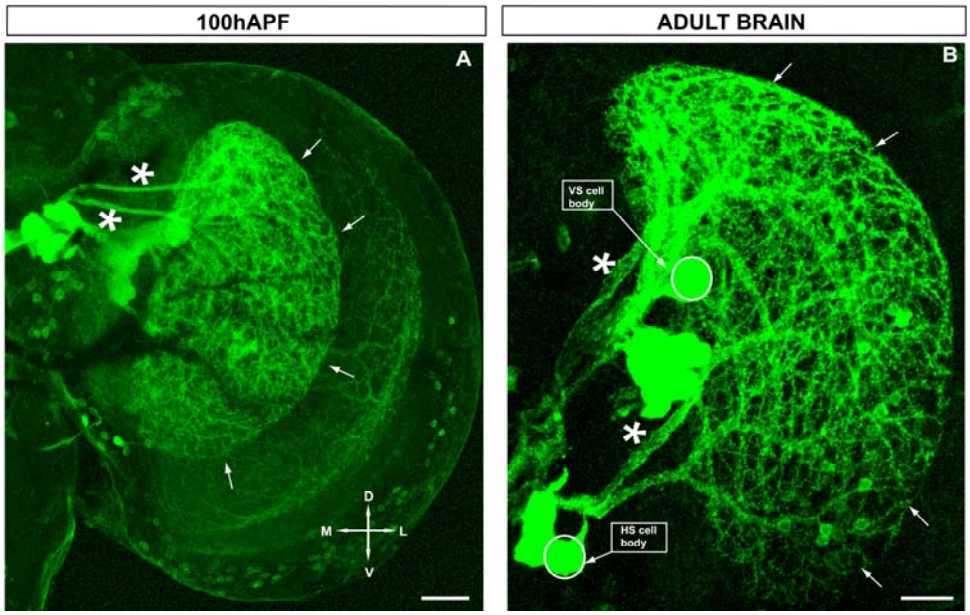

Figure 3.7: Final dendritic arborization pattern of LPTCs. A: View at 100hAPF. Scale bar= 50μm. B: View of an adult animal brain. Scale bars= 20μm. Cell bodies indicated by arrowheads, position of LPTCs dendrites by arrows, and proximal segment of dendrites by white star. L, lateral; M, medial; D, dorsal; V, ventral.

Refinement Phase

The growth of the brain and the maturation of the neuropiles result in a gradual increase of the area covered by the LPTC dendritic trees throughout the pupal stages. The architecture of the 90hAPF (Figure 3.6 A, B) dendritic tree differed from the one at 70hAPF (Figure 3.5 B) - the dendritic tree covered a bigger area and was more densely packed. Unidentified cell bodies remained speckled in the dendritic tree. Comparing to the growth phase and reorganization phases (30h-70hAPF), the processes of the final tuning of LPTC (70h-110hAPF) appear to be very accurate and balanced. Nevertheless, neither mild continuous outgrowth of new branches nor their retraction can be excluded at this point.

In order to show the level of stereotypy of the dendritic tree at a particular developmental phase I presented the outline of LPTCs dissected from two different animals at 90hAPF (Fig.3.6 A, B). Since the cell bodies project from the dendritic plane on their pedunculus sometimes not all of them are included in the images.

Stability Phase

As already mentioned, the characteristics of the tree remained relatively stable from 90hAPF (Figure 3.6 A, B) until eclosion (Figure 3.7 B). To estimate the minor alterations that presumably occur in that time window, the imaging would need to be performed at more frequent time points. Ultimately, time lapse experiments would give relevant insights into these issues. For the experiments where I attempted to culture the developing *Drosophila* brains I adapted the protocol from Gibbs and Truman (1998). Based on the obtained preliminary results I can state that the *Drosophila* brains grow in the dish and the GFP signal can be obtained with high resolution (data not shown). However, the set up for the time lapse imaging requires further optimalization.

Figure 3.7 A shows the 100hAPF dendritic pattern in the context of the entire optic lobe. It helps to imagine the surface and volume occupied by the LPTCs. Finally, a view of the dendritic pattern from a young adult (110hAPF=10h after eclosion) with a complete,

highly condensed network of branches is presented in Figure 3.7 B. To accomplish the developmental line of *Drosophila* I examined also the dendritic pattern at several time points in the adult fly. The dendritic patterns from 4d/ 8d/ 2 week old animals did not show obvious alterations (data not shown).

Development of LPTCs- Summary

The above time point experiments demonstrated that the differentiation process of the LPTC dendrites can be divided into critical developmental phases that the neurons undergo before achieving their final form. Essentially, the initial directional growth is followed by reorganization and then the final refinement. The steps in the differentiation of LPTC dendrites are summarized in Figure 3.8.

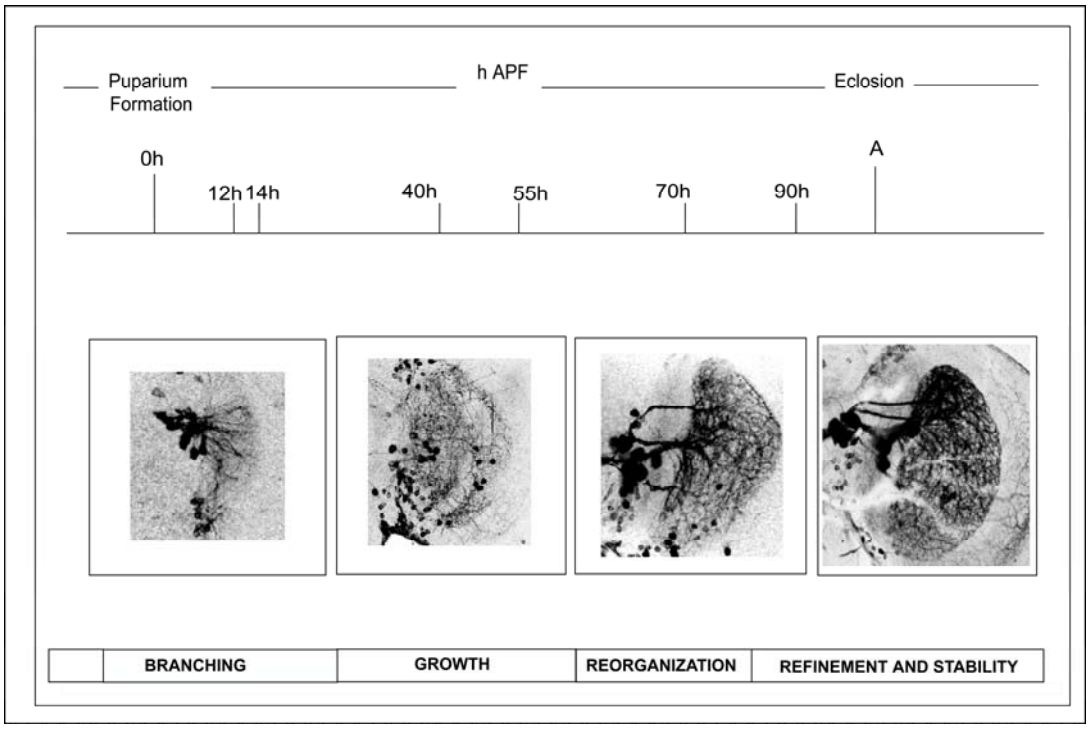

Figure 3.8: A gallery of events in the life line of LPTCs. The bars at the bottom of the gallery symbolize the major developmental steps. Images are in inverted grey scale to improve the contrast.

Subcellular Localization of Actin

For identification of the phases in LPTC dendritogenesis I used the membrane reporter mCD8-GFP. To learn how actin and tubulin participate in the development and which effects they have on the rearrangements crucial for the appropriate dendritic growth (eg. formation of new branches, retraction, pruning, refinements) I first visualized their subcellular localization in the adult neurons.

I generated fly lines containing transgenic elements (see Methods), that enabled me to overexpress actin-GFP and tubulin-GFP in the LPTCs. A strikingly distinct localization of these two molecules could be observed (Figure 3.9 B, C). Tubulin-GFP was present in the primary dendritic branches and the thick bundles of axonal extensions that hypothetically are stable structures in comparison to motile dendritic branch tips. However, as revealed by higher resolution images higher order branches also contain tubulin-GPF (Figure 3.12 M, N, O). Actin-GFP expression is strongly enriched in the spine- like protrusions that decorate dendritic branchlets and the dendritic shaft but it was also detected in dendritic trunks of all orders (Figure 3.12 H, I, J).

These observations were in agreement with findings from vertebrate primary cultures (Matus, 2000).

Having obtained data for adult neurons, I wanted to define how these cytoskeletal molecules localize during dendrite differentiation. The question was whether they were located in the same or different subcellular compartments as at adult stages. I chose to investigate actin-GFP and could observe that the LPTC dendritic tree expressing actin-GFP at 70hAPF differs from the adult one (Figure 3.10). This indicates that reorganization of actin occurs even within the last 30h of the pupal development. This observation was surprising since at 70hAPF the overall scaffolding of the dendritic tree is established (Figure 10. C) and is only followed by a phase of minor refinements. Actin seemed to be localized in the primary branches whereas it was not yet present at its terminal destination at the branch tips. As a consequence, in the DB331UAS-actin-GFP flies (Figure 3.10 A), the presence of actin-enriched dendritic termini was less frequently observed at 70hAPF than in adult flies (Figure 3.10 B).

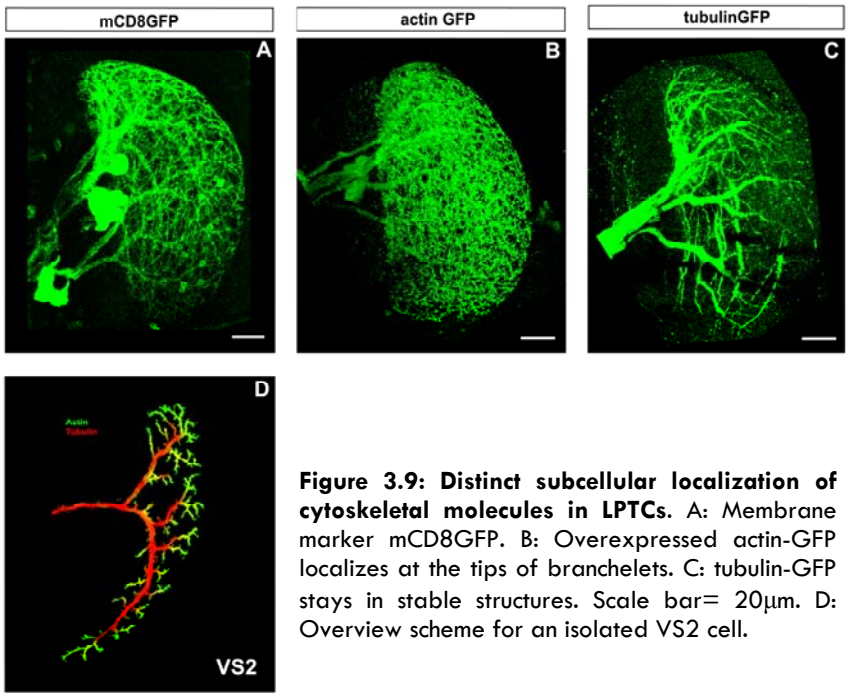

Figure 3.9: Distinct subcellular localization of cytoskeletal molecules in LPTCs. A: Membrane marker mCD8GFP. B: Overexpressed actin-GFP localizes at the tips of branchelets. C: tubulin-GFP stays in stable structures. Scale bar= 20μm. D: Overview scheme for an isolated VS2 cell.

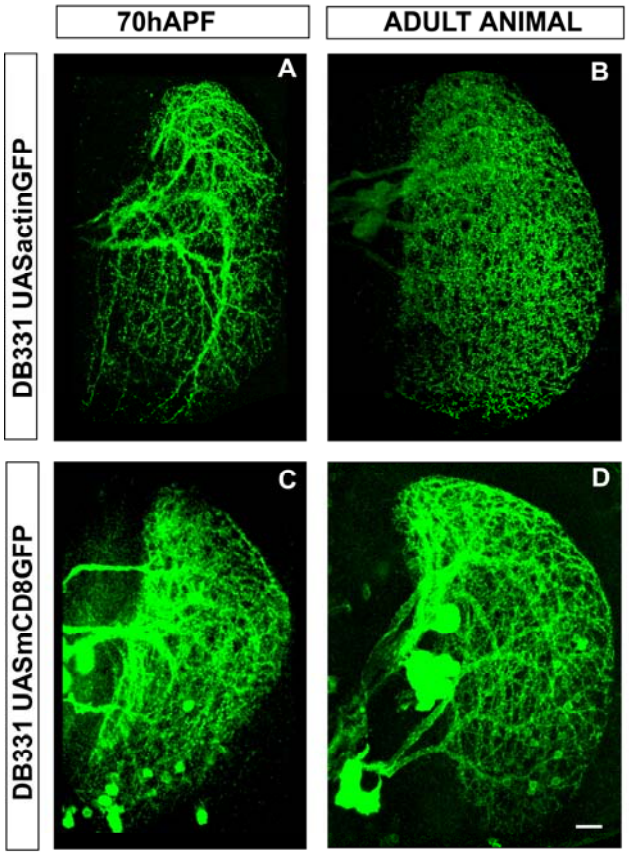

Figure3.10: Compartmentalization of actin in LPTCs at 70hAPF. A: Presence of actin-GFP mainly in the primary branches at 70hAPF, B: Final distribution of actin-GFP, C: Transmembrane marker labels dendritic tree at 70hAPF which reminiscent of the final form, D: LPTCs architecture in the adult animal. Scale bar= 20μm.

However, the overall area covered by the 70hAPF DB331 UAS-actin-GFP (Figure 3.10 A) dendritic tree is comparable with the area covered by 70hAPF DB331 UAS-mCD8GFP (Figure 3.10 C). Further analysis would be required to identify the time point when the spine-like structures appear on the branches.

The preliminary analysis of actin-GFP expression pattern in animals up to 70hAPF showed that actin delocalizes throughout LPTC dendritogenesis. Based on these findings one could speculate that actin actively participates in dynamic developmental processes; however the number of examined animals was too low to draw specific conclusions.

The intriguing observations described above attracted my interest in actin as a molecular player in the arrangement of the fine architecture of LPTC dendrites.

Conclusions

Time point experiments allowed me to obtain descriptive data of the differentiation of LPTC dendrites. This led to defining and characterization of the main phases of LPTC outgrowth. These results will serve as reference data for time lapse studies.

Subcellular distribution of cytoskeletal molecules in the mature *Drosophila* LPTC neurons appeared similar to the distribution in the vertebrate systems. Moreover, it determined the focus of my further studies.

The outcome of this part of my studies clearly opened a broad field of issues to be deciphered in more detail. To highlight a few of them including real time imaging of LPTC differentiation in cultured brains, developmental studies on the impact of actin-GFP and tubulin-GFP as well as their interactors, or on the known mutant fly lines (eg.Cdc42). It was clear that each of these projects would have required extensive analysis. I restricted my studies to only one among them.

LPTC Dendrites bear Spine-Like Protrusions

While analyzing the subcellular localization of actin-GFP, I discovered its accumulation in the fine dendritic termini. These observed terminal dendritic profiles looked like potential

Drosophila counterparts of vertebrate spines. The novel observation of actin- enrichment led to a set of experiments to check if those protrusions also bear other spine characteristics apart from the morphological resemblance and are functionally homologous to vertebrate spines.

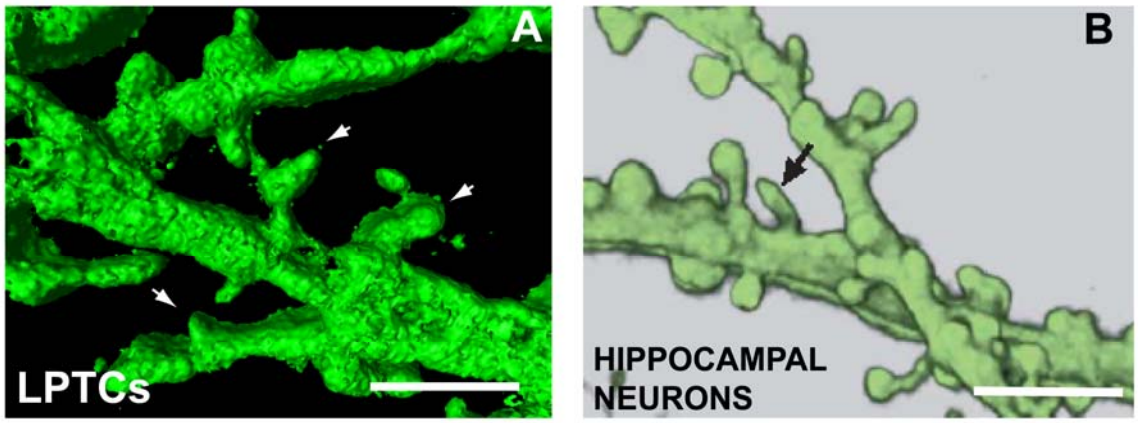

Figure 3.11: Morphological similarities between dendrites of LPTCs and hippocampal neurons. A: Close-up of a LPTC dendrite. Volume rendered (AMIRA) confocal image. Single spines marked with arrows. B: Close-up of dendrites from a CA1 hippocampal slice. Volume rendered (IMARIS) two- photon image. Scale bars=2µm. Modified from Nagerl *et al* (2004).

High resolution images of the dendritic branchlets revealed that the spine- like protrusions on LPTCs (Figure 3.11 A) indeed strongly resembled spines present on CA1 hippocampal neurons (Figure 3.11 B). Encouraged by this observations I aimed to further investigate this matter and check if LPTC spine- like structures form synapses and are genetically modifiable. So far there had been no thorough analysis of spines in *Drosophila*, only indications of the presence of spine-like processes along LPTC dendrites (Scott et al., 2003a).

LPTC Spine- Like Structures are enriched in Actin

To get a more accurate insight into the morphology of LPTC spine- like protrusions, as well as their cytoskeletal organization I generated animals expressing a membrane-tagged version of GFP (mCD8-GFP), actin- GFP or tubulin- GFP (Figure 3. 12 A-E) and

cytoplasmic mRFP (monomeric Red Fluorescent Protein) (Figure 3.12 A, C,) under the control of DB331.

On the dendrites of both VS (Fig. 3.12 A, D, G) and HS cells I could detect the presence of spine- like protrusions, similar to what has been reported by Scott et al. (2003). These spine- like structures were present on all dendrite orders, though only sporadically emerging from the main primary dendritic branches (5-10% of a total number) and in large numbers on fine branchlets (third order dendrites and higher; Figure 3.12 C, D; arrows). This observation fully agreed with the previous reports about the presence of spine- like profiles on the cobalt– filled and silver intensified visual interneurons in *Calliphora* (Hausen et al., 1980).

Spine- like structures on the VS1 neurons

To be able to exploit the LPTCs for genetic analysis of spine- like structures I needed a fast and robust assay system. I developed a simple protocol (see Methods) to extract quantifiable data from a single cell without the need of generating single- cell clones. All quantifications were carried out on VS1 (Figure 3.12, C, H, M) - the most distal neuron among LPTCs, since its dendrites are highly stereotyped and individually identifiable. A middle region of the ventral branch of VS1 was selected for quantification since it has very little overlap with neighboring LPTC dendrites. I could thus more easily and reliably obtain data from a higher number of neurons and animals than previously achieved, due to the low (0.5%) frequency of single cell clones (my data and (Scott et al., 2002)). Actin strongly accumulated in spine- like structures, showing a clear enrichment in comparison to the dendritic branches (Figure 3.12 H, I, arrows). A similar localization was observed upon overexpression of *GMA* (Figure 3.13), a GFP- tagged version of the actin- binding fragment of moesin (Edwards et al., 1997), which is a faithful reporter of actin organization (Dutta et al., 2002).

In contrast, tubulin-GFP was mainly localized in the primary and secondary branches, and proportionally less abundant in high order branches (Figure 3.12 1K- N). Tubulin-GFP appeared completely excluded from the spine- like structures (Figure 3.12 M- O).

Taken together, these data demonstrate that LPTCs possess spine- like structures that are enriched in actin and devoid of tubulin.

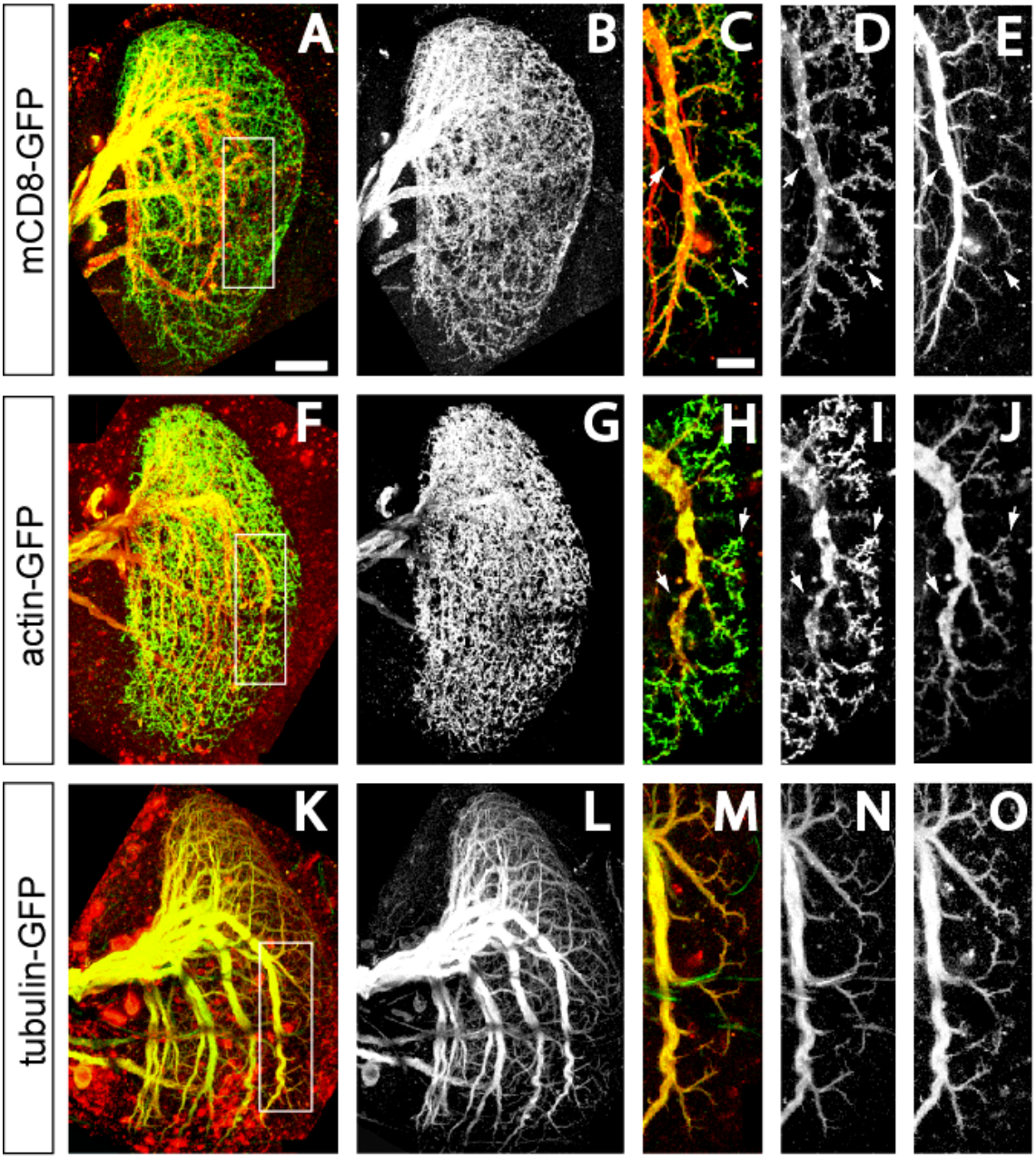

Figure 3.12: *Drosophila* Lobula Plate Tangential Cells have spine-like structures that are enriched in actin. LPTCs expressing membrane tagged GFP (*mCD8-GFP*, A- D), *actin-GFP* (F- I) or *tubulin-GFP* (K- N) together with soluble *mRFP* to visualize the morphology of the neurons (A, C, E, F, H, J, K, M, O). The color panels show a merge of the mRFP signal (red) and the respective GFP-tagged construct (green). Note the high enrichment of actin in spine- like structures (white arrows H- J), and the absence of tubulin in these structures (M- O). Scale bars: A, B, F, G, K, L =20 µm, C- E, H- J, M- O = 10 µm .

Density and Length of LPTC Spine- like Structures

I quantified the length and the density of the spine- like structures in z projections of confocal stacks and obtained an average length of the processes of 1.1 μm (Figure 3.13 G). I counted spine- like structure numbers on volume-rendered 3D reconstructions of confocal optical section series of dendritic branches (see Methods), which allowed observing even short processes with high resolution. I calculated an average density of 1.2 sls/μm (Figure 3.13 H, and see below), which is about twice as high as previously reported for single cell clones. This is most likely due to the use of 3D reconstructions, since I obtained similar values as previously reported (Scott et al., 2002) when counting from projection images (0.6 spines/μm in DB331UAS mCD8-GFP flies).

Actin Overexpression does not affect Spine- like Structures

I wanted to verify whether overexpression of actin-GFP or GMA affects LPTC spine- like structure morphology or density, as has been reported recently in vertebrate neurons (Johnson and Ouimet, 2006).

I compared spine- like structures of LPTCs overexpressing actin-GFP (Figure 3.13, E) or *GMA* (Figure 3.13, F) with spine- like structures of LPTCs overexpressing mCD8-GFP (Figure 3.13 D). In each case I co- expressed a membrane- tagged RFP, myrRFP (Figure 3.13A- C) and quantified both length (Figure 3.13, G) and density of spine- like structures (Figure 3.13, H) in the red channel (myrRFP). Neither the density nor the length of dendritic spine- like structures was modified upon overexpression of actin-GFP or GMA in these neurons. Hence, actin-GFP and GMA were shown to be reliable reporters for actin localization that will be used in further experiments.

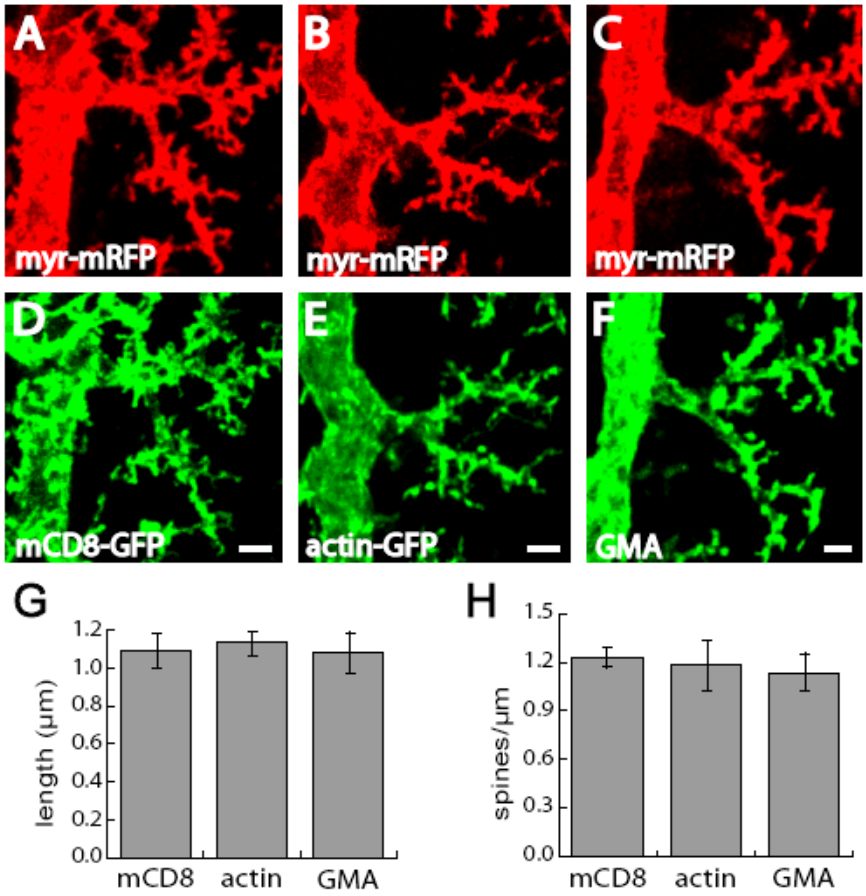

Figure 3.13: Overexpression of actin- GFP or GMA does not alter density of LPTC spine- like structures. Similar dendritic fragments of VS1 are shown for three animals (A&D, B&E, C&F) heterozygous for *UAS-mRFP* (A-C) and either *UAS-mCD8GFP* (D), *UAS-actin-GFP* (E) or *UAS-GMA* (F). No differences in length(G) or density of spine- like structures could be detected between these three genotypes. Quantification of length and density of spine- like structures (H). >500 sls from 5 animals were analyzed per data point. Scale bars= 2 µm.

Morphological Categories of LPTC Spine-Like Structures

Vertebrate spines are transient structures, capable of morphological modifications even in the adult brain (Lendvai et al., 2000; Majewska and Sur, 2003; Holtmaat et al., 2005). Any attempt to classify spine morphology underestimates their diversity.

However, in order to understand possible correlations between their shapes and function or maturation level, they have previously been divided into morphological categories. The original classification was based on ultrastructural data (Fiala and Harris, 2001;

Matsuzaki et al., 2004; Nagerl et al., 2004; Noguchi et al., 2005). These studies indicate that indeed the shape of a spine may reflect its function.

The morphology of the LPTC spine- like structures is also varied (Figure 3.14). In projections of optical section stacks we found that the vast majority of LPTC spine- like structures fall in four categories. These are: *stubby* protrusions (44.5%), when the diameter of the neck was similar to or greater than the total length of the protrusion (Figure 3. 14 A, B); *thin* spines- like structures (24.3 %), when the length was greater than the neck diameter, and the diameters of the head and the neck were similar (Figure 3.14 A, C); *branched* spine- like structures (16.7 %), spine- like protrusions with up to three heads from a single neck (Figure 3.14 A, D); and *mushroom* shaped spine- like protrusions (14.6 %), when the diameter of the head was greater than the diameter of the neck (Figure 3.14 A, E).

Approximately 10% of all protrusions were longer than 3 µm and hence were classified as filopodia.

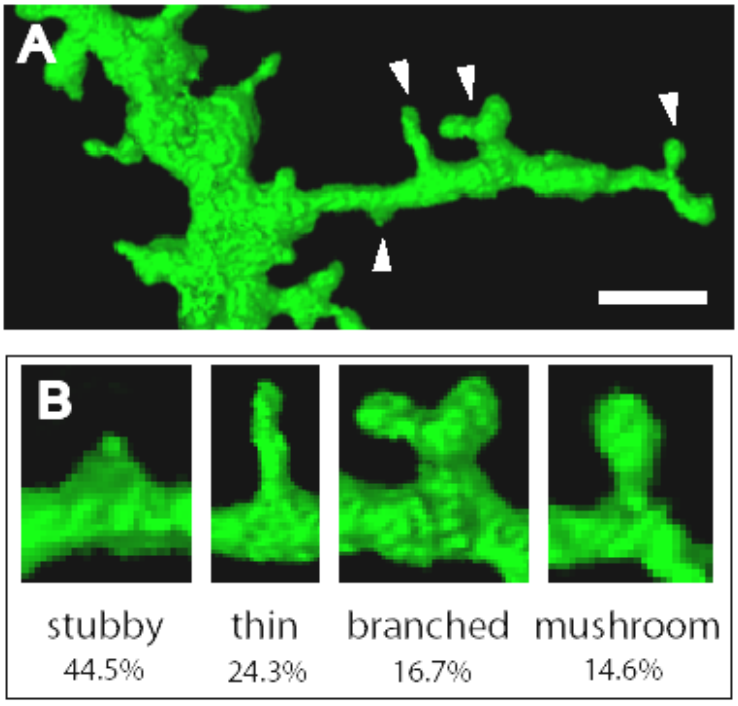

Figure 3.14: Classification of dendritic spine- like structures on LPTC neurons.
(A) 3D reconstruction of a representative small dendritic branchlet of the medial region of VS1 showing the different types of dendritic spine- like structures. Selected spine- like structures are marked with arrowheads and magnified in the subsequent panels indicating four categories: stubby, thin, branched, and mushroom shaped, according to their length and the ratio of their maximum head and minimum neck diameter. (B). The numbers in B represent the percentage of each category. >600 spine-like structures from 5 animals were analyzed. Scale bar = 2 µm.

Thus, LPTC spine- like structures fall into the previously described spine categories. Similarly to what has been reported in other systems (Fiala and Harris, 2001), the

percentage of the potentially mature, mushroom- shaped spine- like structures is approximately 15% of the total number of spine- like structures.

Synaptic Input onto LPTC Spine- Like Structures

Mature dendritic spines are sites of synaptic input (Hering and Sheng, 2001). To address whether the processes that I have observed are functional homologues of vertebrate spines, we tested whether they are sites of synaptic input, as well. I performed immunofluorescence staining on *in toto* brains of adult flies expressing actin-GFP specifically in LPTCs, using antibodies against the pre- synaptic markers synaptotagmin (Littleton et al., 1993; Wu and Bellen, 1997) and syntaxin (Schulze and Bellen, 1996) (data not shown) followed by confocal microscopy optical sectioning (Figure 3.15). With each of the antibodies I obtained a punctuate staining in the neuropils (Figure 3.15), which was absent in the negative controls (secondary antibody only, data not shown). Puncta representing pre- synaptic sites were present both along the length of primary and secondary branches (not shown) and juxtaposed to the spine- like structures (Figure 3.15 A). To estimate the subset of spine- like structures in contact with a pre- synaptic terminal, I counted the number of sites of close proximity between actin-GFP and the presynaptic marker staining in confocal projections (Figure 3.15 C). To test whether there is a real juxtaposition I analyzed the contacts in single optical planes (Figure 3.15 D-F) and finally generated 3D reconstructions of dendrite tree fragments and rotated them: only the contact sites that were maintained at all angles of rotation were counted.

Based on these data I could state that 52% of all spine- like structures are sites of synaptic input. The same percentage was obtained with staining against a second presynaptic marker, syntaxin (data not shown).

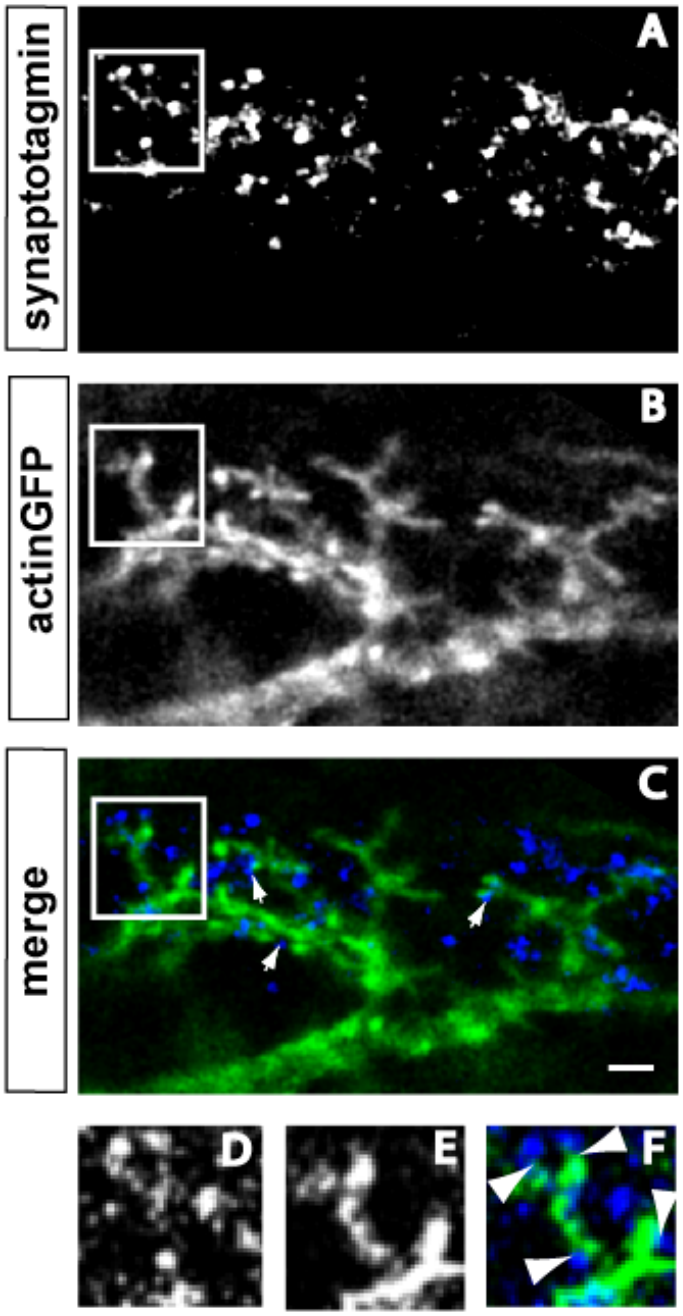

Figure 3.15: LPTC spine- like structures are sites of synaptic input. Projection of a confocal stack through a representative fragment of VS1, stained with anti-synaptotagmin antibody (A) and expressing actin-GFP (B). Sites of juxtaposition between pre-synaptic terminals and actin- enriched spine- like structures were detected in individual confocal planes and are indicated by arrows in the merge (C). (D-F) Single optical sections through the areas boxed in (A-C), showing the juxtaposition of spine- like structures and pre- synaptic dots (arrowheads). (D) anti-synaptotagmin antibody staining, (E) actin-GFP and (F) merge. Scale bar = 2 µm.

Ultrastructure of LPTC Spine- Like Structures

I further tested whether LPTC spine- like structures are sites of synaptic input by analyzing their ultrastructure in immuno- electron microscopy experiments that allowed us to unambiguously identify the LPTCs in brain sections (Figure 3.16). I identified LPTC spine-like structures in serial brain sections: only the processes of up to 3μm in length that were included within 6 sections (50 nm/ section) were considered as spine- like structures. Initially, in this experiment I used DB331 UAS-HRP-CD2 flies (data not shown). However, due to the strong antibody signal against HRP the identification of the synaptic elements was significantly hampered. I overcame this issue by using the UAS-mCD8GFP reporter and performed staining against CD8. In order to narrow down the number of cells labeled I used the 3A driver in this experiment that marks VS cells (see Methods). Finally, the 3A-UAS-mCD8 flies allowed for specific labeling and detection of LPTCs neurons (VS cells mainly).

I found that indeed in all cases (n=5) where I identified spine- like structures based on the serial section EM, T-bars (Prokop and Meinertzhagen, 2006), indicators of active synaptic zones (Zhai and Bellen, 2004; Wagh et al., 2006), were present immediately next to the spine- like structures (Figure 3.16 C, D, arrowheads). The T- bars were localized on the spine- like structure heads in 4 examples (Figure 3.16 D, arrowheads and star), but they were also found on the neck and on shaft of spine- like structures. In two instances I could clearly observe the presence of synaptic vesicles at a T-bar pre-synaptic to a spine –like structure (Figure 3.16 D, Inset).

These findings indicate that spine- like structures present on LPTCs can form active synapses. In several examples, the base of the spine –like structure was marked by the presence of a mitochondrion (Fig. 3.16 E, m), which is essential for spine formation and function in hippocampal neurons (Li et al., 2004). I have not detect the presence of a spine apparatus (Sala, 2002) within LPTCs.

In summary, the spine- like structures observed on LPTCs are morphologically strikingly similar to vertebrate spines; they are enriched in actin, devoid of tubulin and are sites of synaptic input.

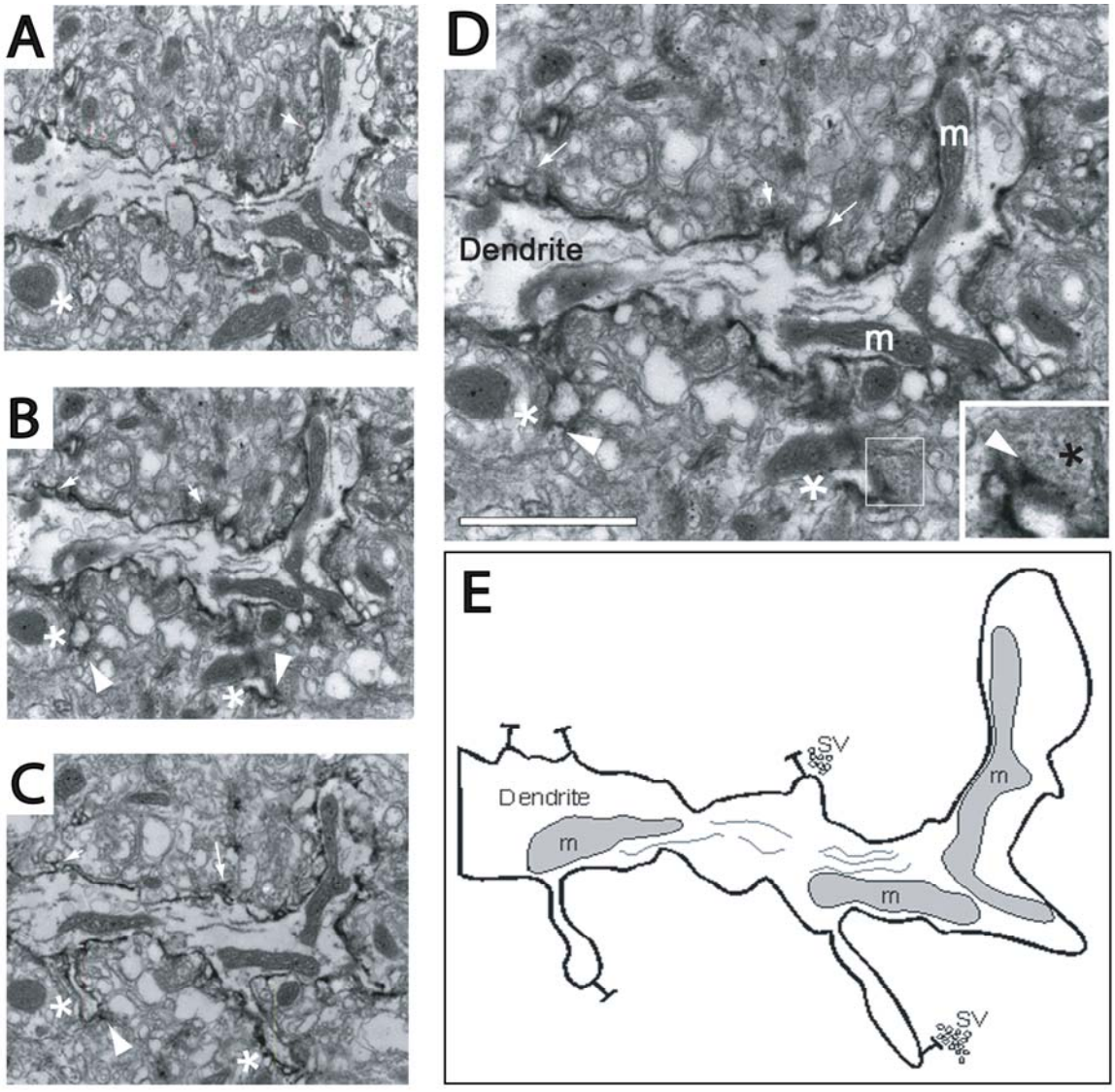

Figure 3.16: Ultrastructure of spine- like structures in specifically labeled LPTC dendrites. (A - C) serial sections through an LPTC dendrite expressing mCD8-GFP and labeled with anti- mCD8 antibody (dark precipitate along the membrane). The white asterisk marks one spine- like structure through all the sections. (D) Higher magnification of the section shown in B. Note the T bars along the dendritic shaft (white arrows) and on the spine- like structures (white arrowheads). Inset: high magnification of a T bar on a spine- like structure with synaptic vesicles around it (black star). (E) Scheme representing a reconstruction of the group of six sections. Mitochondria (m); T bars (T) surrounded by synaptic vesicles (sv). Scale bar = 2µm

Genetic Modifications of LPTC Spine- like Structures

To support my assertion that spine- like structures on *Drosophila* LPTCs closely resemble vertebrate spines I tested whether genes known to affect spine morphology and density in vertebrates would also affect these processes in flies. I looked for candidate genes that could alter spine parameters.

dFmr1 Does Not Affect Spine Morphology

First, I wanted to check if d*fmr1*, the *Drosophila* homolog of fmr1 (fragile X mental retardation 1) (Wan et al., 2000), a gene in which spine morphology in mice and patients is specifically altered, also affects LPTCs spine- like structures.

The human disease is caused by the silencing of the *fmr1* gene, which encodes the RNA binding translational regulator FMRP (Jin and Warren, 2000). In FraX patients and *fmr1* knockout mice, loss of FMRP results in an increase in density and malformation of dendritic spines. The studies done in both peripheral (Zhang et al., 2001) and central (Pan et al., 2004) nervous system of *Drosophila* showed that dFMRP acts as a negative regulator of neuronal complexity in axons and dendrites.

I analyzed morphology of spine- like structures upon overexpresion of a full UAS-dfmr1 construct (Wan et al., 2000) under the control of the DB331 Gal4- driver. I also tested the morphology of spine- like structures in flies homozygous for a deletion mutant allele of this gene- $dfmr1^{\Delta 50}$ (Zhang et al., 2001). Surprisingly, I could observe only minor and inconsistent alterations in spine- like structures density. Namely, I detected a 10% increase in density of spine- like structures due to gain of function (Fig. 3.19 C) and a 10% decrease in spine- like structures density in the mutant flies (Fig. 3.19 B). These alterations were very mild and contrary to the observations from the vertebrate system (Comery et al., 1997).

Nevertheless, I decided to reconfirm my data doing MARCM (see Methods) experiments with the $dfmr1^{\Delta 50}$ mutant allele. This null mutant allele was demonstrated (Pan et al., 2004) to cause more severe phenotypes when analyzed on the level of single cell clones

in the mushroom body. With this experiment I could visualize single homozygous mutant GFP labeled neurons in genotypically heterozygous wild type brains.

First, the single cell clones for the control neurons (DB331 UAS-mCD8) were obtained. The frequency of single cell clones (0.5%) (Figure 3.17 A, B) and double cell clones (0.4%) (data not shown) was very low. The heat shock experiment did not reveal the birth order of LPTCs.

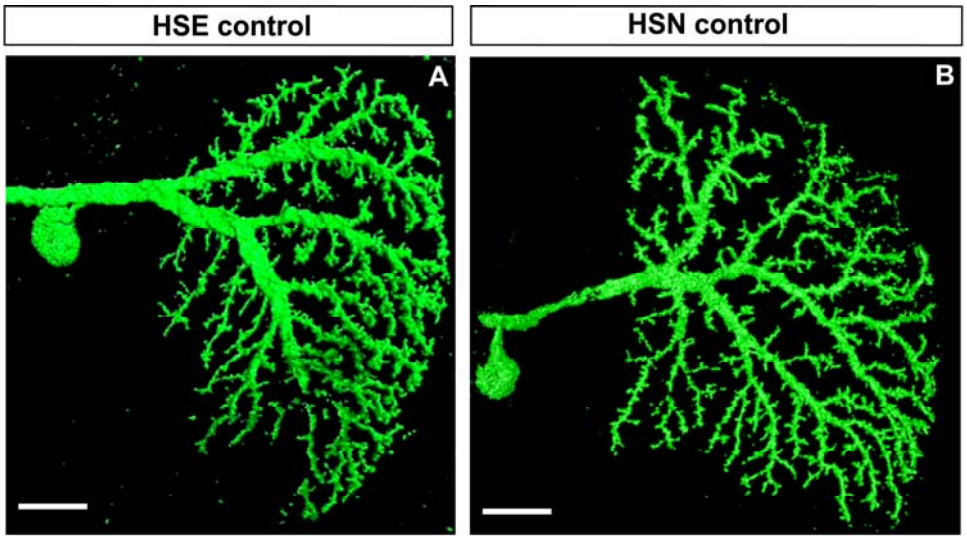

Figure 3. 17: Clonal analysis of spine- like structures morphology. A: HSE control neuron. B: HSN control neuron. Scale bar= 20μm.

As a result, I modified my approach so that the final quantitative analysis was done on the middle region of ventral branch of VS1 neuron from the multiple clones (frequency= 4%) for both wild type (Figure 3.18 A) and mutant conditions (Figure 3.18 B).

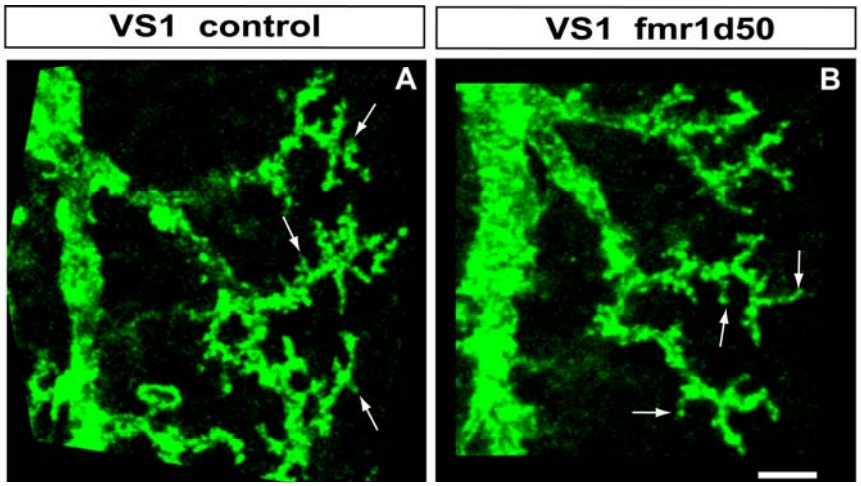

Figure 3. 18: Analysis of spine- like structures morphology on the VS1 neuron in the multiple cell clones. A: control VS1 neuron. B: fmr1d50 neuron. Scale bar= 2μm.

The architecture of the dendritic trees was not affected in either of the genotypes. I observed no significant difference between *dfmr1*$^{\Delta 50}$ null mutant allele neurons (0.99 spines/μm; n=6; total number of spines= 566; p=0.9076 by t-test) in comparison to control neurons (1.01 sls/μm; n=6; total number of sls= 507). The results from the MARCM analysis of the dendritic tree did not differ from the outcome of previously obtained quantifications done on the entire dendritic tree. In short, no significant change in spine density was detected.

It remains unclear if other spine parameters such as length and/ or distribution into categories were altered, as some of the images especially of dfmr1 overexpression might suggest. To answer this question further detailed examination of the images would need to be carried out.

dRac1 Modulates LPTC Spine Morphology

As a second candidate with the potential to modify LPTC spine- like structures I chose *dRac1*. The effects of Rac1 on spines in vertebrates are particularly well characterized (Govek et al., 2005; Tada and Sheng, 2006). I overexpressed full length *dRac1* (FL; rac1.L (Luo et al., 1996; Nakayama et al., 2000; Tashiro and Yuste, 2004; Wiens et al., 2005)) as well as dominant negative (DN; rac1.N17 (Luo et al., 1994)) and constitutively active (CA; rac1.V12 (Luo et al., 1994)) versions of *dRac1* specifically in the LPTCs under the control of DB331 Gal4-driver, and coexpressed *GMA* to visualize the dendritic trees. Since overexpression of CA *dRac1* led to lethality at pupal stages we could only analyze the effects of FL and DN *dRac1* (Figure 3.20). I found the overall dendritic architecture in both genotypes to be similar to the wild type condition: neither position nor branching patterns of primary and secondary order dendrites were affected. This is not surprising since alteration of Rac activity does not affect in vertebrates either dendrite structure of pyramidal neurons or of cerebellar Purkinje neurons (Luo et al., 1996; Nakayama et al., 2000). I did, however, notice a difference in spine- like structure morphology and an increase in density of spine- like structures. To quantify these observations I performed analysis of spine- like structures density as described above. As a control I used flies expressing *myr-mRFP* instead of either FL or DN *dRac1*, together with *GMA*.

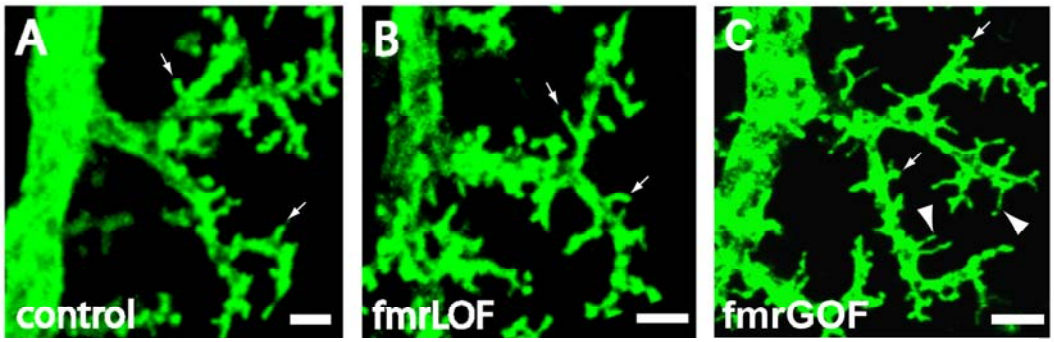

Figure 3. 19: Morphology of LPTC spine- like structures is not altered upon *dfmr1* GOF and LOF. A: Pattern of a control DB331-UAS-GMA, B: Pattern of mutant neurons (*dfmr1* LOF), C: Overexpression of w1118; P{ UAS-Frr1.Z}3. Note presence of diverse classes of spine- like structures in all genotypes (arrows). Larger number of filopodia- like protrusions in fmr1 GOF (arrowheads). LOF= Loss of function, GOF= Gain of function. Scale bar= 2µ.

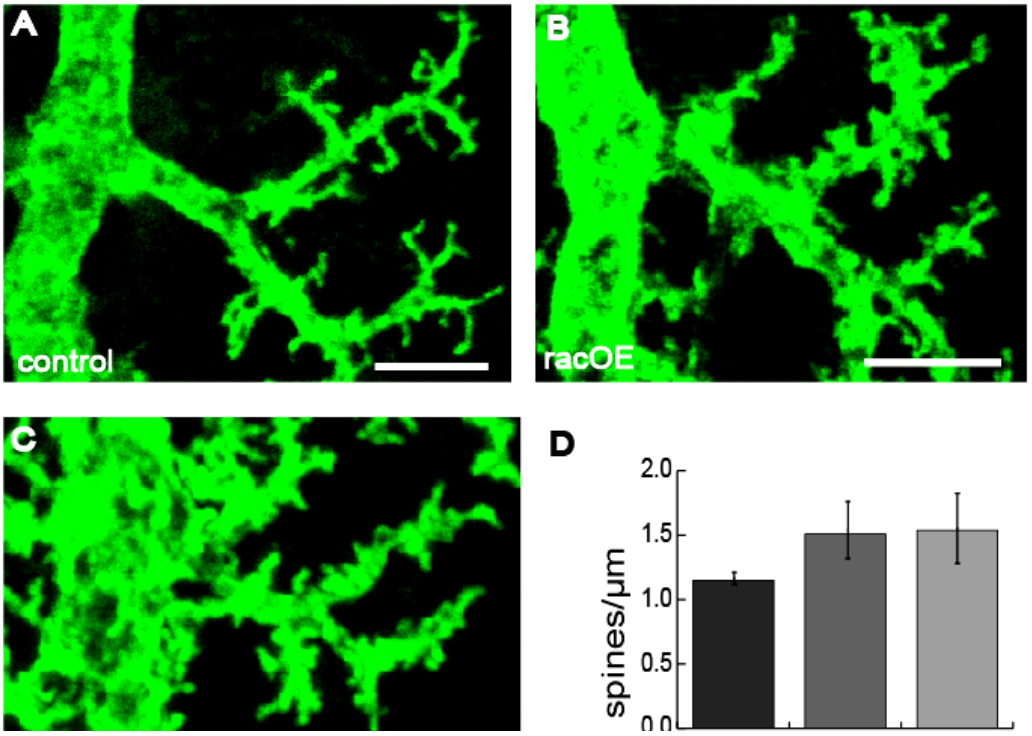

Figure 3.20: Density of spine- like structure is modulated by Rac1. (A-C) Similar dendritic fragments of VS1 are shown for three animals that were heterozygous for UAS-GMA (used for imaging) and either UAS-myr-mRFP (control, A), UAS-rac1.L (B) or UAS-rac1.N17 (C), respectively. (D) Quantification of spine- like structures density. >500 spine- like structures from 5 animals were analyzed per data point. T-test $p < 0.007$ in both cases. The presence of a large number of spine- like structures on the primary branch in (C) is due to overlap of another higher order branch. (OE) =overexpression, (DN) = dominant negative. Scale bars= 5µm.

I found the density of spine- like structures to be increased by ~30% upon overexpression of either FL *dRac1* (1,54 spines/ μm; n=5; p=0.0064 by t-test) or DN *dRac1* (1,51 spines/ μm; n=5 ; p=0,0051), in comparison to the control (1,15 spines/ μm; n=5). Moreover, spine- like structures were shorter and less well defined. Although opposite effects might be expected upon overexpression of FL and DN proteins, both genotypes appeared indistinguishable and yielded similar results in the quantification (see Discussion).

I conclude that Rac1 is involved in regulating morphology and density of spine- like structures in *Drosophila* LPTCs as previously reported for vertebrates.

Spine- Like Structures on Other *Drosophila* Neurons

Intrigued by the presence of spine- like structures on LPTCs, I then examined whether they are more widespread in the adult *Drosophila* CNS.

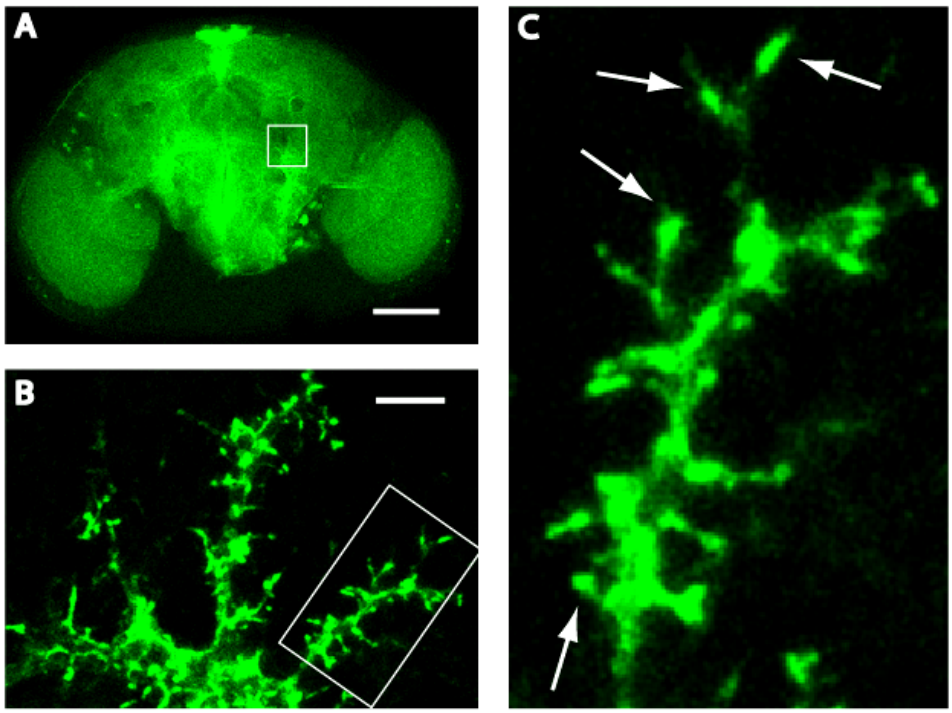

Figure 3.21: Presence of spine- like structures on giant fiber dendrites. (A-C) Actin-enriched spine- like structures along the dendrites of the giant fiber, visualized by overexpressing GMA in the neurons. (A) Localization of the dendrite tree in the adult brain; scale bar = 100 μm. (B) Detail of the dendrite tree; scale bar= 5 μm. (C) High magnification of the dendrite branch included in the box in B. Actin-enriched spine- like structures are indicated by arrows.

I started by looking at the localization of GMA in other described neuronal types. As suggested in previous work (Fayyazuddin et al., 2006), I could demonstrate the presence of spine- like structures along the dendrites of the giant fiber, a large nerve involved in escape behavior (Figure 3.21, A-C).
The dendrites of these neurons are covered with a high number of spine- like structures that are actin- enriched (Figure 3.21 C, arrows).

Motility of LPTC Spine- like Structures

Since motility is one of the prominent characteristics of vertebrate spines (Segal, 2005), I wanted to check if LPTC spine- like structures also have the ability to change their shape over time. Since motility is driven by rearrangements in the actin cytoskeleton, my preceding data suggested that LPTC spine- like structures might be motile.
I optimized the set up designed to visualize the potential dynamics of spine– like structures (see Methods). For these sets of experiments I used late pupal (P8-P10 stages= 75-100h APF) and adult dissected out DB331-UAS-GMA brains. In all movies I could perform imaging on a VS1 close up for a maximum of 3h (n=6 movies; ΔT of taking confocal z stacks within an imaging session= 5-20min; z stacks of 1-3 optical slices were taken; data not shown). Based on these preliminary data, I exclude that LPTC spine- like structures are morphing as reported for cultured hippocampal neurons (Fischer et al., 1998) within the time frame observed (<3h).

Due to technical difficulties (inability to immobilize floating brains in a dish), I was able to follow a single spine- like structure over time only in three cases. Extended imaging was excluded because of the same problem. Retraction or formation of spine- like structures *de novo* was not detected. It would call for further analysis to learn if such changes happen over much longer periods of time (10-20h) as reported in the vertebrate neurons. It remains unsolved if culturing of brains potentially affects motility of spine- like structures. It has been reported in *Drosophila* (Gibbs and Truman, 1998; Brown et al., 2006) that the

development of explanted nervous system slows in culture and 2 hours of time *in vitro* is roughly equivalent to 1 hour *in vivo*.

Conclusions

I showed that spine- like structures present on the dendrites of LPTCs strongly resemble vertebrate spines in several aspects. Their morphology and cytoskeletal organization faithfully mirrors that of hippocampal neurons. I also provided evidence that they are sites of synaptic contacts and that more than half of them (52%) have presynaptic partners. The ultrastructural analysis of LPTC spine- like structres showed that the heads of these protrusions are equipped with the T- bar structures on which synaptic vesicles dock. This finding indicates that LPTC spines form functional synapses. I also demonstrated that LPTC spine- like structures are at least susceptible to some of the genetic factors known to affect vertebrate spines. On the other hand, it remains unclear why no alteration has been detected in single cell MARCM clones in null alleles of the *dfmr1* gene that is a homolog of a vertebrate spine- specific gene. In order to verify whether the actin cytoskeleton induces motility in LPTCs spine- like structures, further analysis needs to be carried out.

4 Discussion

I used genetic methods combined with confocal microscopy to visualize the differentiation and cytoskeletal organization of the dendrites of Lobula Plate Tangential Cells in the intact *Drosophila* brain.

Using immunohistochemical techniques as well as electron microscopy I tested whether actin enriched spine- like protrusions observed on the surface of the LPTCs bear additional features of the vertebrate spines. Below I will discuss the meaning of the obtained results in the context of the existing data. I will point out the caveats and the perspectives.

The form of a neuron's dendritic arbor determines the set of axons with which it may form synaptic contacts, thus the establishement of connectivity within neuronal circuits. In order to build up a specific dendritic morphology the interactions between extrinsic and intrinsic factors must be precisely orchestrated (Jan and Jan, 2003).

Dendrites are active participants in synapse formation. Hence, investigating dendritogenesis should help to understand how the neuronal connections are formed and maintained as well as to find out the commonalities between dendrite development and synaptic plasticity (Jan and Jan, 2001).

Dendritic dynamics during the developmental periods of targeting and synaptogenesis has been addressed in several model systems. Development of cultured hippocampal neurons for instance has been thoroughly described (Bradke and Dotti, 2000). Time lapse *in vivo* studies on the tectal neurons of zebrafish (Niell et al., 2004; Mumm et al., 2006) and *Xenopus* (Cline, 2001) demonstrated that the dendritic developmental processes in intact nervous tissue are very dynamic and that the outgrowth and retraction events highly overlap. Few studies from vertebrate systems also confirm a high level of dendritic dynamics as shown in pyramidal neurons within developing hippocampal tissue slices (Dailey and Smith, 1996) and in isolated retina tissue (Wong et al., 2000). The dynamics

includes both active growth and turnover (resorption) of dendritic branches and spiny protrusions (Dailey and Smith, 1996).

Analyses of vertebrate and invertebrate nervous systems have shown that principal functional features of their neurons share a great deal of similarity. The essential developmental properties are likely to share a common evolutionary origin which makes them potentially translatable between insects and vertebrates (Laurent, 1999). Some of the issues raised during *Drosophila* neurogenesis mirror those that occur in the vertebrate system (reviewed in Grueber and Jan, 2004; Sanchez-Soriano et al., 2005). This facilitates advances in the field of dendrite development through comparative research in *Drosophila* (Jan and Jan, 2003). The gaps in our understanding of the molecular and mechanistic basis of the developmental processes of dendrites have already been partially filled by employing genetically amenable *Drosophila* PNS multiple dendrite dendritic arborization (md-da) neurons as a model system (Jan and Jan, 2001; Andersen et al., 2005).

The insights into cellular and molecular regulation of formation of CNS dendrites gained in *Drosophila* might also have relevance beyond species borders. Examples of such insights have been reported already for motoneuronal dendrites (Baines et al., 2002; Kim and Chiba, 2004), olfactory interneuron dendrites in the adult brain (Zhu and Luo, 2004) and mushroom body neurons (Reuter et al., 2003).

Genetic tools for studying *Drososphila* allow labeling desired subsets of neurons (Brand and Perrimon, 1993) or even single neurons (Lee and Luo, 1999) and offer the possibility to examin exclusively in these specific neurons for instance the involvement of cytoskeletal molecules and their regulators throughout differentiation or mutant analysis (Keller-Peck et al., 2001). *In vivo* imaging of intact *Drosophila* CNS as well as PNS nervous tissue has been applied to follow the developmental aspects of neurons such as hormonal control and pruning (Brown et al., 2006; Williams and Truman, 2005 respectively).

Why study Dendritogenesis in LPTCs?

While *Drosophila* PNS md- da neurons receive sensory input from the body walls that they contact, they do not form synapses and thus cannot provide insight into how the neuronal

circuits are formed. To analyze the development of CNS neurons that are an integral part of neuronal circuitries I chose a subset of Lobula Plate Tangential Cells present in the optic lobe of *Drosophila*. The dendritic architecture of VS and HS cells ought to determine their receptive fields, since the lobula plate is organized retinotopically (see Introduction) (Borst and Haag, 2002). It means that the elaborate dendrite patterns of LPTCs supposedly reflect their function. Moreover, the tissue specific Gal4 driver lines to visualize them were readily available. Another significant benefit from the application of LPTCs system is that these neurons can be observed in the intact brain *in toto*, which allows obtaining developmental data that reflect the actual *in vivo* condition.

The clearly postsynaptic character of dendritic parts of LPTCs has been demonstrated by the ultrastructural analysis. Axons have been demonstrated in the same study to have both pre- and postsynaptic character (Hausen et al., 1980).

The physiological properties of *Calliphora* LPTCs (see Introduction) may also be relevant in *Drosophila*. Studies on *Drosophila optomotor blind* mutant, where the number or morphology of LPTCs is impaired, revealed that these neurons are similarily to counterpart neurons in *Calliphora* involved in guiding the optomotor responses of the fly (Bausenwein et al., 1986). However, due to the differences in the dendritic field coverage and in the number of VS neurons, such a comparison should be made with caution (Rajashekhar and Shamprasad, 2004). Still, the knowledge about the function of LPTCs could in the future be linked with the morphological and developmental data.

Clonal analysis has shown that LPTCs arise in the late larval stages (Scott et al., 2002). The Gal4-driver DB331 used in the current experiments allows visualization of LPTCs in third instar larvae (Figure 3.2). The drawback of Gal4- DB331 usage, though, is that the specificity of its expression pattern is limited. As shown for developmental phases (Figure 3. 5 A) and the adult animal (Figure 3.7 A), the expression pattern includes columnar neurons of the medulla. Since the dendritogenesis of columnar neurons has not been described, interpretation of images from the developmental stages using Gal4- DB331 driver line cannot exclude the presence of columnar neurons.

The UAS mCD8GFP reporter employed in this study, labels the entire membrane of neurons, thus provides a reference marking for subcellular localization of cytoskeletal molecules.

The initial dendritic extensions of LPTCs are asymmetric and directed laterally as seen at 12hAPF (Figure 3.3 B). The directionality of LPTC dendrite growth could potentially be

induced by the chemotrophic cues such as Semaphorins, a family of phylogenetically conserved secreted abd transmembrane glycoproteins. Semaphorin 3A (Sema3A) has been demonstrated to pattern pyramidal neurons during development. Apical dendrites of pyramidal neurons were demonstrated to grow towards the source of Sema 3A, whereas their axons were repelled by the same ligand (Polleux et al., 2000). Hypothetically, chemotropic cues may induce a polarized cellular response also in LPTCs.
On the other hand, dendritogenesis of *Drosophila* sensory neurons was shown to be regulated by transcription factors that play a role in multiple genetic programs (Parrish et al., 2006). Alternatively, the differentiation of LPTC dendrites could be contact-mediated and involve cell adhesion molecules (Kimura et al., 2006). It is likely that both intrinsic and extrinsic factors contribute simultaneously to the LPTC dendrites morphogenesis.

LPTC dendritic extensions already at early developmental stages (Figure 3.4 A) form branches. How does the branching happen? It remains open whether the new branches are added interstitially *de novo* to the dendritic shaft or if they emerge after splitting of previously existing ones as demonstrated to be typical for the *Drosophila* class I and class IV of md-da sensory neurons respectively (Sugimura et al., 2003).

At the following developmental phase around 40h- 55h after puparium formation (APF) a dense pattern of extensively intermingling extensions was observed (Figure 3.4 B, C). The overall structure constitutes of many disorganized multidirected extensions as revealed by 3D animations (data not shown) of the early pupal stages (Figure 3.4 B, C). The gap separating layers of VS and HS cells in the adults does not appear yet. These lateral projections present at 40h- 55hAPF stage could either represent intra- connections among LPTCs or columnar neurons. The role of either type of lateral projections remains unclear.

Why do developing LPTC arbors (Figure 3. 4 B, C) first add extensive numbers of branches that then retract? One might think that the most efficient way to grow an arbor is to add branches and keep them. However, some data indicate, that the rapidly growing arbors decrease their dynamics when they stop growing (Cline, 2001). It is possible that branches 'test' the environment for synaptic partners. The release of the neurotransmitters has been shown to stimulate the dendritic exploration of retinal ganglion cells when

afferents approach, increasing the probability of close contact (Wong et al., 2000). It remains speculative whether a neurotransmitter release from the columnar medullar neurons upstream partners of LPTCs drives their growth.

Another mechanism contributing to the establishement of specific patterns of neuronal connections is targeting of neuronal extensions to particular synaptic laminae. Dendritic lamination has been thought to occur largely by pruning of inappropriately placed arbors. In contrast, recent *in vivo* time lapse study of the retinal ganglion cell dendrites in zebrafish have shown growth patterns implicating dendritic targeting as a mechanism for contacting straight the appropriate synaptic partners (Mumm et al., 2006).
Nevertheless, the comparison of dendritic patterns at 40hAPF (Figure 3.5 A) and 70hAPF (Figure 3.4 B) would rather suggest that in case of LPTCs development, pruning of subsets of branches is involved in the establishment of the correct connections and formation of primary branches. Alternatively, the outgrowth of proximal segment of primary branches around 60-70hAPF could move the entire dendritic tree laterally.

At 70hAPF (Figure 3.5 B) the neurite; a stalk- like connection that isolates cell body from the main neuronal axis is seen for the first time (Figure 3.5 B). Such a physical isolation of somata, resulting in ectosomatic polarity of a neuron could be a common property of *Drosophila* neurons. *Drosophila* ventral nerve cord motorneurons have been demonstrated to be unipolar with only one primary neurite emanating from the cell body, in contrast to vertebrate heteromultipolar motorneurons where postsynaptic dendrite and presynaptic axon both emanate from the soma (Sanchez-Soriano et al., 2005). Nonetheless, the mechanism of formation of pedunculus and its role for LPTCs growth and function remains to be resolved.

My results have highlighted to some extent the developmental stages of the dendritic tree of *Drosophila* LPTCs. The dendritogenesis of the LPTCs can be divided into several phases (Figure 3.8). I monitored the neurons from their birth until maturation inside intact brains.

Next, my interest focused on the involvement of cytoskeletal players in LPTCs dendritogenesis. I started my investigation with assessing the subcellular localization of actin and tubulin in the mature LPTC neurons (Figure 3.9). Then, I followed the subcellar localization of actin throughout pupal stages (data not shown) and analysed the changes at the last developmental phase between 70h- 110h APF (Figure 3.10). The scaffold pattern from the neurons at 70hAPF (Figure 3.10 C) resembles the adult one (Figure 3.10 D). Potential fine differences in dendritic tree dimensions could be revealed by the quantifications of the field of dendritic arborization. On the other hand, the distribution of actin-GFP at that stage (Figure 3.10 A) appeared different from the one in adult neurons (Figure 3.10 B) which could mean that the system is not yet fully mature at 70hAPH. Actin-GFP is at 70hAPF already present at the tips of dendrites but to a lower extent than in the adult animal (Figure 3.10 B), suggesting that cytoskeletal reorganization processes continue in the time window from 70h- 110hAPF. It cannot be excluded that the distinct actin-GFP distribution at different developmental stages is partially due to uneven expression levels throughout the development.

The data on the varying distribution of actin within the developmental phases leave open space for time lapse investigations. LPTCs appear as a suitable genetic system to test the effect of the gain and loss of function of cytoskeletal players and their interactors in the processes crucial for dendritogenesis like branch formation. Moreover, developmental studies on mutants could reveal how dendritic mutant phenotypes gain their malformed shape.

Deciphering how the LPTCs dendrites become integrated into the neuronal circuitry would profit from simultaneous labeling of the upstream partners of LPTCs. Unfortunately, the available driver lines for T4 and T5 medullar neurons (Strausfeld and Lee, 1991; Bausenwein and Fischbach, 1992) are not yet fully characterized.

Conclusions

I analyzed and described the dendritogenesis of LPTCs in intact *Drosophila* brains. This system can be used to conduct further detailed investigations into the involvement of cytoskeletal players in dendritic growth.

LPTC Spine- like Structures are Actin Enriched

Actin and tubulin together with their regulatory molecules define the morphology of neurons. As shown for cultured hippocampal neurons the stable shaft of the dendrites mostly comprises microtubule bundles whereas actin is highly enriched in the motile spines (Matus et al., 1982) (Figure 1.4).

A large body of literature covers the organization and function of actin in vertebrate dendritic spines (Engert and Bonhoeffer, 1999; Matus, 2000; Lieshoff and Bischof, 2003; Zito et al., 2004), whereas much less is known about the distribution of actin in other regions of the dendrite including their tips. For instance, class III md- da neurons in *Drosophila* possess actin enriched filopodia- like structures along their dendrites (Andersen et al., 2005). In insect mushroom bodies actin is enriched in the Kenyon cells (KC) dendritic areas, in comparison to the pedunculus and lobe neuropil (Frambach et al., 2004).

The current study revealed localization of actin-GFP and tubulin-GFP within entire trees of LPTCs to be compartmentally distinct and differed from the pattern of a membrane marker mCD8GFP (Figure 3.9 A). Tubulin-GFP was present mainly in the trunks of the primary branches (Figure 3.9 C). While, actin-GFP was highly enriched at the tips of high order branches (Figure 3.9 B). Higher magnification images show that weaker tubulin expression was in fact detectable also in the high order branches (Figure 3.12 M, N, O), however, actin became accumulated at the tips of the fine branchelets (Figure 3.12 H, I, J).

One of the caveats of the usage of Gal4- UAS system is that it produces overexpression conditions that may differ from the actual expression pattern of the endogenous protein. I wanted to rule out that the observed spine- like protrusions accumulating actin-GFP are an artifact created due to increased amount of the molecule. This concern appears in the context of a study in *Drososphila* larvae md- da neurons, where UAS-actin-GFP overexpression (with the construct used in the current study), revealed filopodia along the dendrites, which were not detected using UAS-GFP that labels the cytoplasm (Andersen et al., 2005). A recent study in organotypic hippocampal slices also demonstrated that overexpression of actin *per se* leads to a significant increase in spine density on CA1 pyramidal cells (Johnson and Ouimet, 2006). However, the quantifications of density and length of spine- like structures present on LPTCs (Figure 3.13) did not expose any

significant differences among genotypes upon overexpression of mCD8GFP, actin-GFP or moesin-GFP (GMA) that binds intracellular actin (Edwards et al., 1997). Meaning that in the LPTCs overexpression of actin *per se* does not alter density of spine- like structures. As a consequence I could reliably use UAS-actin and UAS-GMA constructs in the further assays.

An alternative way to visualize actin is phalloidin detection, which binds actin as shown in cultured neurons and in the insect mushroom body calyxes (Forscher and Smith, 1988; Frambach et al., 2004). However, since actin is ubiquitous within a nervous system such a staining does not allow obtaining high resolution images.

The spine- like structures on LPTC dendrites morphologically resemble vertebrate spines (Figure 3.11) and can be classified into four classes of shapes (Figure 3.14). Classifying spines into morphological subgroups underestimates their heterogeneity (Lippman and Dunaevsky, 2005), but on the other hand it facilitates the analysis of their potential maturity and function (Hering and Sheng, 2001; Noguchi et al., 2005). Similar to hippocampal spines (Hering and Sheng, 2001) LPTC spine- like structures can be generally classified into four categories of shapes: mushroom, thin, stubby and branched (Figure 3.14). Even though the distribution into particular morphological groups is different than in vertebrate spines, in both cases the subset of mushroom shaped spines appears as 15% (Harris et al., 1992). Importantly, mushroom shaped spines based on the physiological data are suggested to be the mature ones (Tada and Sheng, 2006). It remains unclear if the higher percentage of branched LPTC spines correlates with a higher number of spines that undergo head- partitioning upon excitation as reported for vertebrate spines. An alternative explanation for the presence of branched spines is a fusion of neighboring spines (Yuste et al., 2000).

It would be interesting in this regard to investigate if the synaptic contacts are located preferentially on the mushroom shaped spine- like structures and also how the subsets of different classes of spine- like structures change through the development.

Why study Spines in *Drosophila*?

Preferential localization of actin-GFP to the terminal dendritic protrusions that appear in different morphological flavors led me to address whether the analyzed structures could have features of vertebrate spines beyond the morphological such as formation of synapses or motility. The reason why this question is important is because the molecular analysis of spines could profit from introduction of a genetically amenable system such as *Drosophila* CNS neurons.

The middle region of the VS1 cell represent a stereotyped and quantifiable system (see Methods), which offers the possibility of finding new genes that are crucial for spinogenesis and spine maintenance. Screens aimed at finding new genes were already done for axon and dendrite morphology in the mushroom body neurons (Reuter et al., 2003), and for axonal connectivity in the visual system (Newsome et al., 2000).

Moreover, introducing LPTC spine- like structures provides an opportunity to analyze the life history of spine –like structure formation and dynamics. Even though recent data provide *in vivo* insights into vertebrate spine growth mechanisms (Knott et al., 2006), still little is known about the relationship between spine addition and synapse formation.

Dendritic spines have attracted considerable interest because they are suggested to be the cellular effectors of such processes as learning and memory (Yuste and Bonhoeffer, 2001). It is widely assumed that the formation of long- term memories requires activity dependent long- lasting morphological alterations in neuronal networks, which might take place in the neuronal spines (Bonhoeffer and Yuste, 2002; Matus, 2005). Indeed, the number of spines can be modified in response to long-term potentiation (LTP) induction (Engert and Bonhoeffer, 1999; Nagerl et al., 2004) and by experience *in vivo* (Lendvai et al., 2000; Holtmaat et al., 2005). Spines in the calycal interneurons of honeybees were shown to be capable of plastic rearrangements upon nursing and foraging experiences (Coss et al., 1980).

Learning in *Drosophila*

Insects, namely *Drosophila* and honeybee are established model systems used to uncover the molecular and cellular basis of learning and memory processes (Coss et al., 1980; Menzel, 1983; Heisenberg, 1989). *Drosophila* in this regard serves as a particularly genetically helpful model system.

The literature stresses the importance of the *Drosophila* mushroom body (MB) in olfactory learning and memory (Davis, 1993). The cyclic adenosine monophosphate (cAMP) cascade have been shown to be implicated in the regulation of the fiber numbers in the peduncle of the MBs, thus in the structural plasticity of the *Drosophila* brain (Balling et al., 1987). Flies reared under enriched conditions had more KC fibers then their deprived siblings (Technau, 1984). It has also been shown that in fly pairs the volume of the MB calyx depends on the sex of the partner (Heisenberg et al., 1995). In contrast to MBs, cAMP signaling seems not to regulate the plasticity of the size of the lobula plate. It suggests that mechanisms regulating structural plasticity in MB and the optic lobes are based on molecular and cellular mechanisms that are quite different (Barth et al., 1997).

Processes with spine morphology have been reported on dendrites of different types of neurons in several types of insects, including *Musca*, *Calliphora*, cricket and bee (Pierantoni, 1976; Hausen et al., 1980; Cajal and Sanchez, 1983; Farris et al., 2001; Strausfeld and Okamura, 2006). *Manduca* motor neurons, for instance, appear to have spine- like protrusions capable of formation of synapses (Weeks and Truman, 1985; Truman and Reiss, 1988). Moreover, in cricket mushroom bodies the presence of synapses on processes that resemble spines has been shown by electron microscopy (Frambach et al., 2004).

In *Drosophila* the presence of spines was suggested by several studies, which note spine-like processes in LPTCs (Scott et al., 2003a, b) and the presence of synaptic contacts onto small spine- like protrusions in lateral horn neurons receiving input from the mushroom body Kenyon cells (Yasuyama et al., 2003). Laminar neurons (Gorska-Andrzejak et al., 2005) and Kenyon cells present in the MBs (Ito et al., 1998) appear to have spine- like protrusions of regular shape and density as well.

In addition to the spine- like structures on LPTCs, the current study demonstrated actin enriched spine- like structures on the dendritic extensions of the giant fiber neurons (Figure

3.21 C). These dendrites have been previously demonstrated to be postsynaptic and to bear excitatory receptors (Fayyazuddin et al., 2006).

However, the word 'spine' has been applied to insect neurons on largely intuitive grounds. None of these studies have demonstrated in a coherent way either the cytoskeleton organization of spine- like structures or that the observed structures possessed other essential characteristics of spines, such as the presence of synaptic inputs.

Are LPTC Spine- like structures Plastic?

I have postulated that the system of LPTC spine- like structures could be used to look for novel genes as well as to study the formation and maintenance of spines. Would it be also suitable to study plasticity? Are the LPTC spine- like structures capable of plastic rearrangements? As reported by Scott *et al* (2003) the development of *Drosophila* LPTCs is independent of the visual experience (Scott et al., 2003b). The experiments on dark reared animals indicated no role for visual experience in the development of the VS dendrites, and no significant alteration in the dendrite or spine- like structures proprieties. Spontaneous activity from photoreceptor does not play a role in the formation of fully complex dendrites in *Drosophila* LPTCs (Scott et al., 2003b). On the other hand, it is possible that the direct upstream partners of LPTCs, possibly T4 and T5 neurons (Strausfeld and Lee, 1991; Bausenwein and Fischbach, 1992), would need to be completely silenced to stop synaptic input to LPTCs (Mizrahi and Libersat, 2002).
The results of Scott *et al* (2003) have bolstered the previous findings from *Calliphora* where no differences (in respect to dendritic pattern and dendritic terminae) were detected in cells from animals dark reared or reared under illumination with stroboscopic flashes in comparison to cells from control animals kept under normal rearing conditions (12h daylight/12h darkness) and animals Golgi impregnated directly after emergence were analyzed. Therefore the dendritic patterns of the LPTCs are fully developed at emergence and seem to remain constant irrespective of visual experience or visual deprivation of the animal (Hausen, 1984).

A "hard-wired" visual map in the fly brain has been postulated by Hiesinger *et al* (2006) based on recent findings from the quantitative ultrastructural analysis of photoreceptor synapses in *Drosophila* mutants affecting synapse formation. Photoreceptors were shown to form a precise and constant number of afferent synapses independent of neuronal activity and partner accuracy. Thus, cell- autonomous control of synapse numbers are a part of a intrinsic developmental program of activity- independent steps (Hiesinger et al., 2006).

On the other hand, there exist examples showing that structural, biochemical and physiological plasticity actually does occur in the fly visual system. Critical periods, meant as the time windows during development in which the anatomy and physiology of the visual system are mutable or plastic, have been reported in vertebrate systems (Daw, 1995), but seem to play a crucial role also in the fly visual system (Barth et al., 1997).

The volumetric changes detected in most neuropile regions of *Drosophila* indicate that the brain is continuously reorganized throughout life in response to specific living conditions. It has been shown that various parts of the brain including the optic lobe are plastic in 8d-16d old flies (Heisenberg et al., 1995).

As an example of structural plasticity, the number of L2 laminar synaptic profiles is changed during the first 4d of adulthood as a consequence of rearing in different light regimes (Kral and Meinertzhagen, 1989). The lamina is ready to grow to its full size during the first day after eclosion, but only provided that light is available. At least for the first 5d the lamina stays sensitive to light deprivation (Barth et al., 1997). Lobula plate and lamina monopolar cells may have other time windows for their sensitivity to visual stimulation.

Taken together, the cases of structural plasticity in the insect visual system are very rare. The above listed examples might indicate that in contrast to the vertebrate visual system (Karmarkar, 2006, See also Introduction) fly visual system remains incapable of plastic changes.

Structural changes are the crudest ones to be expected, though. It remains elusive if synaptic connections and the physiological properties of tangential cells from deprived animals are also unaffected. Such functional alterations have been observed in the visual interneurons in locusts (Bloom and Atwood, 1980) and in mechanosensory system in crickets (Murphey, 1977). Biochemical or physiological plasticity might be more common in the insect visual system. A few examples would support this hypothesis: dark rearing of *Musca*

domestica during the first 5d after emergence was shown to increase light and contrast sensitivity (Deimel E., 1992) and contact with a structured environment in the first days after eclosion was demonstrated to determine the capability of C*alliphora* to differentiate patterns (Mimura 1986).

In conclusion, based on the experiments conducted on LPTCs in *Drosophila* (Scott et al., 2003b) and C*alliphora* (Hausen, 1984) as well as on the indications suggesting that the fly visual system is hard wired (Hiesinger et al., 2006) LPTC spine- like structures are rather not a relevant system for studying plasticity. Additionally, studying the physiology of LPTC spines is experimentally limited.

Are the synaptic contacts on LPTC Spine- like structures active?

Synaptic contacts can be detected by immunocytochemistry using antibodies recognizing molecular components of the synapses (Figure 1.7). In general, proteins involved in synaptic transmission in *Drosophila* appear well conserved when compared with their vertebrate homologues (Wu and Bellen, 1997). In the vertebrate system both post- (anti-PSD95 (Shiraishi et al., 2003)) and presynaptic (anti- synapsin (Zagrebelsky et al., 2005)) molecules can be identified simultaneously, which allows almost unambiguous assessment of the presence of synaptic contacts .

In the *Drosophila* central nervous system immunohistochemical detection of synaptic contacts is limited to analysis of presynaptic components (Figure1. 8) e.g. vesicle associated molecules (Littleton et al., 1993). Dlg (homolog of PSD-95, *disc-large-1,* membrane associated guanylate kinase,) a postsynaptic marker, allowed reliable results exclusively in the neuromuscular junctions (NMJs) (Chen and Featherstone, 2005).

On the basis of antibody staining against the presynaptic marker synaptotagmin (Figure 3. 15) I have demonstrated that around 52% of the spine- like structures are sites of synaptic contacts. It might indicate that in comparison to vertebrate slices, a slightly lower number of LPTC spine- like structures have presynaptic partners (Zagrebelsky et al., 2005). A detected gradient of antibody within the nervous tissue indicated an impaired penetration of the antibody. It could suggest that the actual number of synaptic contacts onto LPTC spine- like structures is higher than detected. Neither the prolonged incubation with the antibody nor attempts to further permeabilize the nervous tissue significantly

altered the percentage of neurons making contacts. The staining was performed on the brains *in toto* that is relatively thick (app.200μm). On the other hand, the tangential neurons are located relatively close to the brain dorsal surface; so that for the efficient staining the antibody did not need to penetrate more then 40μm which is relativity thin in comparison to the hippocampal slices that are 300-400μm thick. The function of the spine-like structures that do not form synapses remains uncertain.

An alternative way of detecting individual active zones is the usage of an antibody nc82 that stains the Bruchpilot molecule (Brp) (Wagh et al., 2006), a structural protein present at all *Drosophila* synapses (see Introduction). It might give the possibility to report the proportion of syt- positive contacts with Brp- positive puncta.

Interestingly, recent studies in *Manduca* using staining anti- synaptotagmin revealed that the different types of filopodia in the developing motor neurons can form synaptic contacts (Evers et al., 2006).

Final proof of synaptic transmission relies on electrophysiological recordings, which could not be conducted due to technical limitations. However, in order to rule out false synaptic connections I conducted the analysis of LPTC spine- like structures at the ultrastructural level (Figure 1. 6). I could unambiguously demonstrate by serial section EM analysis of the brain samples where the LPTCs were specifically labeled, the presence of postsynaptic densities decorated with presynaptic T- bars on each of the identified spine- like structure heads (n= 6) as well as on the dendritic shafts (Figure 3.16).

T- bars together with calcium channels are known to be key components of the *Drosophila* active zones (Kittel et al., 2006) (Figure 1.7). *Drosophila* T- bars consist of a platform and a pedestal which is a docking site for the vesicles. Functional counterparts of *Drosophila* T-bars are present in many species including humans (Zhai and Bellen, 2004). The presence of T- bars is essential for the functioning of synapses in *Drosophila*, as reported in the NMJ (Meinertzhagen et al., 1998), in the tetrad synapses between photoreceptor and lamina monopolar cells (Meinertzhagen, 1996) as well as in the mushroom bodies (Yasuyama et al., 2002).

Importantly, I was able to show T- bar structures surrounded by synaptic vesicles (Figure 3.16 D, inset) which might suggest that the synapses at the LPTC spine- like structures are capable of synaptic transmission.

Comparison among the appearance of T- bars present on the LPTC dendrite shaft with the T- bars on the head or neck of spine- like structures showed no obvious difference (Figure 3.16). The number of serial section electron microscopy examples (n=6) in which I identified spine- like structures on LPTCs was not sufficient to verify if there is any difference among appearance or number of synaptic vesicles present at T- bars localized at the shaft versus the T- bars localized at the spine- like structure head and neck. In all examples of spine- like structures detected by EM, the base of the spine was marked by the presence of a mitochondrion (Figure 3.16). Consistent with reports that mitochondria are essential for spine formation and function in the hippocampal neurons (Li et al., 2004). On the other hand neither the presence of smooth endoplastic reticulum (SER), nor so called 'spine apparatus' (Westrum et al., 1980) was detected in any of the cases. It might be due to the sample preparation of the immunohistochemically labeled brain slices. All Purkinje cell spines and about 50% of the spines on hippocampal CA1 cells are equipped with SER whereas only some pyramidal neurons have spine apparatus. Both the SER and spine apparatus are usually associated with larger vertebrate spines and are absent in the small spines (Spacek and Harris, 1997). As SER is known to play a role in Ca^{2+} managing, differently sized spines may have different ways to control calcium homeostasis (Andrews et al., 1988). It remains unclear if the LPTC spine- like structures are devoid of Ca^{2+} handling machinery or if its absence on the EM images is due to technical limitations. However, in *Calliphora* it has been shown using *in vivo* Ca^{2+} imaging upon visual stimuli that Ca^{2+} entry is voltage dependent and only secondary to the synaptically induced depolarization (NMDA-like currents are not known to exist in these circuits) (Single and Borst, 1998; Laurent, 1999).

Genetical Manipulations of LPTC Spine-like Structures

I proposed to ultimately use the *Drosophila* LPTC spine- like structures as a system to screen for novel genes important for spine formation and dynamics.

First, I wanted to verify the hypothesis that similar genes might be important in controlling spines in both vertebrate and insects. I thus checked the effect of *Drosophila* homolog of Fragile X Mental Retardation Syndrome (*dFmr1)* (Figure 3.17 and 3.18). Fmr1 is a widely expressed RNA-binding translational regulator with many potential targets. Mutation in

the *fmr1* gene leads to Fragile X syndrome which is a most common inherited mental retardation disease. One of its landmarks is increased number and malformation of the spines (Fiala et al., 2002). *Drosophila* FMR model has revealed novel mechanistic insights into the disease. In general, dfmr1 is a global negative regulator of neuronal architectural complexity in motor (Zhang et al., 2001), sensory (Lee et al., 2003) and central interneurons (Pan et al., 2004). *dfmr1* dendritic mutants appear to display more prominent phenotypes than mouse *fmr1* knockouts which greatly facilitates the analysis (Zhang and Broadie, 2005). However, my study was the first that examined the effect of *dfmr1* on the level of spines of *Drosophila* CNS dendrites.

The primary analysis of *dfmr1* was done for overexpression and loss of function conditions using a full length UAS-Fmr1 construct (Figure 3.17 C) and the deletion mutant allele *dfmr1Δ50* (Wan et al., 2000; Zhang et al., 2001) (Figure 3.17 B). Surprisingly, no alterations were revealed in density and length of spine- like structures in either of the cases when using the standard quantification protocol (see Methods). No major alterations in dendrite architecture were detected either. Overexpression of the full length UAS-Fmr1 construct might lead to a higher number of filopodia- like protrusions (Figure 3.17 C) as based on the qualitative estimation. Therefore it is likely that the distribution of spines within categories is different, and that only one stage of a spine's lifetime is increased in number or duration. Accurate analysis of spine- like structure categories distribution could reveal whether this hypothesis is correct.

In order to test a cell- autonomous effect of *dfmr1*, I generated single cell mutant *dfmr1Δ50* clones of LPTCs using the MARCM technique (Lee and Luo, 1999) in the otherwise wild type background (Figure 3.18). The mutant allele *dfmr1Δ50* (Zhang et al., 2001) used in the current study has been previously reported to show more evident phenotype when analyzed on the single cell level in the mushroom body neurons (Pan et al., 2004).

As mentioned already, the clonal analysis was severely hampered by the fact that the time window of LPTCs appearance is not precisely identified (Scott et al., 2002) and that LPTCs emerge in a random manner. Eventually, the extremely low frequency of single and double cell clones (0.5% and 0.4% respectively) brought me to quantifications of middle region of VS1 neuron in the multiple cell clones (Figure 3.19). No difference in spine- like structure density was detected. There are two potential explanations for the lack of

dfmr1Δ50 phenotype either *dfmr1Δ50* allele does not have a cell- autonomous function in LPTCs or there is no effect of *dfmr1Δ50* on LPTC spines.

Whether LPTC spine- like structures are more susceptible to other existing *dfmr1* mutant alleles, like *dfmr1*^{3}, that have been demonstrated to affect synaptic plasticity in MB neurons (McBride et al., 2005) remains to be resolved.

The attempt to identify the presence of FMRP within the lobula plate using the antibody against FMRP did not bring conclusive results. This might indicate that dfmr1 is not expressed in LPTCs.

A lack of phenotype in LPTC spines overexpressing UAS-Fmr1(Wan et al., 2000) as well as other genes regarded as potential 'positive controls' to test the system, such as calcium/calmodulin- dependent protein kinase II (CaMKII), known to be implicated in regulation of both morphological and elecrophysiological plasticity (Wang et al., 1998) may be due to several issues. Possibly, a single copy of a Gal4- DB331 driver is not efficient enough to produce amounts of a desired molecule that would lead to a phenotype. The UAS-actin GFP construct used in the current study did not change spine-like structure density and length in comparison to the membrane marker (Figure 3.13). This is in contrast to already mentioned recent findings from the vertebrate system where the actin overexpression in the organotypic hippocampal cultures *per se* leads to a significant increase in the spine density on CA1 pyramidal cells (Johnson and Ouimet, 2006). The other explanation could be that the produced phenotypes are too subtle to be detected by testing only density and length of spine- like structures and not spine categories.

Next, I investigated the effect of one of the small GTPases- Rac1 on spine- like structures density. The small GTPases are crucial regulators of the actin cytoskeleton and Rac1 is a prominent regulator of spine morphology and density (Luo et al., 1996; Nakayama et al., 2000; Tashiro and Yuste, 2004). Overexpression of a constitutively active (CA) version of Rac1 in murine cerebellar Purkinje cells (Luo et al., 1996) and in rat pyramidal neurons (Nakayama et al., 2000) leads to an increase in spine density and a reduction of spine length (Luo et al., 1996). In contrast, overexpression of dominant negative (DN) Rac1 in rat pyramidal neurons or hippocampal neurons results in progressive elimination of dendritic spines (Nakayama et al., 2000, Zhang and Macara, 2006).

In the current experiments on *Drosophila* spine- like structures both the overexpression of wild- type full length *dRac1* or of a DN version of Rac1(Luo et al., 1994), which possibly acts through sequestering rate-limiting GEFs (Ridley et al., 1992), surprisingly led to the same result, a 30% increase in the number of the spine- like structures (Figure 3.19 B,C). It is possible that high level overexpression of Rac1 could produce also a dominant-negative effect by down-titrating GEFs common to other GTPases. It is worth noting that in *Drosophila* the same axon guidance phenotype was obtained upon overexpression of DN and CA *Rac* (Luo et al., 1994).

In this context, my data suggest that blocking Rac1 activity leads to an increase in the number and a decrease of the length of spine- like structures in LPTCs, but further experiments with single *Rac1*, *Rac2* and *Mtl* mutants (Hakeda-Suzuki et al., 2002; Ng et al., 2002) should solve this issue.

Thus, my studies have demonstrated a proof of principle that the spines present on LPTCs are genetically modifiable and can be used to test novel molecular candidates.

Differences between LPTC Spine- like Structures and Vertebrate Spines

Spines were originally identified and named by Ramon y Cayal (1911) on the basis of their shape. The name '*Espinas*' was supposed to reflect their resemblance of flower thorns.

I have demonstrated that spine- like structures present on the LPTCs are actin enriched and that their morphologies can be classified into four categories. Moreover, I have shown that they form synapses and can be genetically modified. These novel findings open the possibility to use the LPTC spine- like structures as a system to look for novel molecules important for spine maintenance. The mentioned criteria indicate that LPTC spine- like structres can, at least in some regards, be treated as the counterparts of vertebrate spines. Below I present some characteristics that make the LPTC spine- like structures different from vertebrate spines.

One of the basic differences between the vertebrate and invertebrate CNS is that the most common excitatory transmitter in the vertebrate CNS is glutamate but in the insects it is acetyl choline (Sanchez-Soriano et al., 2005).

As reported in *Calliphora* immunostainings for nAChRs and GABARs can be observed in close vicinity to lobula plate tangential cells (Brotz et al., 2001). NMDA receptors are not known to exist on those neurons (Laurent, 1999). The presence of GABARs and AChRs on LPTCs in *Calliphora* has been proposed to be associated with the function of LPTCs that are capable of directional selective responses to visual motion stimuli (Brotz and Borst, 1996). In the model proposed by Brotz and Borst (1996) two detector subunits are connected schematically with the LPTC dendrite. Within each detector subunit, light signals from two neighboring retinal locations are correlated with each other. These signals are proposed to modulate acetylcholine and GABA receptors, respectively, located on dendrites of LPTCs. This represents a cellular implementation of a substraction of subunit output signals leading to an enhanced direction selectivity of postsynaptic LPTCs (Brotz and Borst, 1996).

In contrast, vertebrate spines are normally decorated with excitatory glutamate-NMDA and AMPA receptors (Sala, 2002). The same spines can also have an inhibitory input (Knott et al., 2002), however essentially spines represent the main unitary postsynaptic compartment for excitatory input (Sala, 2002). Glutamate receptors 'report' synaptic activity and therefore it is believed that they might be involved in the activity dependent regulation of spines (McKinney et al., 1999; Shimizu et al., 2000).

Moderate levels of intra spine calcium (such as provided by release from the SER, or by AMPA-receptor- mediated depolarization and influx through voltage-gated calcium channels) promote spine stability/growth, whereas high calcium levels (such as those observed after a prolonged activation of NMDA receptors) induce shrinkage or collapse. A similar dichotomy in the effects of postsynaptic calcium elevation has been invoked to explain the different calcium requirements of long-term depression (LTD) and LTP. Thus, NMDA receptors are hypothesized to underline associative learning as shown for vertebrate system (Shimizu et al., 2000) as well as olfactory learning in *Drosophila* (Xia et al., 2005).

Moreover, in the vertebrate system NMDA receptors are the factors crucial for plasticity in the visual cortex. They are activated by sensory signals that govern plasticity; antagonists to NMDA receptors reduce plasticity, and there are more of them in the visual cortex at the peak of the critical period for plasticity than later in development (Daw, 1995).

Although the comparison between fly species should be done with caution, one can hypothesize that *Drosophila* LPTC dendrites also bear ACh and GABA receptors as it is

shown in *Calliphora* (Brotz et al., 2001). The difference in the type of receptors present on vertebrate spines and LPTC spine- like structures may be correlated with differences in the eventual function of both types of dendritic protrusions.
Interestingly, there are reports suggesting that the two classes of excitatory synapses AChRs and NMDARs might in fact not be too different in their molecular composition since they use the same scaffolding proteins (Parker et al., 2004).

Speculations about Function of LPTC Spine- Like Structures

Discussed above similarities and differences between vertebrate spines and *Drosophila* LPTC spine- like structures brings some insight into the potential function of LPTC spine- like structures.

Spine- like structures significantly increase the surface of LPTC dendrites providing site for receptors and vesicle docking as revealed by the electron microscopy studies (Figure 3. 16).

Retinotopic organization of *Drosophila* visual system indicates that the overall morphology of LPTCs together with presence of spine- like structures on their dendrites mirrors well their function which is to integrate the input coming from the upstream columnar bushy neurons T4 and T5, as revealed by studies in the counterpart *Calliphora* LPTCs (Borst and Haag, 2002). The shape of spine- like structures on LPTCs may indicate that they represent partially autonomous subunits where compartmentalization of biochemical and electrical input signals occurs (Nimchinsky et al., 2002).

LPTC spine- like structures could linearize the summation of neighboring synaptic inputs by preventing saturation effects. Every point in the visual field should count as much in the sense of integrating inputs. Meaning, the neighboring point should not inhibit each other, which they would do if one increases locally the membrane potential and that decreases the driving force of the other LPTC spine- like structure.

Final Thoughts

In the first part of my work I showed the development of LPTC dendrites in the intact brain tissue. Then, I demonstrated distinct localization of actin- GFP and tubulin- GFP within LPTCs in the adult animals as well as provided preliminary insights into the involvement of actin in the development of LPTC dendrites. These findings open the possibility to examine molecular basis of LPTC dendritogenesis in more detail using time lapse imaging.

Second part of my work was devoted to analysis of actin enriched spine-like protrusion present at LPTC dendrites. In addition to morphological quantitative analysis I have demonstrated that LPTC spine-like structures form synapses and I demonstrated that they can be genetically manipulated. The resemblance of LPTC spine- like structures to vertebrate spines made the system suitable to perform a genetic screen aiming to find novel genes important for spine formation and maintenance. Moreover, LPTC spine- like structures can serve as a system to investigate in the intact brain tissue the formation of spines.

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