Chapter 1

Introduction

Information processing in the nervous system is basically the interplay between biochemical reactions affecting the neuronal membrane's ionic conductivity and the temporal and spatial integration of the transient changes of electrical potentials it thereby evokes. However, it is an ongoing debate whether the spatial structure of neurons is just a hindering necessity to wire synaptic partners in dense neuropils or whether neuronal branching can inherently optimize neuronal computation by exploiting its passive integration and filter properties. Examples supporting either extreme position can be found: in rat hippocampal pyramidal neurons synaptic conductivity is increased with greater distance to the soma, effectively compensating passive signal attenuation and therefore leveling influences of neuronal structure (Magee and Cook, 2000). On the other hand, the computation of image velocity in lobula plate tangential cells in the blowfly is improved by the specific passive signal integration of their dendritic geometry (Single and Borst, 1998).
If neuronal structure has an important impact on behavioral performance, its developmental morphogenesis should be tightly regulated. Answering the following questions in a joint effort might therefore clarify whether neuronal structure subserves adequate computation: How large is the variability in neuronal geometry? Are there rules for the distribution of synaptic input sites and transmitter types over the dendritic tree? What are the developmental factors shaping dendritic morphology? How does a specific synapse distribution and neuronal geometry affect neuronal computation? Tackling the above questions requires as a common but essential prerequisite methods for precisely and quantitatively analyzing neuronal geometry and the localized distribution of proteins along its spatial extent.
Confocal or two-photon laser scanning microscopy offers excellent possibilities to acquire 3-dimensional image stacks of fluorescently labeled structures at submicron resolution. Deploying fluorophores with distinct spectral excitation and emission wavelengths makes

it possible to discriminate multiple selectively stained structures within the same specimen. Neurons can be labeled with fluorescent neuronal tracers, and proteins can be selectively stained by immuno-cytochemistry in fixed tissue. It is even possible to image ongoing neuronal growth and the interaction with proteins in living tissue by introducing genetically engineering DNA or RNA constructs into the host organism, and deploying the cell's own protein synthesis machinery to build fluorescently tagged proteins.
Computer programs exist to visualize 3-dimensional image data. Available automatic reconstruction programs however can only approximately quantify the coarse neuronal geometry. Furthermore, their application is restricted to well stained neuronal structures. To assess the geometry of complete neuronal trees from confocal image stacks, including thin and weakly stained structures close to background intensity so far requires the experimenter to manually chart midlines, diameters and the topology of all neuronal branches in 3 dimensions. This is a time-costly process, which effectively restricts the analysis to only small sample sizes, and the results strongly depend on the individual experimenter's perception.
Analyzing the localization of cell-cell contacts, synaptically localized proteins or surface molecules along the neuronal geometry is close or beyond optical resolution and therefore demands a respectively high precision in defining the neuronal boundary, which can not be reached with manual approaches. To avoid inflicting individual interpretation and the low reproducibility of manual approaches, algorithmic and highly automated evaluation methods are essential preliminaries to achieve the necessary accuracy. In order to analyze large sample numbers and therefore allow statistics testing, a higher evaluation speed, as compared to manual approaches, is of similar importance.

1.1 The goal of the thesis

In this thesis I aimed to investigate the role of dendritic filopodia during postembryonic remodelling of dendritic architecture with respect to synaptogenesis and steering dendritic growth. The size of both, filopodia and synapses is close to or just beyond optical resolution of confocal microscopy. To evaluate the structural interplay between both demands to fully exploit the respective microscopic measuring precision. Because no previously available evaluation method fulfills this essential criterion, the first aim of this thesis was to develop tools to quantify neuronal geometry and neuronal surfaces at full optical resolution by 3-dimensional reconstruction from confocal image stacks. Extensive automation should help to speed up data evaluation and should make it predominantly independent of an experimentor's individual perception. Using the exact description of neuronal shape,

quantification of additional fluorescent signals along the neuron's 3-dimensional projection axes should be made assessable in relation to the neuronal surface or volume.
The developed methods should then be applied to investigate the role of dendritic filopodia for the postembryonic remodelling of dendritic architecture of the Motorneuron 5 of *Manduca sexta* with respect to synaptogenesis and steering dendritic growth.

The results are presented in three chapters based on accepted publications and one manuscript ready for submission:

Chapter 2 describes the development of reconstruction algorithms for the precise measurement of neuronal geometry and to determine the neuronal boundary, and is published as:

SCHMITT S, EVERS JF, DUCH C, SCHOLZ M, OBERMAYER K (2004) *New methods for the computer-assisted 3-D reconstruction of neurons from confocal image stacks.* Neuroimage 23: 1283-1298.

Chapter 3 deals with the full automation of the reconstruction algorithms described in chapter 2 and the development of quantification methods to evaluate the distribution of fluorescently labeled proteins, i.e. their localized concentration along neuronal projections at full optical resolution. This chapter is published as:

EVERS JF, SCHMITT S, SIBILA M, DUCH C (2005) *Progress in functional neuroanatomy: Precise automatic geometric reconstruction of neuronal morphology from confocal image stacks.* Journal of Neurophysiology 93: 2331-2342.

Chapter 4 analyses the interplay of dendritic filopodia with presynaptic axons during postembryonic development, applying previously established methods and is ready for submission as:

EVERS JF, MUENCH D, DUCH C (2005) *Synaptogenic control of dendritic filopodia morphogenesis.*

An additional chapter shows work in progress which will need further labor after this thesis before being published:

Chapter 5 Computational analysis of the dendritic signal integration at different developmental stages of the Motorneuron 5 of *Manduca sexta.*

1.2 Filopodia on developing neurons

One essential function of neurons is to link separate synaptic regions and thus to form neuronal networks. How neurons find the correct path over long distances in the developing embryonic neuropil is attributed to the interplay of the detection of non-diffusible surface molecules and diffusible factors mediating chemo-attraction or repulsion along gradients. As long as neurons grow along fasciculated neuron bundles, axonal growth cones have a plain morphology. However, axonal growth cones with prominent filopodia (thin and highly motile, actin-rich protrusions from the surface of developing neurons) can be found at pioneer neurons *in vivo* (Sanchez-Soriano and Prokop, 2005) and in cultured neurons (Kater and Shibata, 1994; Zheng et al., 1996). A highly dynamic behavior of filopodia is also demonstrated *in situ* at growing dendrites in the central nervous system, continuously extending and retracting at a rate of up to several micrometers per minute (Dailey and Smith, 1996; Lohmann et al., 2005; Portera-Cailliau et al., 2003; Ziv and Smith, 1996). This dynamic property gives strong indications suggesting a searching behavior that stretches out in order to better sample the surrounding environment. Clearly, this behavior increases the probability of finding synaptic partners or so called "guidepost cells" which could influence the neuron's sensitivity for different guiding cues.
Filopodia, however, are also shown to be the growth cone's sensing organs for diffusible factors, mediating chemo-attractive or -repellent growth cone turning (Bentley and Toroian-raymond, 1986; Gundersen and Barrett, 1980; Marsh and Letourneau, 1984; McCaig, 1986; Zheng et al., 1994, 1996). Their length and high surface-to-volume ratio make them excellent structures for probing the growth cone's chemical environment. Filopodia are therefore discussed in heavily diverging contexts: localized targeting and discrimination between multiple possible partner cells at small neuropil sub-volumes (for example: synaptogenesis), and steering net growth at a relatively large-scale orientation, assuring that the correct places in the neuropil are occupied.
Most of our knowledge about the role of filopodia and their regulation has been established from work on axonal growth cones, which is probably due to their good accessibility in cell culture and at peripheral muscles. In recent years, however, the understanding of filopodia on growing dendrites has grown remarkably, mainly due to tremendous advances in life imaging of growth processes *in situ* (Konur and Yuste, 2004a,b; Lohmann et al., 2005; Niell et al., 2004; Portera-Cailliau et al., 2003; Yuste and Bonhoeffer, 2004).
Transient changes in intracellular calcium concentration are demonstrated as a common regulatory factor for axonal (Gomez et al., 2001; Kater and Mills, 1991; Robles et al., 1999, 2003) as well as dendritic (Lohmann et al., 2005, 2002; Meberg et al., 1999) filopodia.

Graded levels of calcium concentration are shown to generate a complete range of filopodia behavior: low concentrations induce filopodia sprouting; medium concentrations stabilize filopodia, and high concentrations lead to retraction/collapse of filopodia (Kater and Shibata, 1994). However the elicitors for calcium elevations obviously diverge at different kinds of filopodia, namely those which sense diffusible factors or those that detect surface molecules upon contact.

In developing dendritic trees, filopodia-dependent dendritic growth and synaptogenesis can take place simultaneously, as has been shown for vertebrate neurons (Fiala et al., 1998; Lohmann et al., 2005, 2002; Niell et al., 2004; Vaughn, 1989) and invertebrate neurons (Duch and Levine, 2000; Libersat and Duch, 2002). Therefore it is appealing to assume functional divergent filopodia within one dendritic tree at a given developmental stage. Indeed, two morphologically distinct types of dendritic filopodia have recently been shown in pyramidal cells of the optic tectum in rats during embryonic development, reacting differently to induced neuronal activity (Portera-Cailliau et al., 2003). However, it is unknown how different types of dendritic filopodia interact with presynaptic axonal endings during ongoing synaptogenesis in development. Furthermore, filopodia have so far only been investigated in the embryonic development of dendritic trees. The role of dendritic filopodia in the postembryonic remodelling of neuronal architecture has not yet been examined. In this thesis the role of dendritic filopodia during postembryonic dendritic remodelling should therefore be addressed with regard to possible divergent functions: axonal sampling and steering growth.

1.3 Metamorphosis of holometabolous insects: a model for postembryonic development

A drastic example for postembryonic remodelling is the metamorphosis of holometabolous insects. During pupal life, adult behavior such as walking, flight, mating and oviposition supersedes larval behavior such as feeding and crawling. Accordingly, these changes in behavior are accompanied by dramatic alterations of the neuronal circuitry and the motor system (reviewed in (Consoulas et al., 2000; Levine et al., 1995)). Most larval muscles undergo programmed cell death at the onset of metamorphosis and are replaced with adult muscles which are derived from myoblasts. The majority of adult sensory neurons are also born post-embryonically. Within the central nervous system, unused larval neurons undergo programmed cell death, many neurons persist and several interneurons are newly born during metamorphosis.

In contrast, most if not all adult motorneurons are already born during larval life and survive metamorphosis, they however undergo extensive remodelling of their axonal and dendritic processes during pupal development (Consoulas et al., 2002; Duch et al., 2000; Duch and Levine, 2000; Kent et al., 1995; Kent and Levine, 1993; Libersat and Duch, 2002). With the retraction of larval arborizations, most synaptic connectivity is dismantled (Duch and Mentel, 2004; Gray and Weeks, 2003; Jacobs and Weeks, 1990; Streichert and Weeks, 1995). Starting in early pupal life at the time of maximum dendritic regression, motorneurons begin with massive dendritic outgrowth (Duch et al., 2000; Libersat and Duch, 2002). The formation of new dendrites is accompanied by an alteration of ionic conductivity and the formation of new synaptic contacts, integrating into newly emerging adult networks (Duch and Levine, 2000). It seems reasonable that the structural remodelling of persisting motorneurons during postembryonic development is causally related to their respecified function in adult behaviour (Levine et al., 1995), however this has not yet been demonstrated.
Insect motorneurons can be identified on the basis of their axonal projection and the location of their cell body. This allows conducting repeated quantitative neuro-anatomy and intracellular physiology from identical cells. Therefore the postembryonic development of motorneurons during insect metamorphosis is well suited as a model to elucidate the developmental mechanisms of postembryonic modification of neuronal architecture.

1.4 An identified neuron which persists during insect metamorphosis, the Motorneuron 5 of *Manduca sexta*

One striking example for the re-specification of larval motorneurons during metamorphosis is the postembryonic remodelling of the Motorneuron 5 (MN5) of *Manduca sexta*. During larval life, MN5 is a typical slow motorneuron innervating a slow contracting dorsal body wall muscle involved in crawling behavior. During pupal life, however MN5 is remodeled to a fast motorneuron, innervating one of five muscle bundles of the main down-stroke flight muscle, the dorsal longitudinal muscle (DLM).
The remodelling of the dendritic field of MN5 is accompanied by changes of ionic conductivity, synaptic connectivity and neuronal architecture, what happens in three successive developmental episodes. During late larval life, larval dendrites begin to retract, accompanied by a dismantling of larval synapses. This retraction is under hormonal control, but it can be altered by neuronal activity (Duch and Mentel, 2004). Dendritic growth

is devided into two successive periods. Reaching maximum dendritic retraction in early pupal life, the dendrite starts with massive outgrowth, densely decorated with filopodia (Libersat and Duch, 2002). This initial dendritic growth phase is accompanied by heavy synaptogenesis (Duch and Levine, 2000). Only 3 days later in development, all major dendrites are laid out, which coincides with the loss of filopodia. During the following 2 weeks of metamorphosis, dendritic growth is restricted to high-order branching at the dendritic perimeter, probably for structural and synaptic refinement (Libersat and Duch, 2002).

The dramatic functional switch of the MN5 during metamorphosis involves heavily divergent requests to neuronal computation. The slow larval MN5 integrates volleys of synaptic potentials and answers with tonic depolarization and bursts of action potentials to induce graded muscle contraction. The adult MN5 however has to fire precisely timed action potentials at about every 30 milliseconds (∼30 Hertz), whereas every single action potential evokes a full power twitch of the respective flight muscle bundle. To generate stable flight, the muscle bundle however must contract synchronously with the other 4 portions of the DLM. Therefore, the remodelling of dendritic architecture of the MN5 during metamorphosis is ideal for investigating whether the MN5's quite opposite larval and adult behavioural demands on neuronal computation are met by a co-operative structure-function interrelation.

References

Bentley, D. and Toroianraymond, A. (1986). Disoriented pathfinding by pioneer neuron growth cones deprived of filopodia by cytochalasin treatment. *Nature*, 323(6090):712–715.

Consoulas, C., Duch, C., Bayline, R. J., and Levine, R. B. (2000). Behavioral transformations during metamorphosis: remodeling of neural and motor systems. *Brain Res. Bull.*, 53(5):571–583.

Consoulas, C., Restifo, L. L., and Levine, R. B. (2002). Dendritic remodeling and growth of motoneurons during metamorphosis of *Drosophila melanogaster*. *J. Neurosci.*, 22(12):4906–4917.

Dailey, M. E. and Smith, S. J. (1996). The dynamics of dendritic structure in developing hippocampal slices. *J. Neurosci.*, 16(9):2983–2994.

REFERENCES

Duch, C., Bayline, R. J., and Levine, R. B. (2000). Postembryonic development of the dorsal longitudinal flight muscle and its innervation in *Manduca sexta*. *J. Comp Neurol.*, 422(1):1–17.

Duch, C. and Levine, R. B. (2000). Remodeling of membrane properties and dendritic architecture accompanies the postembryonic conversion of a slow into a fast motoneuron. *J. Neurosci.*, 20(18):6950–6961.

Duch, C. and Mentel, T. (2004). Activity affects dendritic shape and synapse elimination during steroid controlled dendritic retraction in *Manduca sexta*. *J. Neurosci.*, 24(44):9826–9837.

Fiala, J. C., Feinberg, M., Popov, V., and Harris, K. M. (1998). Synaptogenesis via dendritic filopodia in developing hippocampal area ca1. *J. Neurosci.*, 18(21):8900–8911.

Gomez, T. M., Robles, E., Poo, M. M., and Spitzer, N. C. (2001). Filopodial calcium transients promote substrate- dependent growth cone turning. *Science*, 291(5510):1983–1987.

Gray, J. R. and Weeks, J. C. (2003). Steroid-induced dendritic regression reduces anatomical contacts between neurons during synaptic weakening and the developmental loss of a behavior. *J. Neurosci.*, 23(4):1406–1415.

Gundersen, R. W. and Barrett, J. N. (1980). Characterization of the turning response of dorsal root neurites toward nerve growth-factor. *J. Cell Biol.*, 87(3):546–554.

Jacobs, G. A. and Weeks, J. C. (1990). Postsynaptic changes at a sensory-to-motoneuron synapse contribute to the developmental loss of a reflex behavior during insect metamorphosis. *J. Neurosci.*, 10(4):1341–1356.

Kater, S. B. and Mills, L. R. (1991). Regulation of growth cone behavior by calcium. *J. Neurosci.*, 11(4):891–899.

Kater, S. B. and Shibata, A. (1994). The unique and shared properties of neuronal growth cones that enable navigation and specific pathfinding. *J. Physiol Paris*, 88(3):155–163.

Kent, K. S., Consoulas, C., Duncan, K., Johnston, R. M., Luedeman, R., and Levine, R. B. (1995). Remodelling of neuromuscular systems during insect metamorphosis. *Am. Zool.*, 35(6):578–584.

Kent, K. S. and Levine, R. B. (1993). Dendritic reorganization of an identified neuron during metamorphosis of the moth *Manduca sexta* - the influence of interactions with the periphery. *J. Neurobiol.*, 24(1):1–22.

Konur, S. and Yuste, R. (2004a). Developmental regulation of spine and filopodial motility in primary visual cortex: Reduced effects of activity and sensory deprivation. *J. Neurobiol.*, 59(2):236–246.

Konur, S. and Yuste, R. (2004b). Imaging the motility of dendritic protrusions and axon terminals: roles in axon sampling and synaptic competition. *Mol. Cell. Neurosci.*, 27(4):427–440.

Levine, R. B., Morton, D. B., and Restifo, L. L. (1995). Remodeling of the insect nervous system. *Curr. Opin. Neurobiol.*, 5(1):28–35.

Libersat, F. and Duch, C. (2002). Morphometric analysis of dendritic remodeling in an identified motoneuron during postembryonic development. *J. Comp Neurol.*, 450(2):153–166.

Lohmann, C., Finski, A., and Bonhoeffer, T. (2005). Local calcium transients regulate the spontaneous motility of dendritic filopodia. *Nat. Neurosci.*, 8(3):305–312.

Lohmann, C., Myhr, K. L., and Wong, R. O. (2002). Transmitter-evoked local calcium release stabilizes developing dendrites. *Nature*, 418(6894):177–181.

Magee, J. C. and Cook, E. P. (2000). Somatic epsp amplitude is independent of synapse location in hippocampal pyramidal neurons. *Nat. Neurosci.*, 3(9):895–903.

Marsh, L. and Letourneau, P. C. (1984). Growth of neurites without filopodial or lamellipodial activity in the presence of cytochalasin-b. *J. Cell Biol.*, 99(6):2041–2047.

McCaig, C. D. (1986). Electric-fields, contact guidance and the direction of nerve growth. *Journal of Embryology and Experimental Morphology*, 94:245–255.

Meberg, P. J., Kossel, A. H., Williams, C. V., and Kater, S. B. (1999). Calcium-dependent alterations in dendritic architecture of hippocampal pyramidal neurons. *Neuroreport*, 10(3):639–644.

Niell, C. M., Meyer, M. P., and Smith, S. J. (2004). In vivo imaging of synapse formation on a growing dendritic arbor. *Nat. Neurosci.*, 7(3):254–260.

Portera-Cailliau, C., Pan, D. T., and Yuste, R. (2003). Activity-regulated dynamic behavior of early dendritic protrusions: evidence for different types of dendritic filopodia. *J. Neurosci.*, 23(18):7129–7142.

Robles, E., Gomez, T. M., and Spitzer, N. C. (1999). Global and local growth cone calcium transients promote axon turning at substrate borders in vitro. *Mol. Biol. Cell*, 10:451A.

Robles, E., Huttenlocher, A., and Gomez, T. M. (2003). Filopodial calcium transients regulate growth cone motility and guidance through local activation of calpain. *Neuron*, 38(4):597–609.

Sanchez-Soriano, N. and Prokop, A. (2005). The influence of pioneer neurons on a growing motor nerve in *Drosophila* requires the neural cell adhesion molecule homolog fasciclinii. *J. Neurosci.*, 25(1):78–87.

Single, S. and Borst, A. (1998). Dendritic integration and its role in computing image velocity. *Science*, 281(5384):1848–1850.

Streichert, L. C. and Weeks, J. C. (1995). Decreased monosynaptic sensory input to an identified motoneuron is associated with steroid-mediated dendritic regression during metamorphosis in *Manduca sexta*. *J. Neurosci.*, 15(2):1484–1495.

Vaughn, J. E. (1989). Fine-structure of synaptogenesis in the vertebrate central nervous-system. *Synapse*, 3(3):255–285.

Yuste, R. and Bonhoeffer, T. (2004). Genesis of dendritic spines: insights from ultrastructural and imaging studies. *Nat. Rev. Neurosci.*, 5(1):24–34.

Zheng, J. Q., Felder, M., Connor, J. A., and Poo, M. M. (1994). Turning of nerve growth cones induced by neurotransmitters. *Nature*, 368(6467):140–144.

Zheng, J. Q., Wan, J. J., and Poo, M. M. (1996). Essential role of filopodia in chemotropic turning of nerve growth cone induced by a glutamate gradient. *J. Neurosci.*, 16(3):1140–1149.

Ziv, N. E. and Smith, S. J. (1996). Evidence for a role of dendritic filopodia in synaptogenesis and spine formation. *Neuron*, 17(1):91–102.