Imaging cellular mechanisms of presynaptic structural plasticity

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To my beloved family

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Abbreviations

*	Significant, $p < 0.05$
**	Significant, $p < 0.01$
***	Significant, $p < 0.001$
ACSF	Artificial cerebrospinal fluid
AM	Acetomethyl
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
Ani	Anisomycin
AZ	Active Zone
BDNF	Brain-derived neurotrophic factor
BME	Basal medium
CA1	Cornu ammonis 1
CA3	Cornu ammonis 3
CamKII	Calmoldulin dependent protein kinase
CREB	cAMP response element binding protein
DHPG	Dihydroxyphenylglycine
DUB	Deubiquitinating enzyme
EPSC	Excitatory postsynaptic current
EPSP	Excitatory postsynaptic potential
fEPSP	Field excitatory postsynaptic potential
HBSS	Hanks balanced salt solution
Lacta	Lactacystin
LFS	Low frequency stimulation
L-LTD	late-LTD
L-LTP	late-LTP
LTD	Long-term depression

LTP	Long-term potentiation
NIH	National Institute of Health
NMDAR	N-Methyl-D-Aspartate receptor
NS	Not significant
PKA	Protein kinase A
PKC	Protein kinase C
PPR	Paired pulse ratio
PSD	Postsynaptic density
PTV	Piccolo transport vesicle
SEM	Standard error of mean
SNAP	Soluble NSF attachment proteins
STV	Synaptic vesicle proteins transport vesicle
UPS	Ubiquitin proteasome system
v/v	Volume / volume
VGCC	Voltage gated calcium channels
VGluT	Vesicular glutamate transporter protein

1 Summary

Activity-dependent plasticity of postsynaptic structures has been intensely studied in recent years, but to date presynaptic morphological plasticity remains poorly understood. In my thesis, I set out to investigate the structural and cellular mechanisms of structural plasticity of axonal varicosities, the morphological specializations of presynaptic terminals. To this end, I investigated the role of protein synthesis and degradation for presynaptic structural plasticity in hippocampal neurons under baseline conditions and during the induction of functional synaptic plasticity. To study the link between structural and functional plasticity on the single synapse level, I investigated the ability of stable and dynamic axonal varicosities to form functional synapses.

Combining time lapse two-photon imaging and electrophysiological recordings in organotypic hippocampal slices, I investigated the effects of pharmacological blockade of protein synthesis and protein degradation on the gain and loss (turnover) of axonal varicosities during baseline conditions and long-term depression (LTD), a well-studied experimental model of synaptic plasticity. Moreover, I simultaneously surveyed the dynamics of a fluorescently tagged presynaptic marker protein (VGluT-1) and the morphological plasticity of volume-labeled axonal varicosities.

I observed that pharmacologically blocking either protein synthesis or proteasome-dependent protein degradation impaired the LTD-induced turnover of axonal varicosities, while leaving their turnover during baseline conditions unaffected. Distinct types of morphological dynamics mediated the structural plasticity of axonal varicosities, and LTD-induction selectively influenced their *de novo* growth and straight loss, while it did not affect other forms of their structural dynamics. In further experiments, I demonstrated that more than 90% of morphologically identified varicosities colocalized with a presynaptic marker protein, and the majority of dynamic axonal varicosities accumulated the synaptic marker within four hours after their appearance. Conversely, most of the observed examples did not contain the synaptic marker protein prior to their disassembly.

The data in this thesis indicate that the size of axonal varicosities can be regulated gradually or step-wise by distinct types of structural plasticity. Furthermore, the results imply the use and recycling of preexisting proteins for the turnover of presynaptic structures under baseline conditions. This finding is in agreement with the existence of a pool of presynaptic proteins that may be shared amongst multiple varicosities. Moreover, my data indicate the requirement for additional - yet unknown - factors that are necessary for LTD-induced presynaptic structural plasticity.

Colocalization of a synaptic marker protein with axonal varicosities indicates that most stable varicosities represent functional presynaptic release sites, and that dynamic varicosities can rapidly acquire the potential to form functional synapses. The results suggest that morphological changes temporally bracket functional changes, i.e. synaptic marker proteins accumulate after the formation of new varicosities and disappear before the disassembly of existing varicosities. In summary, my thesis provides novel insights into the structural and cellular mechanisms of presynaptic plasticity as well as into its functional consequences.

2 Introduction

2.1 Synaptic plasticity

Learning and memory refers to the ability of the brain to encode, store and retrieve information. It is widely believed that this striking capability relies on the plastic nature of the neuronal network that allows the brain to adapt and rewire itself in response to experience. Patterns of synaptic activity can persistently modify the strength of synaptic transmission (synaptic strength) in an input-specific manner in a phenomenon termed synaptic plasticity. The concept of synaptic plasticity was first introduced by Donald Hebb nearly 60 years ago: 'When an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic changes takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased' (Hebb 1949). However, it took more than twenty years until the first experimental evidence of synaptic plasticity was provided experimentally. In 1973, Bliss and Lømo discovered that high synaptic activity can result in a persistent increase of synaptic strength, a phenomenon refered to as long-term potentiation (LTP) (Bliss and Lomo 1973). LTP proved to have a functionally opposing counterpart: Long-term depression (LTD), the activity-dependent, input-specific decrease of synaptic strength (Lynch 1977). LTP and LTD are the most extensively studied experimental paradigms of synaptic plasticity and occur in a wide range of species. Indeed, most synapses studied in the brain so far have the ability to undergo LTP and LTD (Cooke and Bliss 2006), and a large body of data reports their importance for learning and memory *in vivo.* To date, LTP and LTD are believed to be cellular correlates of learning and memory.

2.1.1 Long-term depression

LTD refers to the decrease of synaptic strength as result of neuronal activity. It is input-specific, cooperative and associative, thereby fulfilling the most important characteristics of a memory storage and learning mechanism (Kemp and Manahan-Vaughan 2007). LTD is found in at least six subforms that are not mutually exclusive: electrically and

chemically induced, homosynaptic and heterosynaptic, and *N*-methyl-d-aspartate receptor (NMDAR) dependent and NMDAR independent LTD (Kemp and Manahan-Vaughan 2007). Homosynaptic LTD refers to a depression of the synaptic pathways at which the plasticity was induced, whereas heterosynaptic LTD modifies synapses that were inactive during plasticity induction. Initially, LTD was discovered in its heterosynaptic form, when LTP-inducing stimuli delivered to one hippocampal pathway elicited LTD in a separate, unstimulated pathway (Lynch 1977). This heterosynaptic LTD was also reported in the dentate gyrus *in vivo* a few years later (Levy and Steward 1979). The first presumed evidence of homosynaptic LTD followed in 1980, when synaptic depression was induced by low-frequency stimulation (Barrionuevo, Schottler et al. 1980). However, this finding was misleading as it referred to depotentiation, the functional reversal of previously potentiated synapses to baseline levels after LTP. Depotentiation differs from LTD as it exclusively occurs at recently potentiated synapses, cannot be induced at naive synapses and engages different molecular mechanisms than LTD. The first evidence of homosynaptic *de novo* LTD was finally reported more than 10 years later in the hippocampus and in the visual cortex (Dudek and Bear 1992; Mulkey and Malenka 1992; Kirkwood and Bear 1994).

Regarding the great variety of LTD types that can vary amongst different neurons, it may be useful to refer to LTD as a general class of cellular and synaptic phenomena (Malenka and Bear 2004). When discussing its induction, expression and functional role it is thus necessary to define at which specific synapse LTD is studied.

2.1.2 LTD induction and expression in CA1 of the hippocampus

Amongst other brain areas, LTD can be expressed in the hippocampus at the synapses of presynaptic CA3 and postsynaptic CA1 neurons. This form of LTD advanced to be the most intensively studied type of LTD in the forebrain, and its mechanistic understanding is probably the most detailed (Malenka and Bear 2004). It is NMDAR dependent an requires the stimulus-induced activation of NMDARs during postsynaptic depotentiation (Dudek and Bear 1992; Dudek and Bear 1993). NMDAR dependent LTD is typically induced by a prolonged repetitive synaptic stimulation, referred to as low-frequency stimulation (LFS) at 0.5 to 5 Hz for a duration of 600 to 900 stimuli (Dudek

and Bear 1992). As the optimal protocol is variable and depends on factors including animal age or the recent history of synaptic and cellular activity (Abraham and Bear 1996; Bear and Abraham 1996), protocols of repeated paired pulses (Lee, Takamiya et al. 2003) or pairing protocols that couple pre- and postsynaptic activity are also used for its induction. All mentioned protocols converge in activating NMDAR and lead to Ca^{2+} influx into the postsynaptic neuron (Mulkey and Malenka 1992). The required Ca^{2+} concentration can also be provided by other sources such as by intracellular stores or via experimental postsynaptic Ca^{2+} uncaging (Yang, Tang et al. 1999). The quantitative, temporal and spatial characteristics of postsynaptic Ca^{2+} concentration provides a critical molecular switch in synaptic plasticity, as different kinetics of its influx leads to LTP instead of LTD. Ca^{2+} influx triggers Calmodulin-dependent kinase II (CamKII) and a serine / threonine phosphatase cascade that includes Protein Phosphatase 1 (PP1) and its substrates Protein Kinase A and C (PKA and PKC). Changes of synaptic transmission efficacy are ultimately achieved by posttranslational modifications and removal of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors at the postsynaptic density (PSD), resulting in a reduced steady-state of AMPA receptors and reduced synaptic transmission (Carroll, Lissin et al. 1999; Lüscher, Xia et al. 1999). Numerous studies have investigated the cellular and molecular postsynaptic expression mechanism of LTD and dissected the essential and modulating postsynaptic pathways of LTD induction. In contrast to postsynaptic mechanisms, little presynaptic changes have been found to date, and NMDAR dependent LTD at the CA3-CA1 synapse is thought to be mostly independent of presynaptic molecular changes.

With regards to this study, it is important to highlight that the CA3-CA1 synapse can also express NMDAR independent LTD. In NMDAR independent LTD, the required Ca^{2+} signal arises from sources such as voltage-gated Ca^{2+} channels (VGCCs) or through release from intracellular stores by activation of metabotrophic glutamate receptors. It is independent of protein synthesis, can be expressed homosynaptically or heterosynaptically and includes a presynaptic contribution (Abraham, Mason-Parker et al. 2006; Pöschel and Manahan-Vaughan 2007). For example, it is accompanied by changes in paired-pulse ratio (PPR), traditionally interpreted as changes in the presynaptic release probability (Nosyreva and Huber 2005), and other presynaptically

caused effects include an increase in the failure rate of evoked postsynaptic currents (EPSCs) and changes in the coefficient of variation of EPSCs (Fitzjohn, Palmer et al. 2001; Anwyl 2006). Along the same lines, a lack of postsynaptic changes has been observed in this form of LTD (Rammes, Palmer et al. 2003). However, the induction protocols and target synapse of NMDAR dependent and NMDAR independent LTD substantially differ. While NMDAR dependent LTD is typically induced by LFS, NMDAR independent LTD is induced by application of DHPG (Dihydroxyphenylglycine) or paired pulses (Snyder, Philpot et al. 2001). Moreover, while NMDAR dependent LTD is expressed at CA3-CA1 and other synapses, NMDAR independent LTD is typically found in the dentate gyrus. The present study induces LTD by LFS at the CA3-CA1 synapse, and thus focuses on homosynaptic, NMDAR dependent LTD.

2.1.3 Physiological relevance of LTD in learning and memory

The bidirectional nature of synaptic plasticity is to date widely accepted as a generally implemented cellular mechanism in memory formation (Bear and Abraham 1996). The importance of LTD becomes obvious not only by its ubiquitous expression, but also by its different functional relevance across the various brain areas. In general, the physiological consequence of synaptic plasticity is closely linked to the function of the brain area itself. To better understand the physiological relevance of hippocampal LTD, it is thus helpful to discuss it within context of the physiological role of the hippocampus.

The hippocampus plays a well-documented role in spatial memory (S.J.Y. Mizumori 1999; Leutgeb, Leutgeb et al. 2005; Kjelstrup, Solstad et al. 2008) as well as in novelty detection (Lisman and Grace 2005) and for object-place associations (Buffalo, Bellgowan et al. 2006). The hippocampus generates necessary input information for the formation of semantic-like memory in the neocortex (Miyashita 2004) and is involved in episodic memory in humans, linking an event to space and time (Shrager, Bayley et al. 2007). To this end, LTD in the hippocampus is particularly important in spatial learning and in forming a cognitive spatial map (Kemp and Manahan-Vaughan 2007). First indications that LTD plays a role in spatial learning derived from comparative studies of LTD and LTP in genetically modified animals and from studies about the influence of novel environments on LTD and LTP. Whereas novel empty space reinforces the

expression of LTP in the CA1 area, presenting an environment that contains novel objects reinforces LTD (Kemp and Manahan-Vaughan 2005; Lemon and Manahan-Vaughan 2006). Conversely, presenting empty space impairs LTD, and the presentation of novel objects impairs LTP. The role of LTD on spatial learning and in the storage of spatial information is further supported by a LTD-impaired knock-out mouse (Etkin, Alarcón et al. 2006). As recent evidence arises that the multiple distinct memory tasks performed in the hippocampus are region specific (Kemp and Manahan-Vaughan 2007), LTD may exert different physiological functions depending on the hippocampal region where it is expressed. In summary, hippocampal LTD plays a role in spatial memory, novelty detection (Vianna, Alonso et al. 2000) and object constellations within an environment (Manahan-Vaughan and Braunewell 1999). Encompassing a broader view, one can speculate that hippocampal LTD is associated with any 'event' that is linked to a spatial location.

2.1.4 Protein synthesis and synaptic plasticity

While the molecular mechanisms that underlie the induction synaptic plasticity have been intensely investigated, the mechanisms involved in its maintenance remain rather poorly understood. Both NMDAR dependent LTD and LTP are two-stage processes that can be discriminated by their sensitivity to protein synthesis blockade. LTP and LTD induction trigger biochemical cascades that result in modifications of AMPA receptor numbers in postsynaptic spines. These processes are sufficient to induce and establish the first, early stage of LTP or LTD and are presumably exerted directly at the activated synapses in a locally restricted and input-specific fashion. The second, late stage of LTD and LTP is characterized by the synthesis of new proteins that consolidate the acquired functional changes. Late-LTP and late-LTD (L-LTP and L-LTD) require, by definition, protein synthesis and last for hours and days (Malenka, Nicoll et al. 1999). Protein synthesis is caused by a local Ca^{2+} rise at the time of plasticity induction that triggers a synapse-to-nucleus signaling cascade involving molecules like Calmodulin (CaM), MAP kinases, Ras and cAMP (Frey, Huang et al. 1993; Kandel 2001; Saha and Dudek 2008). The cascade activates transcription factors like CREB and other transcription factors that in turn lead to transcription and translation of immediate early

genes including c-fos, Homer 1b, Arc and BDNF (Miyashita, Kubik et al. 2008). In addition to initiating transcription, plasticity induction also directly regulates local translation of mRNA into protein in dendrites and spines (Bramham and Wells 2007). Expression of constitutively active forms of transcription factors can facilitate the consolidation of LTP to L-LTP (Barco, Alarcon et al. 2002; Miyashita, Kubik et al. 2008). Conversely, blockade of protein synthesis at the time of plasticity induction blocks the maintenance of LTP (Krug, Lössner et al. 1984; Frey, M et al. 1988; Abraham and Williams 2008), and specific manipulations of the protein synthesis machinery or knock-down approaches of transcription factors such as CREB block both L-LTP and certain forms of memory (Bourtchuladze, Frenguelli et al. 1994).

Like for LTP, multiple evidence for a protein-synthesis dependent late phase has also been derived for LTD. Studies in the cerebellum reported that application of protein synthesis inhibitors prevent L-LTD (Linden 1996), and these findings have been extended to hippocampal slice cultures and in the hippocampus of freely moving rats (Manahan-Vaughan, Kulla et al. 2000; Sajikumar and Frey 2003). Interestingly, inhibition of transcription instead of translation does not inhibit L-LTD, suggesting that mRNAs are already present in the neuron, and maybe even in the dendritic tree or at the spine.

It was long time postulated that once synaptic plasticity is established, it is independent of protein synthesis (Huang, Nguyen et al. 1996). However, it has recently been described that L-LTP is sensitive to protein synthesis under conditions of ongoing synaptic activity. While L-LTP is unaffected by protein synthesis blockade without ongoing test pulse stimulation, L-LTP destabilizes when the test pulses are continued during protein synthesis blockade (Fonseca, Nägerl et al. 2006). This finding indicates that synaptic activity during LTP maintenance utilizes proteins necessary for LTP stabilization, and that their depletion without replenishment leads to LTP decay.

The process of LTP and LTD consolidation strikingly parallels the consolidation of memory formation (Dudai 2002), where establishing new memories proceeds in phases and the received information is processed via short-term memory into long-term memory. The initial consolidation depends on protein synthesis, and once long-term

memory is established it becomes insensitive to protein synthesis blockade. However, when memories are retrieved they become labile again and must undergo a renewed consolidation, called reconsolidation (Nader, Schafe et al. 2000; Dudai 2004; Dudai and Eisenberg 2004; Morris, Inglis et al. 2006).

The importance of protein synthesis and up-regulation of immediate early genes for late-phase LTP and LTD has been intensively studied on the postsynaptic side. In contrast, it is elusive if a parallel process exists on the presynaptic side, and the requirement of presynaptic gene products in L-LTP and L-LTD awaits further study.

2.1.5 Protein degradation and synaptic plasticity

Akin to protein synthesis, protein degradation has been shown to critically regulate synaptic plasticity. Most proteins of eukaryotic cells and neurons are degraded by a highly conserved biochemical pathway that is composed of multiple regulatory and catalytic proteins, the ubiquitin proteasome system (UPS). UPS-mediated protein degradation is a multi-step process (Figure 2-1) that consists of activating ubiquitin and conjugating it with the target protein, elongating the ubiquitin chain at the target molecule, recruiting the protein to the proteasome and finally degrading the protein (Yi and Ehlers 2007).

The degradation process depends on the activity of the ubiquitin ligases E2, E3 and E4 that mediate initiation and elongation of the ubiquitin chain and are counteracted by deubiquitinating enzymes (DUBs) which cleave ubiquitin molecules from targeted proteins. The length of the ubiquitin chain provides an important threshold that regulates the fate of the target proteins: Before a critical chain length is reached, the ubiquitination process is fully reversible, while a longer chain irreversibly results in protein degradation by the proteasome.

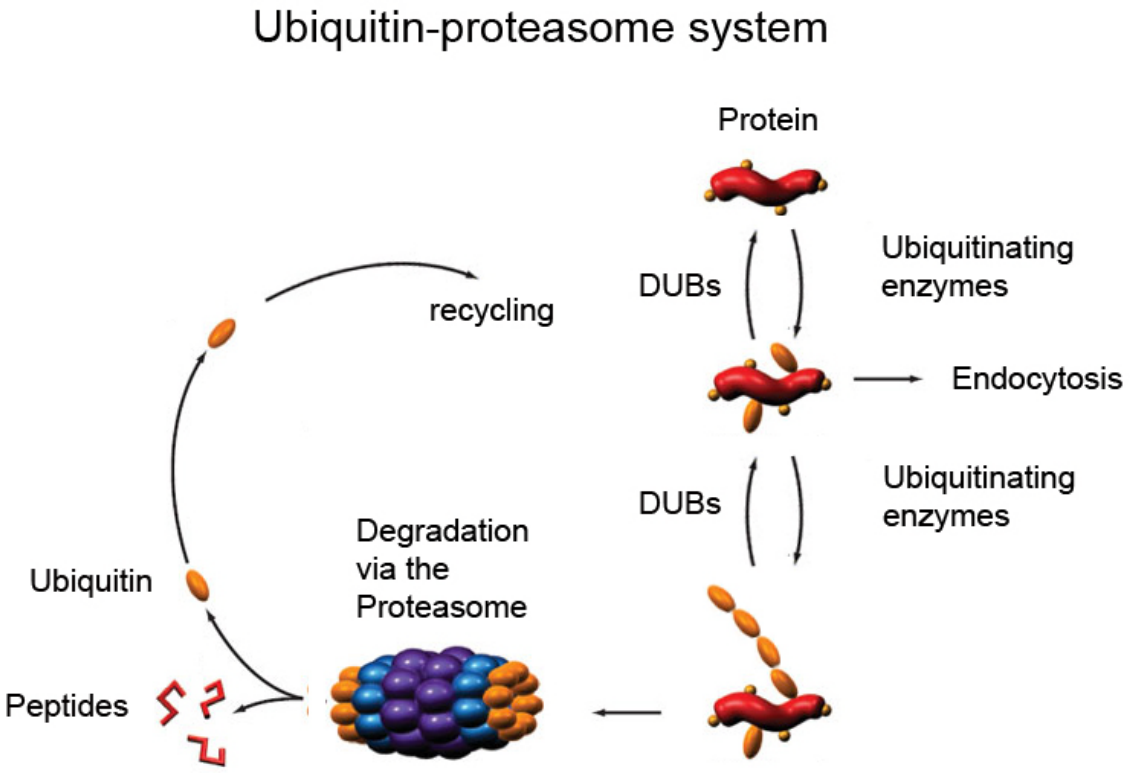

Figure 2-1: The ubiquitin proteasome system (UPS)

Neuronal proteins can undergo ubiquitination by enzymes of the ubiquitin proteasome system. Target proteins can be reversibly ubiquitinated by ubiquitinating enzymes or deubiquitinating enzymes (DUBs). Monoubiquitinated membrane proteins can undergo endocytosis, while polyubiquitinated species become degraded by the proteasome.

Protein degradation critically regulates synaptic plasticity. Blocking protein degradation by the application of the proteasome inhibitors lactacystin or MG132 severely impairs L-LTP (Zhao, Hegde et al. 2003; Fonseca, Vabulas et al. 2006; Karpova, Mikhaylova et al. 2006). Fonseca et al. also showed that both protein synthesis and degradation are required to establish long-term synaptic changes, as L-LTP is blocked by protein synthesis or degradation inhibitors but rescued by inhibiting both processes simultaneously. The data indicates that only a proper balance of synaptic proteins allows the consolidation of LTP, and 'freezing' the composition of postsynaptic proteins

by preventing both sides of protein turnover rescues LTP. For LTD, the requirement for UPS-mediated protein degradation has also been investigated, but yet comparably little is known about the effect of the UPS on LTD. So far, only one study in the literature reports that blocking the proteasome abolishes a pairing-induced form of LTD (Colledge, Snyder et al. 2003), but this finding has not yet been extended to other protocols of LTD induction.

In addition to altering synaptic plasticity, the ubiquitin proteasome system also mediates other cellular processes. When discussing the effect of blocking protein degradation, it should be thus kept in mind that other neuronal and synaptic functions may also be affected. For example, mono-ubiquitination of membranous proteins such as ion channels, G protein coupled receptors, and receptor tyrosine kinases mediates endocytosis (Mori, Claessonwelsh et al. 1995; Shenoy, McDonald et al. 2001). Furthermore, the UPS is important in neuronal development, as pharmacological inhibition of the proteasome leads to irresponsiveness of *Xenopus laevis* axonal growth cones to extracellular guidance cues and compromises the formation of new growth cones in transsected neurons (Campbell and Holt 2001). A proper balance of ubiquitination and deubiquitiation events is critical for proper synaptogenesis in *Drosophila melanogaster,* and further studies reveal a role of the UPS in synapse elimination (Verma, Chierzi et al. 2005). In mammals, proteasomal degradation is involved in the regulation of spine size by degrading a regulator of spine growth during development (Pak, Yang et al. 2001), and in mature neurons inhibition of the proteasome elevates presynaptic vesicle recycling while leaving the release rate and amount of release unaffected (Willeumier, Pulst et al. 2006), indicating an increase in the presynaptic vesicle pool. Interestingly, the effect is independent of protein synthesis and relies on synaptic activity. On the postsynaptic side, activity-dependent remodeling of the PSD composition requires the proteasome (Ehlers 2003), which affects half-life, intracellular trafficking and localization of synaptic receptors and scaffolding molecules like PSD-95 and Shank. An elevation of synaptic activity doubles the number of ubiquitinated postsynaptic proteins and the proteasome itself is presumably redistributed to synapses upon activity (Bingol and Schuman 2006).

2.2 Structural plasticity

Activity-dependent changes in synaptic strength are thought to be a cellular mechanism for learning and memory. Not surprisingly, the neuronal network does not only functionally, but also morphologically reshape upon synaptic plasticity. For example, the addition of new synapses may be required to further increase neuronal connectivity in learning and memory. Conversely, downscaling of weak synapses may result in a loss of synaptic function and possibly also in the loss of the non-functional synaptic structures. By these and other means, morphological plasticity potentially enables the neuronal network to maintain flexibility and adaptivity.

Synaptic structure and function are closely connected in the hippocampus. Postsynaptic terminals of glutamatergic synapses are located in spines in approximately a one-to-one ratio (Nimchinsky, Sabatini et al. 2002), and presynaptic terminals are localized also in approximately a one-to-one ratio in axonal varicosities (boutons) that appear as bulbus-like swellings along the axons. These overall stoichiometric relationships are especially important as they allow for drawing conclusions from structure to function, but taking a closer look, it is important to highlight that these relationships are less stringent on the level of single synapses. While some spines may not bear a PSD, others may have two functional connections to presynaptic terminals. Conversely, axonal varicosities can contain two release sites and postsynaptic partners, or not be opposed to a postsynaptic partner at all.

Structural dynamics follow general, genetically encoded principles as well as activity-dependent mechanisms. The vast majority of morphological dynamics, synapse growth and elimination during development, is mediated by genetically encoded molecular cues and by general activity patterns (Holtmaat, Trachtenberg et al. 2005). Most spines *in vivo* remain transient after development and are influenced by synaptic activity. Recent studies provide insight into the functional consequences of structural plasticity at the single synapse level, and the correlation and causal dependence of structural and functional changes is currently subject of intensive studies (Alvarez and Sabatini 2007).

2.2.1 Postsynaptic structural plasticity

The advent of new imaging techniques enabled a series of studies that substantially advanced the knowledge about structural plasticity. The majority of them focused on postsynaptic spines, small protrusions along the dendritic tree that harbor postsynaptic terminals. Spines are highly dynamic *in vivo* and *in vitro*, and their plasticity is highly regulated by genetic guidelines and synaptic activity (Grutzendler, Kasthuri et al. 2002; Trachtenberg, Chen et al. 2002; Majewska and Sur 2003; Holtmaat, Trachtenberg et al. 2005). The global spine formation pattern during development follows genetic outlines, and the rate of spine formation remains constant for most of the postnatal life in mice. In contrast, the rate of synapse elimination is developmentally regulated, which leads to a net loss of synapses in juvenile mice that slows down in adult mice (Grutzendler, Kasthuri et al. 2002; Holtmaat, Trachtenberg et al. 2005). In more mature networks, synaptic activity predominantly regulates the morphological plasticity of spines including their size, shape and total numbers.

At first, synaptic activity regulates the size of dendritic spines. Spine size is stronlgy related to synaptic function as it scales with size of the PSD, number of AMPA receptors and size of AMPA receptor currents (Harris and Stevens 1989; Matsuzaki, Ellis-Davies et al. 2001; Matsuzaki, Honkura et al. 2004). New protrusions that have not yet formed synapses are smaller than persistent spines (Knott, Holtmaat et al. 2006), and LTP induction leads to an approximately twofold volume increase of spines (Matsuzaki, Honkura et al. 2004). Activity-dependent changes of spine size have been investigated both *in vitro* and *in vivo* and in a variety of LTP protocols (Lang, Barco et al. 2004; Matsuzaki, Honkura et al. 2004; Kopec, Li et al. 2006), and the dynamic nature of spine sizes hints to ongoing functional plasticity of the respective spine (Zuo, Lin et al. 2005). The link between synaptic function and spine size has been well established so that spine enlargement has been lately used as readout for the potentiation status of single synapses (Harvey, Yasuda et al. 2008).

The molecular pathways that link structural and functional spine plasticity have been intensely studied, and LTP induction has been reported to trigger the increase in spine size via CamKIIα, Ras and actin remodeling (Fischer, Kaech et al. 2000; Fukazawa,

Saitoh et al. 2003; Zito, Knott et al. 2004; Holtmaat, Trachtenberg et al. 2005). Synaptic insertion of AMPA receptors is required for spine enlargement both in hippocampal slice cultures and *in vivo*, and spine enlargement in turn leads to AMPA receptor insertion (Kopec, Li et al. 2006; Matsuo, Reijmers et al. 2008). However, synaptic insertion of AMPA receptors is not sufficient to drive structural plasticity and, conversely, the functionality of AMPA receptors, crucial for LTP expression, is not required for LTP-driven spine enlargement (Kopec, Real et al. 2007). These data indicate that although postsynaptic structural and functional plasticity are tightly linked, their expression mechanisms diverge, and that they can be expressed independently.

While an increase in spine size has been observed for LTP, the opposite effect, spine shrinkage, has been reported for LTD (Nägerl, Eberhorn et al. 2004; Zhou, Homma et al. 2004), and is accompanied by actin remodeling and changes in the number of AMPA receptors (Snyder, Philpot et al. 2001; Okamoto, Nagai et al. 2004; Nosyreva and Huber 2005). The cascades which lead to structural and functional plasticity have recently been elucidated, and while the first part of the LTD-signaling cascade is shared amongst structural and functional plasticity – both require NMDAR activation and Calcineurin activity – the downstream pathways involve different molecular players. Spine shrinkage depends on Cofilin activation, whereas functional changes require PP-1 activity (Zhou, Homma et al. 2004), indicating that functional and structural LTD, though intimately connected, do not necessarily mirror each other.

Beside changes in spine size, the shape and number of spines also change in response to synaptic activity. LTP in hippocampal slices leads to the outgrowth of new spines (Engert and Bonhoeffer 1999), and ultimately to the formation of new synapses (Nägerl, Kostinger et al. 2007). This *in vitro* findings have been extended to *in vivo* studies, where spinogenesis in the cortex is enhanced by experience (Trachtenberg, Chen et al. 2002; Holtmaat, Wilbrecht et al. 2006) and followed by synapse formation (Knott, Holtmaat et al. 2006). In LTD, the converse effect of spine removal and synapse elimination has been reported (Nägerl, Eberhorn et al. 2004; Zhou, Homma et al. 2004). Also, the number of bifurcating spines and perforated synapses is increased by LTP (Harris, Jensen et al. 1992; Toni, Buchs et al. 2001), but these structures remain

comparably rare and may represent transient stages of changing the presynaptic partners.

2.2.2 Presynaptic structural plasticity

In contrast to postsynaptic structural plasticity, much less is known about presynaptic structural changes. Recently, the spontaneous plasticity of presynaptic axonal varicosities, the morphological specializations that contain the presynaptic terminals, has been investigated (Toni, Buchs et al. 2001; Krueger, Kolar et al. 2003; Konur and Yuste 2004; Deng and Dunaevsky 2005) and revealed a substantial degree of plasticity *in vitro* and *in vivo* (De Paola, Holtmaat et al. 2006; Majewska, Newton et al. 2006; Stettler, Yamahachi et al. 2006). The few studies addressing activity-dependence of structural plasticity observed different effects, ranging from no effect to an increase in the mobility or an increase in the number of axonal varicosities (De Paola, Arber et al. 2003; Nikonenko, Jourdain et al. 2003; Umeda, Ebihara et al. 2005). Lately, Becker et al. have reported that LTD increases presynaptic structural plasticity (Becker, Wierenga et al. 2008) by elevating the gain and loss (turnover) of axonal varicosities. Intriguingly, the elevated turnover leaves the total numbers of axonal varicosities unchanged, while the number of varicosity-spine contacts of CA3-CA1 neurons is decreased. Although it remains yet elusive how a higher varicosity turnover contributes to a decrease in CA3-CA1 connectivity and synaptic transmission, different scenarios provide possible explanations. For example, LTD induction may increase the fraction of axonal varicosities that target non-CA1 neurons, e.g. inhibitory interneurons, or LTD induction may increase the number of varicosities without postsynaptic partner. Further studies will be required to elucidate this phenomenon and to amend the knowledge about the physiological role of presynaptic structural plasticity.

2.2.3 Axonal varicosities and synaptic vesicles

Presynaptic terminals of hippocampal CA3 neurons are located in 1-2 µm long and about 1 µm wide bulbus-shaped axonal varicosities that are distributed en-passant along axons. They are composed of multiple different proteins (Li and Jimenez 2008) which can be grouped by their function in synaptic vesicle proteins, active zone

molecules, scaffolding molecules and trans-synaptic adhesion molecules (McAllister 2007). At a chemical synapse, a presynaptic terminal is opposed to a postsynaptic terminal, forming a synaptic cleft in which the neurotransmitter is released by the presynaptic neuron and detected by the postsynaptic neuron. The assembly and stability of the synaptic cleft is mediated by trans-synaptic adhesion molecules, and the presynaptic terminal itself is characterized by the presence of hundreds to thousands of synaptic vesicles (SVs) that can fuse and release their neurotransmitter content into the synaptic cleft. Scaffolding molecules form the cytosceletal matrix of the presynaptic terminal and active zone proteins regulate the neurotransmitter release at the active-zone into the synaptic cleft.

Neurotransmitter secretion is a circular process that involves the release of transmitter into the synaptic cleft and the regeneration of synaptic vesicles (Ziv and Garner 2004) (Figure 2-2). A depolarizing axonal action potential of the presynaptic neuron that arrives at the presynaptic terminal can initiate the fusion of a synaptic vesicle with the active zone membrane and the discharge of neurotransmitter into the synaptic cleft. After exocytosis, the synaptic vesicle proteins become recycled and are retrieved by clathrin-mediated endocytosis. They reform vesicles which acidify and must be refilled with neurotransmitter to become available for the next round of exocytosis. The uptake of neurotransmitter to presynaptic vesicles in hippocampal excitatory neurons is conferred by vesicular glutamate transporters (VGluTs). VGluT proteins are essential for refilling synaptic vesicles with glutamate and for neurotransmitter release in glutamatergic -excitatory - synapses (Jean-Luc Boulland 2004). In the hippocampus, VGluT-1 is the predominant isoform of VGluT proteins at the CA3-CA1 synapse.

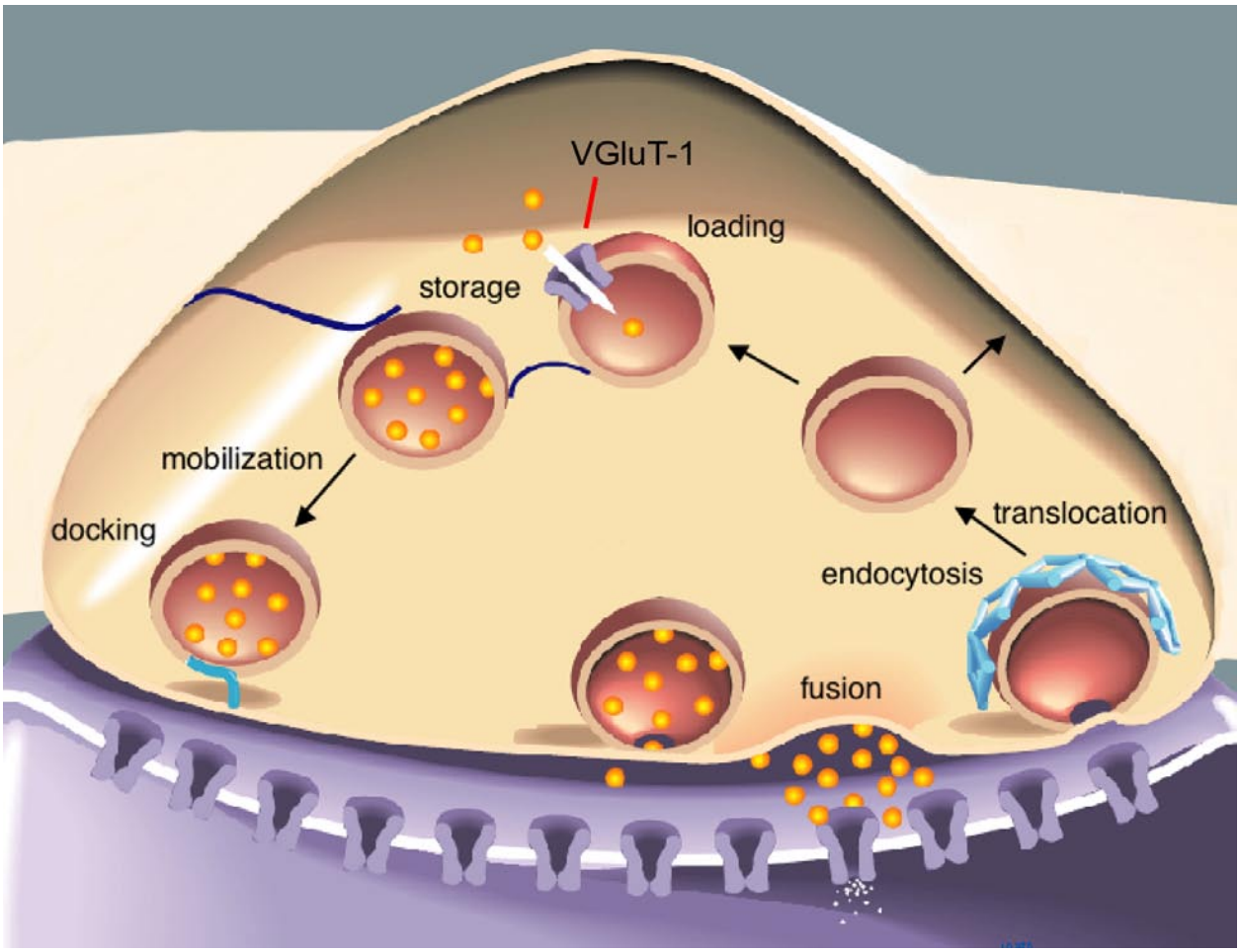

Figure 2-2: Presynaptic vesicles and vesicular glutamate transporters

Presynaptic terminals are located in axonal varicosities along CA3 axons. They contain numerous synaptic vesicles (SVs) that can release neurotransmitter into the synaptic cleft. Upon release, the vesicles get retrieved via endocytosis and must be refilled with the neurotransmitter by vesicular glutamate transporters (VGluT). Adapted from Richmond (Richmond 2007).

2.2.4 Assembly of axonal varicosities

The formation of presynaptic terminals can be described as the assembly of presynaptic proteins to an axonal area where pre- and postsynaptic neurons form a contact. The majority of studies on presynaptic assembly focused neurons in a developmental stage, when the gross of synapse formation takes place. It has been shown that the presynaptic molecules constituting axonal varicosities are present in neurons before most synapses have formed (Fletcher, Cameron et al. 1991; Rao, Kim et al. 1998).

Multi-molecular transport packets represent the assembly units of axonal varicosities before and during synaptogenesis and are recruited to sites of axodendritic contact with rapid dynamics (Shapira, Zhai et al. 2003). The content of these transport packets is heterogeneous, and at least two different types have been investigated in detail: STVs (Synaptic vesicle proteins transport vesicles), which transport synaptic vesicle proteins, and PTVs (piccolo transport vesicles), which transport active zone proteins. (Zhai, Vardinon-Friedman et al. 2001; Sabo, Gomes et al. 2006). PTVs have a homogenous molecular content and appear as 80 nm dense-core particles, while STVs have a more heterogenous appearance that ranges from pleiomorphic, tubo-vesicular organelles (Ahmari, Buchanan et al. 2000) to PTV like small clusters (Kraszewski, Mundigl et al. 1995). Both types of transport packets exhibit a rapid and bidirectional movement along axons in young neurons and divide or coalesce with each other, delivering material for the formation of axonal varicosities. Many laboratories have reported that transport packets can already display important functional presynaptic properties such as vesicle cycling (Kraszewski, Mundigl et al. 1995; Krueger, Kolar et al. 2003) and the capability to release glutamate even before axodendritic contact (Sabo, Gomes et al. 2006).

Besides investigating preclustered assembly units, few studies so far have addressed if single molecules can appear at the axonal varicosity in a continous fashion (Shapira, Zhai et al. 2003; Gerrow, Romorini et al. 2006). While STV and PTV studies favor a quantal assembly of proteins, critical presynaptic constituents like SNAP-25 and syntaxin are difficult to model by quantal insertion, and further evidence supports the dynamic and continous exchange of single molecules amongst presynaptic sites (Tsuriel, Geva et al. 2006).

If the mechanisms of axonal varicosity formation in developing neurons also apply in mature neurons, when most synapses are already formed, is still not fully clear. The present data so far reports multiple possible mechanisms for presynaptic terminal formation in mature neurons. Portions of presynaptic terminals can be mobilized and contribute to varicosity formation (Krueger, Kolar et al. 2003), and single molecules can be dynamically exchanged (Tsuriel, Geva et al. 2006). Which - if any - mechanism

predominates in mature neurons remains elusive to date. Also, if the findings in dissociated cultures hold true in functional neuronal networks or *in vivo* is still elusive.

2.2.5 Synapse formation and disassembly

Synapse formation includes pre- and postsynaptic synaptogenesis as well as the initialization and stabilization of an axodendritic contact. The initial contact formation and stabilization of glutamatergic synapses is mediated by the outgrowth of axonal or dendritic filipodia (Ahmari, Buchanan et al. 2000), of which a subset stabilizes and forms nascent synapses (Ziv and Smith 1996; Sabo, Gomes et al. 2006). Although current models of synapse formation imply that axodendritic contacts can be established anywhere along the axon, little experimental evidence supports this hypothesis, and contact and varicosity formation may alternatively occur at predefined sites along the axon. Two recent reports favor the latter hypothesis, with one of the reports documenting that predefined, distinct axonal sites enable the pausing of presynaptic STVs and stabilize dendritic filopodia (Sabo, Gomes et al. 2006) and the second study reporting that stable sites of postsynaptic scaffold protein accumulation correlate with the formation of presynaptic terminals (Gerrow, Romorini et al. 2006). Further data along these lines describe that preexisting axonal varicosities may be a specific target for spine outgrowth, as many young spines contact preexisting axonal varicosities that are already making synapses with other presynaptic partners. This finding supports the hypothesis that young spines can compete for already present presynaptic varicosities (Knott, Holtmaat et al. 2006; Toni, Teng et al. 2007).

Although synapse disassembly is a crucial process to refine neuronal circuitry (Shatz and Stryker 1978; Trachtenberg, Chen et al. 2002; Goda and Davis 2003; Nägerl, Eberhorn et al. 2004; Zhou, Homma et al. 2004), little conclusive knowledge about synapse disassembly exists. As mentioned before, LTD can trigger synapse disassembly (Bastrikova, Gardner et al. 2008), but the temporal and causal sequence of events remain elusive, especially for central nervous system (CNS) synapses. At the neuromuscular junction of the periphal nervous system, however, it has been shown that presynaptic receptor endocytosis precedes the loss of presynaptic terminals (Akaaboune, Culican et al. 1999). In the CNS, the importance of the complement

cascade in synapse disassembly has been recently described (Stevens, Allen et al. 2007), but still little is known about cellular and molecular aspects of synapse disassembly.

2.3 Objectives of this study

Structural plasticity is a hallmark of the brain, and it allows to modify neuronal circuits in development, learning and memory. Since the recent development of novel imaging techniques allows the observation of morphological changes in neuronal networks with high temporal and spatial resolution, numerous studies have investigated structural plasticity and its link to functional synaptic plasticity. To this end, the effect of the experimental learning paradigms LTD and LTP on structural plasticity has been intensely studied (Engert and Bonhoeffer 1999; Toni, Buchs et al. 1999; Nägerl, Eberhorn et al. 2004; Zhou, Homma et al. 2004; Knott, Holtmaat et al. 2006; Nägerl, Kostinger et al. 2007; Bastrikova, Gardner et al. 2008). Most reports so far have focused on postsynaptic spines, whereas the changes of presynaptic axonal varicosities has only recently been elucidated (De Paola, Arber et al. 2003; Konur and Yuste 2004; Umeda, Ebihara et al. 2005; De Paola, Holtmaat et al. 2006; Stettler, Yamahachi et al. 2006). Therefore, the cellular and structural mechanisms that underlie presynaptic structural plasticity remain mostly unknown to date. While the building blocks for nascent synapses are intensively studied in developing neurons (Ahmari, Buchanan et al. 2000; McAllister 2007), the assembly units of axonal varicosities in mature neurons remain mostly elusive. Moreover, while protein synthesis and degradation are required for the functional expression of LTP and LTD (Frey, M et al. 1988; Colledge, Snyder et al. 2003; Sajikumar and Frey 2003; Karpova, Mikhaylova et al. 2006), their role in presynaptic structural plasticity remains unknown to date.

To this end, I set out to investigate the presynaptic structural plasticity of axonal varicosities of CA3 hippocampal neurons. Using time lapse two-photon imaging together with electrophysiological recordings in organotypic slices, I studied the assembly units of axonal varicosities and their distinct types of presynaptic structural plasticity. Furthermore, I tested if the turnover of axonal varicosities requires the acute synthesis and degradation of proteins or if it can rely on preexisting proteins. Similarly, I tested the requirement of protein synthesis and degradation for LTD-induced structural plasticity. Finally, I observed the functional status of stable and dynamic presynaptic

axonal varicosities by testing for their colocalization with a presynaptic marker protein VGluT-1-Venus. By this means, my thesis aims for contributing to the current knowledge about structural and cellular mechanisms of presynaptic structural plasticity, and about the link between structural and functional presynaptic plasticity.

3 Material and Methods

3.1 Material

3.1.1 Equipment

Microscopy instruments	Supplier
Mira-Verdi Laser System	Coherent, Santa Clara, CA
Mai Tai Laser System	Coherent, Santa Clara, CA
Electro-optical modulator	Polytec, Waldbronn, Germany
Bandpass filter HQ525/50	LOT Oriel, Darmstadt, Germany
Bandpass filter HQ590/55	LOT Oriel, Darmstadt, Germany
Dichroic mirror HQ572LP	LOT Oriel, Darmstadt, Germany
Dichroic mirror CH-700DCXR2638	LOT Oriel, Darmstadt, Germany
Inverted microscope IX70	Olympus, Hamburg, Germany
Scanhaed Yanus II	TILL Photonics, Gräfelfing, Germany
Water-immersion objective, 40x, NA1.2	Zeiss, Oberkochen, Germany
Photomultiplier Tubes R6357	Hamamatsu, Herrsching, Germany

Electrophysiology instruments	Supplier
A/D converter PCI-6052	National Instruments, Austin, TX
Amplifier axopatch 200B	Axon Instruments, Foster City, CA
Stimulus Isolator A360	World Precision Instruments, Sarasota, FL
Glass capillaries, thin wall, outer diameter 1.5 mm	World Precision Instruments,Sarasiota, FL
Silver wire 0.5 mm	World Precision Instruments, Sarisota, FL

Software	Supplier
ImageJ	NIH, Bethesda, MD
Macbiophotonics ImageJ	Macbiophotonics, CA
Matlab R2007B	Math Works, Natick, MA
LabView 8.3	National Instruments, Austin, TX

Other equipment	Supplier
Picospritzer	Parker Hannifin Corp, Fairfield, NJ
Perfusion Pump, Minipulse peristaltic	Gilson, Middletown, WI

3.1.2 Chemicals

Artificial cerebrospinal fluid (ACSF)	Supplier
$CaCl_2$*$2H_2O$	Merck, Darmstadt, Germany
Glucose ($C_6H_{12}O_6$)	Merck, Darmstadt, Germany
HEPES	Sigma, Munich, Germany
Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carbon acid)	Sigma, Munich, Germany
KCl	Merck, Darmstadt, Germany
K-Gluconate ($C_6H_{11}KO_7$)	Sigma, Munich, Germany
Mg^{2+}-ATP	Sigma, Munich, Germany
$MgCl_2$	Merck, Darmstadt, Germany
NaCl	Merck, Darmstadt, Germany
$NaHCO_3$	Merck, Darmstadt, Germany
NaH_2CO_3	Merck, Darmstadt, Germany
Pyruvate ($C_3H_4O_3$)	Sigma, Munich, Germany

Culture media and other solutions	Supplier
BME (basal medium)	Sigma, Munich, Germany
Cytosine β-arabino-furanoside hydrochloride	Gibco, Karlsruhe, Germany
5-Fluorodeoxyuridine	Sigma, Munich, Germany
Hanks balanced salt solution (HBSS)	Gibco, Karlsruhe, Germany
Horse serum	Invitrogen, Karlsruhe, Germany
Kaliumdihydrophosphate (KH_2PO_4)	Merck, Darmstadt, Germany
Kynurenic acid	Invitrogen, Karlsruhe, Germany
L-Glutamine	Invitrogen, Karlsruhe, Germany
Magnesiumsulfate ($MgSO_4$)	Merck, Darmstadt, Germany
Sodiumhydroxide (NaOH)	Merck, Darmstadt, Germany
Uridine	Sigma, Munich, Germany

3.1.3 Media

Artificial cerebrospinal fluid (ACSF)

ACSF was prepared fresh at the day of experiment. It contained 126 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgCl_2$, 20 mM Glucose, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 1 mM Pyruvate and 1 mM Trolox. It was continuously perfused with Carbogen (95% O_2, 5% CO_2) to maintain pH 7.4.

Intracellular solution

Internal solution for patching single cells in VGluT-1 experiments contained 120 mM K-Gluconate, 10mM KCl, 20 mM HEPES, 5 mM NaCl, 12 mM Mg^{2+}-ATP, was adjusted to 300 mOsm and pH 7.2 by adding NaOH and sterile filtered.

Preparation medium

The medium for preparing Gähwiler cultures contained 2.5mM $CaCL_2$, 5.05 mM D-Glucose, 4.96 mM KCL, 0.22mM KH_2PO_4, 1 mM Kynurenic acid, 1.03 mM $MgCL_2$, 0.28 $MgSO_4$, 136.89 mM NaCl, 2,7mM $NahCO_3$, 0.87 mM NaH_2PO_4. It was adjusted to pH 7.2 and sterile filtered.

Gähwiler culture medium

The culture medium for maintaining Gähwiler cultures contained 50% (v/v) BME, 25% (v/v) horse serum, 25% (v/v) HBSS, 1 mM L-Glutamine, 5 mg/ml Glucose and was sterile filtered.

3.1.4 Fluorescent dyes

Dye	Concentration [mM]	Supplier
Alexa fluor 568 hydrazide	0.5	Invitrogen, Karlsruhe, Germany
Calcein red-orange AM ester	0.5	Invitrogen, Karlsruhe, Germany

3.2 Methods

3.2.1 Organotypic hippocampal slice cultures

Hippocampal slices were prepared from C57 BL/6J according to the Gähwiler method (Gahwiler, 1981). Alternatively, hippocampal slices were prepared from knock-in VGluT-1-Venus mice (Courtesy of Etienne Herzog, Max Planck Institute for Experimental Medicine, Göttingen, Germany) for VGluT-1-Venus experiments.

In brief, mice of both sexes were decapitated at postnatal day 5-7, the two hippocampi isolated and cut into transversal slices of 400 µm thickness. After cutting, the slices were kept at 4°C for 30 minutes to allow regeneration of the tissue. Then, single slices were embedded in a plasma clot on glass cover slips, and addition of thrombin induced coaglutination within the following 30 to 45 minutes. Slices were incubated in Gähwiler medium and stored in single tubes, using a roller incubator at a temperature of 35°C. Two days after preparation, mitosis inhibitor was added to the medium for 24 hours to stop further proliferation of glia cells within the slices. Gähwiler medium was partially renewed every three to four days for a total of 10 to 20 days of culturing period. Before the experiments, slice cultures were transferred into a recording chamber and continuously perfused with artificial cerebrospinal fluid (ACSF) at a perfusion rate of 1 ml/min. Temperature was maintained at 35°C, and the solution was continuously perfused with Carbogen (95% O_2, 5% CO_2) to maintain pH 7.4.

3.2.2 Two-photon microscopy

Time-lapse image stacks were acquired using a custom-built 2-photon laser scanning microscope (Figure 3-1). The excitation light from a Mira-Verdi laser system (Coherent, Santa Clara, CA) or a Mai Tai laser system (Newport Spectra Physics, Darmstadt Germany) was routed through a laser scanhead (Yanus II, TILL Photonics, Gräfelfing, Germany), a dichroic mirror (LOT Oriel, Darmstadt, Germany) and a 40x, 1.2 NA water-immersion objective (Zeiss, Oberkochen, Germany) mounted on an inverted IX70 microscope (Olympus, Hamburg, Germany).

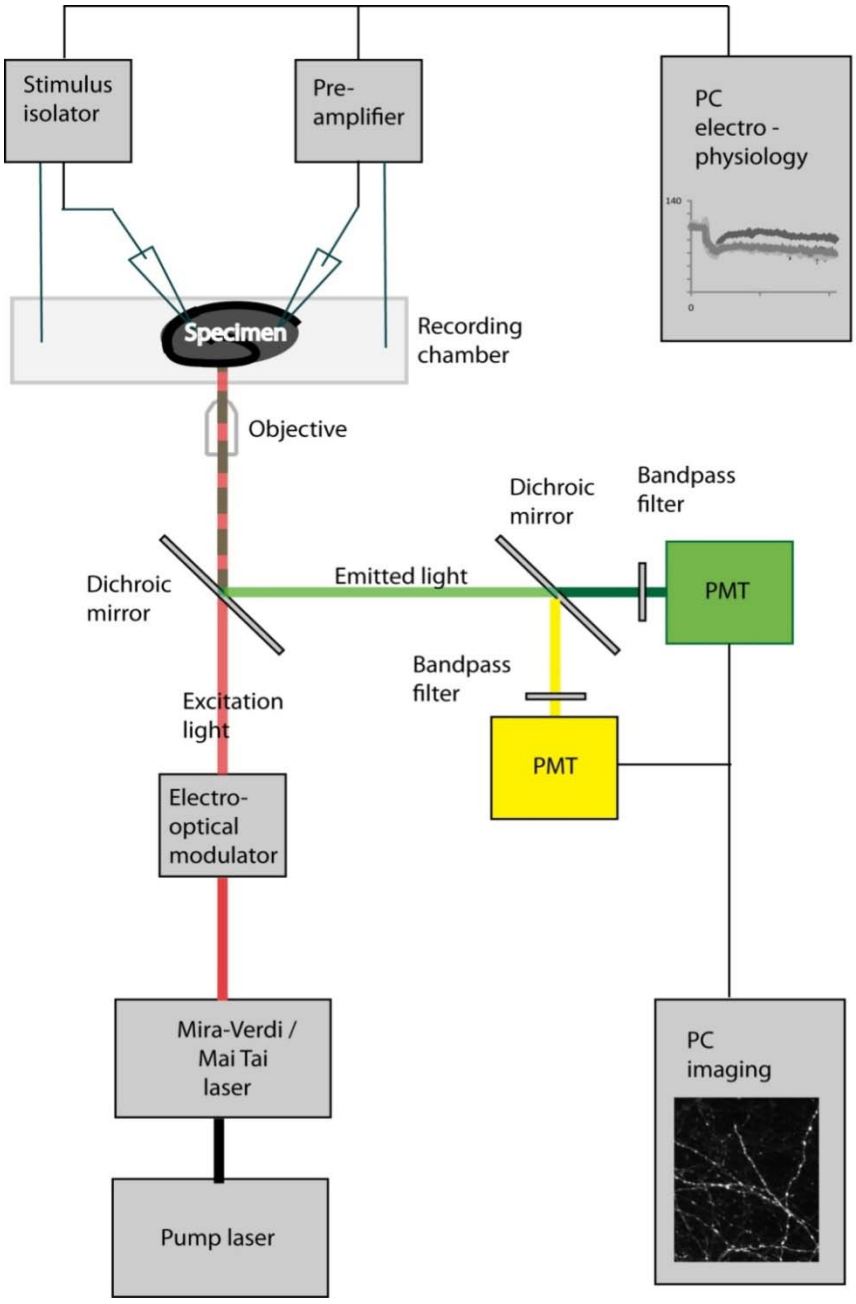

Figure 3-1: Custom-built two-photon setup

Schematic drawing of the two-photon microscope and electrical circuit. The two-photon microscopy enables the acquisition of images (PC imaging), and the electrical circuit enables the recording of field potentials (PC electrophysiology). Details are described in the text.

For morphology experiments in chapter 4.1, the Mira-Verdi laser system was tuned to a wavelength of λ=790 nm. For VGlut-1-Venus experiments in chapter 4.2, the Mai Tai laser was used at λ=910 nm. The power of the excitation light was adjusted before

every experiment by an electro-optical modulator (Polytec, Waldbronn, Germany) and kept constant during the experiments. The emitted fluorescence was split by a dichroic mirror (LOT Oriel) into red and green fluorescence, filtered by adequate bandpass filters (LOT Oriel), and detected by two external photomultiplier tubes (R6357, Hamamatsu, Herrsching, Germany). Image acquisition was performed by a custom-programmed software (LabVIEW 8.2, National Instruments, Austin, TX).

Labeling and imaging parameters

For imaging presynaptic morphology, CA3 pyramidal cells were loaded via extracellular bolus loading. Calcein red-orange AM (Invitrogen, Karlsruhe, Germany) was diluted in ACSF to 0.5 mM final concentration, loaded into a freshly prepared glass micropipette (Havard Apparatus, MA, USA), connected to a Picospritzer (Parker Hannifin Corporation, Fairfield, NJ, USA) and placed in the middle of the cell body layer of the CA3 area. The dye was injected into the tissue by applying gentle and brief pressure pulses (5 to 15 ms, 10 psi) every 15 seconds for 60 to 90 minutes, and dye injection was stopped when about 40 to 80 CA3 neurons were intensely labeled. The concentration of Calcein red-orange AM equilibrated in the distal parts of neuronal processes with a delay of 30 minutes after completion of the loading procedure. It then remained constant for the remaining experiment (4-5 hours).

For VGluT-1-Venus experiments, one or two single CA3 pyramidal neurons were intracellularly loaded for 2-3 min via a patch pipette containing 0.5 mM Alexa Fluor 568 dissolved in internal solution. The labeling of patch-loaded cell equilibrated with 10 minutes delay and then remained constant for the rest of the experiment (4-5 hours).

Image stacks were acquired with a dwell time of 5 µs / pixel without frame averaging, and a constant pixel resolution of 136.7 nM / pixel in all experiments. The imaged x-y dimensions varied between 140 and 210 µm. 3D stacks consisted of z layers with a constant step size of 0.5 µm and a variable numbers of z-planes (20-99). Image stacks were acquired every 30 min.

3.2.3 Electrophysiology

Field excitatory postsynaptic potentials (fEPSPs) were recorded from the cell body layer in the area of CA1 pyramidal neurons, close to or overlapping with the imaging area. CA3 pyramidal neurons were stimulated by 0.2 ms current pulses from a stimulus isolator using a glass micropipette filled with 3 M NaCl, immobilized at the tip by 0.5% agarose. Stimulus strength ranged from 12 to 50 µA and was adjusted to elicit fEPSPs at 60% of the maximal amplitude, ranging from 0.5 to 3 mV. fEPSPs were recorded by an ACSF-filled glass micropipette connected to an Axopatch 200B amplifier (Axon Instruments, Foster City, CA), an A/D converter (PCI-6025E, 16 and 12bit, National Instruments) and a custom-programmed acquisition software (LabVIEW 7.2). Test pulses for pre-baseline and baseline were acquired every 15 s (0.067 Hz). Before the start of an experiment, pre-baseline recordings were taken until fEPSPs had reached stable amplitudes for 30 minutes. Then, the recordings of the experiments started with test pulses that continued throughout the experiment. For LTD-experiments, the stimulation protocol was changed to low frequency stimulation (LFS) after 45 minutes of experiment. The LFS protocol consisted of 900 pulses delivered at 1 Hz and started after electrophysiological recordings of 45 min, when two image stacks were taken. After LFS, the stimulation continued with test pulses for the rest of the experiment.

3.2.4 Pharmacology

For pharmacological experiments, anisomycin (Ani) and lactacystin (lacta) were bath-applied for one or four hours. Anisomycin was dissolved at 7.5 mM in H_20, stored at 4°C and used within 3 weeks. Before the experiment, the solution was diluted into ACSF to a final concentration of 7.5 µM. Lactacystin was dissolved in DMSO / 20% pluronic acid, stored at -20°C and further diluted to 2 µM (0.04% DMSO) in ACSF before the experiments. The perfusion ACSF was replaced with ACSF containing the pharmacological reagents for the duration of application as indicated in the individual experiments. All bath solutions were used once and were not recycled during or after perfusion.

3.2.5 Simultaneous two-photon imaging, electrophysiological recordings and pharmacological blockade

Hippocampal Gähwiler cultures were transferred to an imaging chamber and experiments commenced with normal ACSF containing no pharmaka. A population of CA3 neurons was labeled by extracellular bolus loading as described in 3.2.2. Subsequently, the loading pipette was exchanged with the stimulation electrode. Under two-photon visual guidance, the stimulation electrode was placed in the middle of the labeled CA3 cell population to achieve maximal overlap of labeled and stimulated cells. Also under visual guidance, a CA1 area suitable for imaging and electrophysiological recordings was identified, a recording electrode was placed and electrophysiological recordings performed as described in 3.2.3. After fEPSPs of stable amplitudes were obtained for 30 minutes, stimulus strength was adjusted to 60% and the experiment was started (Figure 3-2).

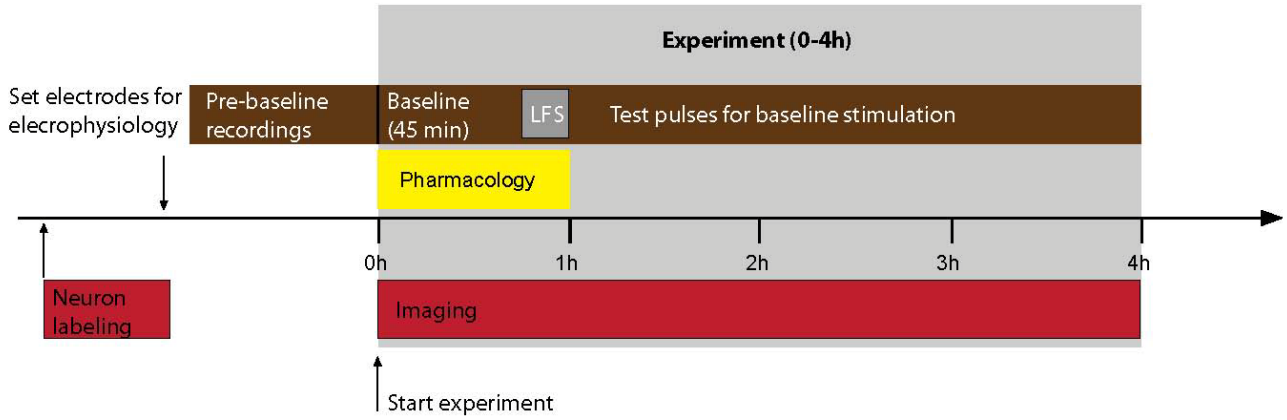

Figure 3-2: Experiment outline

Temporal sequence of imaging, electrophysiology and application of pharmacological inhibitors. Details are described in the text.

At the beginning of the experiment, pharmacological application, baseline recordings and imaging started simultaneously. Therefore, the perfusing solutions were exchanged (if applicable) and the experimental electrophysiological recordings were started together with the first 3D two-photon imaging stack. In case of LTD-inducing experiments, LFS was applied 45 minutes after beginning of the experiments and when

the second imaging stack was completed. After 1 hour, when LFS stimulation was completed, test pulses were resumed and drug-containing ACSF was switched back to normal ACSF. Test pulses and image acquisition every 30 minutes was continued throughout the experiment.

3.2.6 Blind electrophysiological experiments

Blind electrophysiological experiments were performed similar to the experiments described in 3.2.5. Hippocampal Gähwiler cultures were placed into an imaging chamber and were perfused with ACSF containing no pharmacological reagents. Anisomycin, lactacystin or no reagent was added into a separate ACSF by a third person in the absence of the researcher. Stimulation and recording electrodes were placed and test-pulses were applied every 15 seconds to record a pre-baseline. When the elicited fEPSPs maintained stable amplitudes for 30 minutes, the ACSF solutions were exchanged and experimental recordings commenced. 45 minutes later, low-frequency stimulation was applied to induce LTD, and 15 minutes later (after 60 minutes total experiment time), the ACSF solution was changed back to ACSF containing no pharmacological reagents.

3.2.7 VGluT-1-Venus experiments

For VGluT-1-Venus experiments, VGluT-1-Venus hippocampal Gähwiler cultures were transferred to an imaging chamber and perfused with ACSF at 35°C. One or two cells were patch-filled with Alexa 568 hydrazide as described in 3.2.1. Subsequently, the loading pipette was exchanged with the stimulation electrode, a recoding electrode was placed in CA1 and extracellular stimulation with test-pulses every 15 seconds were performed as described. Under visual guidance, an area in CA1 containing labeled axons was selected for two-photon imaging and immediately thereafter, the first 3D two-photon imaging stack was acquired. In total, nine imaging stacks were taken over four hours in 30 minutes intervals. In some cases, LFS was applied 45 minutes after beginning of the experiment.

3.2.8 Analysis and Statistics

Image analysis

3D (x, y, z) image stacks were processed and analyzed using ImageJ (NIH, Bethesda, MD). To reduce noise, images were filtered with a Gaussian blur or a Median algorithm in ImageJ. Within each experiment, three to ten axons were analyzed over a length of 60 - 160 µm. Within the whole image stacks, axons that were located within 5 - 20 z layers (2.5-10 µm) were selected. The corresponding z-layers containing the axon were projected as maximum intensity projection. For each time point, the projected axons were straightened using the straightener tool of ImageJ, and all time points were combined into a kymograph, with the axons stretching horizontally and the time coded vertically. On the basis of this kymographs, varicosity turnover was analyzed blindly in respect to the experimental condition and the direction of time. Varicosity turnover was assessed by eye, and results of the researcher were in some cases blindly double checked by a different person.

Analysis of electrophysiological recordings

Single fEPSPs recorded with the Labview software were analyzed offline with a custom-made analysis software (Labview 7.2). Recordings were filtered with a 400 hz longpass filter and the maximal amplitudes of the fEPSPs were measured. Temporal filtering was performed by averaging four fEPSPs to a single average of 1/minute. The baseline 100% value was calculated according to the amplitudes of the first 30 minutes of fEPSPs within the experiments. The experiments were only subjected to analysis when they displayed stable electrical properties during the four hour recording period. For experiments on baseline varicosity turnover, the fEPSP amplitudes were not allowed to decline to less than 80% of the average amplitudes of the baseline. For low-frequency stimulation, experiments that failed to display the functional properties of LTD or the properties obtained in the series of blind experiments, were also discarded.

Statistics

Data are reported as means ± SEM (standard error of the mean) unless stated otherwise. The statistical significance of the electrophysiological effects was calculated over the whole length of the experiments. One or two-tailed, unpaired t-tests were performed on groups of 15 minutes of the temporally filtered data (1 / min). For imaging data, varicosity turnover was assessed per axon, and turnover rate was normalized to four hours and 100 µm. Pharmacological conditions were assessed by comparing the groups of axons with one- or two-tailed, unpaired t-tests (Excel software) or a single-factor ANOVA (Matlab software). The following significance levels were applied: Error probability $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

4 Results

In this thesis, I investigated the structural and cellular mechanisms of baseline and activity-dependent presynaptic structural plasticity and its potential for altering synaptic transmission. In chapter 4.1, I examined effects of acute protein synthesis and degradation blockade on baseline and LTD-induced structural dynamics of presynaptic varicosities. In chapter 4.2, I investigated the functional status of presynaptic varicosities using the synaptic marker protein VGluT-1-Venus.

4.1 Protein synthesis and degradation regulate activity-dependent presynaptic structural plasticity

To date, little is known about the cellular mechanisms that govern the structural plasticity of presynaptic axonal varicosities. Therefore, I set out to investigate structural and cellular mechanisms of activity-dependent presynaptic morphological plasticity in CA3 hippocampal neurons. Using time lapse two-photon imaging together with electrophysiological recordings in organotypic slices, I examined the morphological characteristics of presynaptic varicosity assembly and disassembly. Moreover, I tested the effects of pharmacological blockade of protein synthesis and protein degradation on the plasticity of axonal varicosities under constitutive conditions and during LTD.

4.1.1 Monitoring structural dynamics of axonal varicosities together with functional properties of CA3-CA1 synapses

I combined two-photon time-lapse microscopy and electrophysiological extracellular field recordings within the same experiments to simultaneously visualize morphological varicosity turnover and functional properties at CA3-CA1 synapses (Figure 4-1). To observe the turnover of axonal varicosities at CA3 axons (Schaffer collaterals), a population of CA3 cells in organotypic Gähwiler cultures was morphologically labeled using a cell-permeable AM-ester (Calcein red-orange AM) that was applied by pressure injection into the CA3 area.

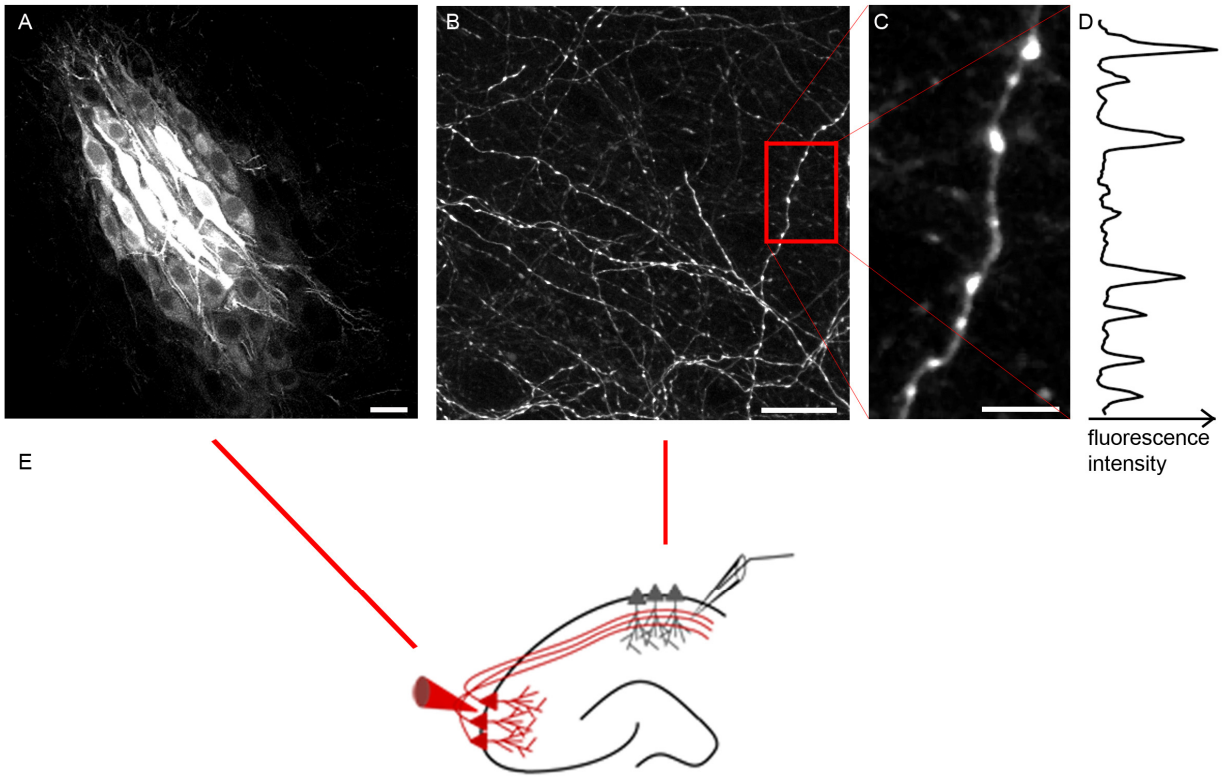

Figure 4-1: Visualizing Schaffer collateral axons and axonal varicosities in hippocampal Gähwiler slices by two-photon imaging

A population of CA3 cells was morphologically labeled by pressure-pulse injection of a cell-permeable morphological tracer (Calcein red-orange AM). Axons and axonal varicosities were readily discernable in CA1. (A) Labeled CA3 population. (B) Axons of cells in (A) in the CA1 area, potentially contacting unlabeled postsynaptic neurons and forming axonal varicosities. (C) Close up of axonal varicosities. (D) Intensity plot marking axonal varicosities. (E) Schematic overview. Scale bars: (A) 20 µm. (B) 10 µm. (C) 5 µm.

The labeled cell population of neighboring CA3 neurons typically consisted of 40 to 80 cells per experiment (Figure 4-1 A). CA3 axons were readily discernable in CA1 and formed axonal varicosities, presumably innervating unlabeled postsynaptic target CA1 cells (B, C). Single axons were selected for image analysis (see methods), and all individual time points were assembled side by side in a kymograph where the structural changes of single varicosities were easy to follow over time (Figure 4-2 D).

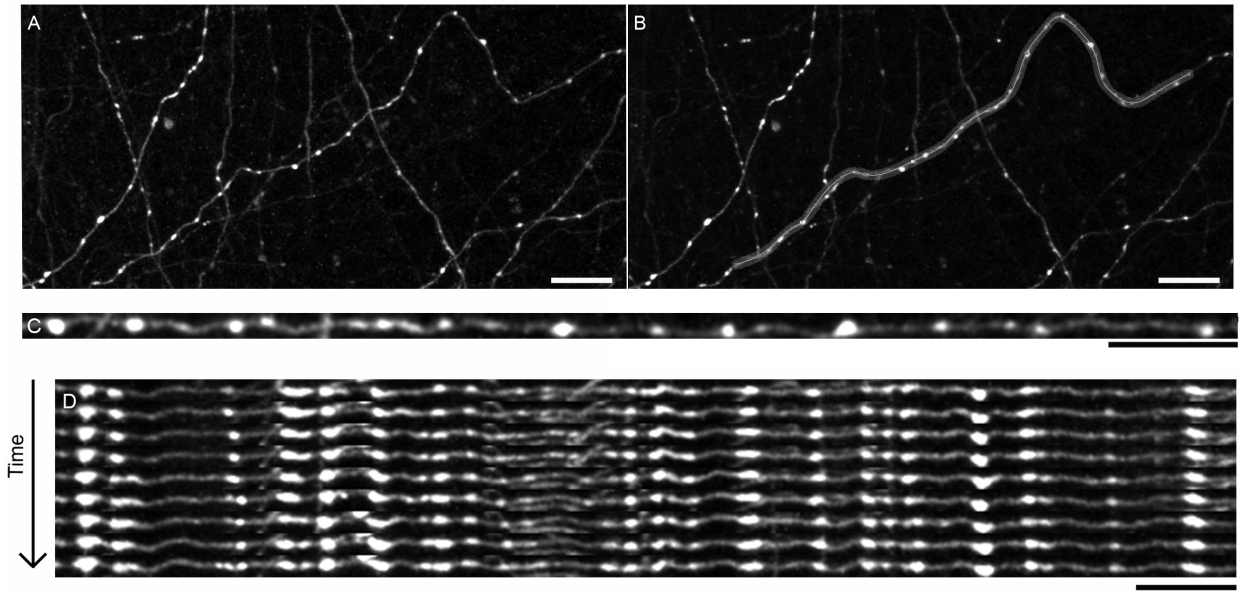

Figure 4-2: Analysis of varicosity turnover

To analyze varicosity turnover, single axons were selected, spatially isolated, projected in two dimensions and straightened. All time points were aligned into a kymograph. (A) Single axon. (B) Overlay of selected axons and straightening curve (C) Axon in (B), straightened. (D) Example kymograph. Scale bars: (A) 10 µm. (B) 10 µm. (C) 5 µm. (D) 5 µm.

To prevent the analysis from a possible bias of the researcher, all further analyses were performed blindly regarding the time and the experimental conditions. In the kymographs, axonal varicosities as well as other axonal volumes were readily visible along the axons and were selected by size and stability criterions. Axonal varicosities that were gained or lost during the experiment are referred to as 'plastic' or 'dynamic' axonal varicosities. All axonal varicosities had to exceed a minimum size of 0.376 μm^2, and their two dimensional projection characteristically included an area of 0.94 ± 0.03 μm^2, while their fluorescence intensity typically exceeded the axon intensity 7.79 ± 0.3 times the standard deviation of axon intensity (n = 10 axons, 164 varicosities). Axonal varicosities had to be detectable for at least two consecutive time points (30 min), either at the beginning or at the end of the experiment. The latter criterion was chosen according to the recent finding of Becker et al. that new varicosities exhibit action-potential dependent voltage-gated Ca^{2+} entry into varicosities after approximately 30 minutes, indicating their potential for neurotransmitter release (Becker, Wierenga et al. 2008). Moreover, axonal varicosities had to remain at the same location along the axon

(± 0.04 µm / 4 hours). Structures that fulfilled the same size criteria as axonal varicosities but were not stably localized were classified as axonal volumes. Axonal volumes displayed an average relocalization of 1.9 ± 0.08 µm per experiment, and a average maximum velocity of 1.18 ± 0.07 µm between single time points. To ensure the correct identity of the axonal volumes, all structures that relocated more than 4 µm within the experiment were not subjected to analyses.

To simultaneously assess the synaptic transmission of the labeled synapse population, I performed extracellular field stimulation of the labeled CA3 cell population. A stimulation electrode was placed into the center of the dye-loaded CA3 neurons, and cells were stimulated with test pulses (see methods for details). To ensure a highest possible overlap of labeled and stimulated cells, placing of the stimulation electrode was performed under two-photon guidance and targeted the center of the labeled CA3 population. The electrical stimulation of presynaptic cells evoked postsynaptic field potentials in CA1 (fEPSPs, Figure 4-3). Field potentials displayed typical amplitudes of 0.5 - 2 mV and remained stable, yet displayed a progressive decline during the experiment which is consistently reported in electrophysiological field recordings (Frey, M et al. 1988; Sajikumar and Frey 2003; Fonseca, Nägerl et al. 2004; Abraham, Mason-Parker et al. 2006; Fonseca, Nägerl et al. 2006; Fonseca, Vabulas et al. 2006).

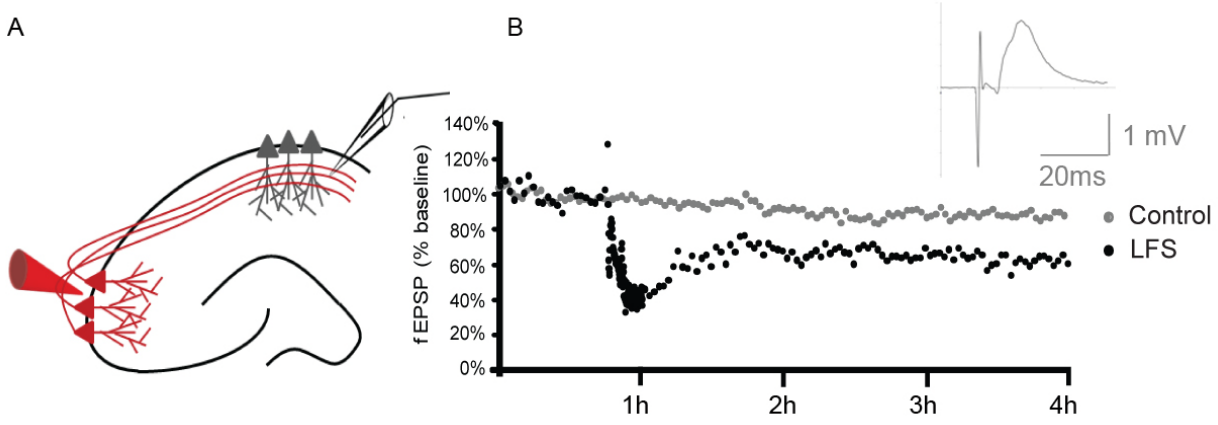

Figure 4-3: Electrophysiological recordings in the CA1 area of hippocampal Gähwiler cultures

The labeled CA3 population was stimulated extracellularly, and resulting postsynaptic responses (fEPSPs) of CA1 cells were measured. fEPSPs document stable recordings. (A) Overview. (B) Example recordings of a control stimulation (grey) and LTD stimulation (black). Inset: Example fEPSP.

4.1.2 Distinct types of structural dynamics

To characterize the remodeling of axonal membranes in morphological plasticity, I surveyed the characteristics of assembly and disassembly of axonal varicosities under baseline conditions. Therefore, I labeled hippocampal CA3 neurons in organotypic slice cultures and simultaneously recorded postsynaptic field potentials (fEPSPs) within the CA1 area as described before. I analyzed the time course of the positions and sizes of discernible axonal varicosities and axonal volumes on individual axons. Consistent with previous reports, axonal varicosities appeared and disappeared spontaneously at roughly similar rates, their net number remaining largely constant (Deng and Dunaevsky 2005; Becker, Wierenga et al. 2008). I could observe distinct types of structural changes which frequently led to the gain or the loss of axonal varicosities (Figure 4-4).

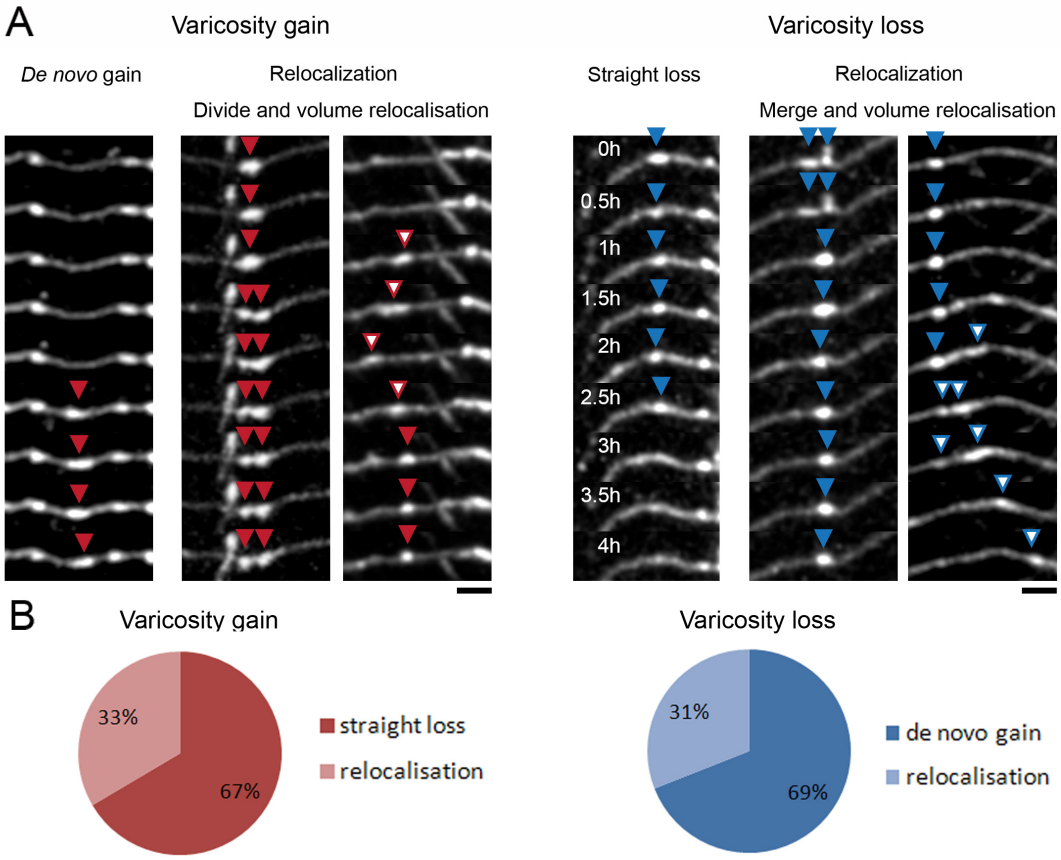

Figure 4-4: Distinct types of structural dynamics contributed to varicosity turnover

Varicosities emerged by de novo formation, by dividing or by relocalization of axonal volumes. Likewise, they disappeared by straight loss, by merging or by relocalization of axonal volumes. (A) Time-lapse examples. (B) Relative fractions. Scale bars: 2µm.

New varicosities emerged by *de novo* formation, reflecting the straight appearance of a bulbus-shaped structure on a dim axonal site, either rapidly reaching a stable size or continuously growing over hours (Figure 4-4). Furthermore, axonal varicosities also emerged by relocalization processes, which either appeared as splitting of an existing varicosity into two or by stabilization of motile axonal volumes into a stable axonal varicosity. Likewise, I detected varicosities that disappeared by straight loss of the structure or by relocalization processes, that reflected the merging of existing varicosities or the dispersion of existing varicosities into smaller volumes. In addition, small or transient changes in the size and position of axonal volumes were also visible that did not affect the number of axonal varicosities and were therefore excluded from analysis.

To assess how important the various types of structural rearrangements are for the loss or gain of axonal varicosities, I determined how often each type could be observed. *De novo* formation was frequently responsible for the appearance of a varicosity (69%), while relocalizations were responsible for the formation of the remaining 31% of new varicosities. Likewise, more axonal varicosities disappeared by straight loss (67%) than by relocalizations (33%)(Table 1).

Varicosity turnover	total varicosity loss	total varicosity gain	loss by relocalization	gain by relocalization	straight loss	*de novo* gain	n (axons)
Relative contribution	100%	100%	33%	31%	67%	69%	32
varicosities / 100 µm	1.01	1.52	0.34	0.47	0.67	1.05	32

Table 1: Relative fractions of the different types of varicosity turnover

In summary, I here report that the turnover of presynaptic varicosities in mature hippocampal networks was mediated both by relocalization of axonal volumes and by *de novo* formation and straight loss. The two processes provided distinct mechanisms that both contributed to the turnover of axonal varicosities. Relocalizations were suggestive for the use of preexisting material for the assembly of axonal varicosities, and the recycling of their constituents upon their disassembly. It was therefore of interest to test if varicosity turnover can rely on use and recycling of preexisting material, or alternatively, if it requires the acute synthesis or degradation of proteins.

4.1.3 Effect of protein synthesis and degradation on baseline structural plasticity of axonal varicosities

To test whether the synthesis of new proteins is required for the structural plasticity of axonal varicosities under baseline conditions, I carried out time lapse experiments in the presence of the drug anisomycin, which specifically inhibits the translation of mRNA into proteins. This allowed me to investigate whether the structural turnover of axonal varicosities can occur in the absence of protein synthesis. To this end, I bath applied anisomycin for one hour at the beginning of the experiment and analyzed how the loss and gain of varicosities was affected. At the same time I carried out field potential recordings to monitor the strength of synaptic transmission between CA3 and CA1 pyramidal neurons. The latter assured that synaptic transmission remained unaltered during the experiment and documented the healthy condition of the investigated neuronal population.

Interestingly, the structural dynamics were unaffected when protein synthesis was blocked for one hour, irrespective of whether the time window I analyzed was restricted to the period of pharmacological blockade or was extended by a few hours (Figure 4-5, Table 2).

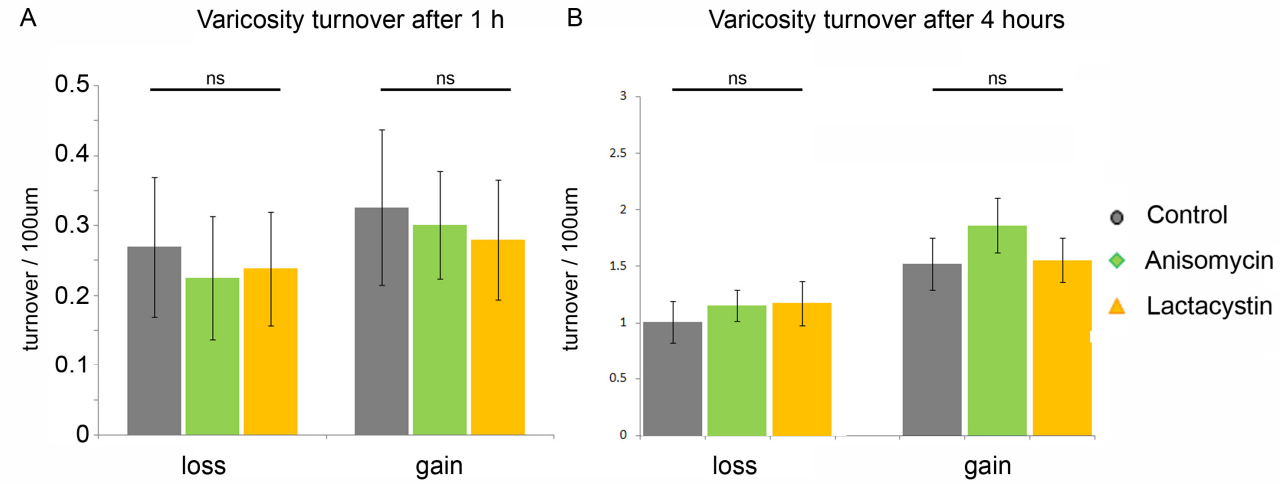

Figure 4-5: Varicosity turnover was unchanged by pharmacological blockades after one or four hours

The application of anisomycin or lactacystin for one hour did not alter presynaptic turnover, irrespective when the turnover of axonal varicosities was assessed. (A) Varicosity turnover after 1 hour. (B) Varicosity turnover after 4 hours. ns: not significant.

Table 2: Turnover of axonal varicosities were unchanged after one and four hours

Varicosity turnover after 1 hour	total varicosity loss	total varicosity gain	standard error loss	standard error gain	n (axons)	significance test	significance loss (p value)	significance gain (p value)
control	0.27	0.33	0.09	0.10	32			
Anisomycin	0.22	0.30	0.08	0.11	33	control / ani	0.86	0.70
Lactacystin	0.24	0.28	0.09	0.08	31	control / lacta	0.72	0.80
Varicosity turnover after 4 hours	total varicosity loss	total varicosity gain	standard error loss	standard error gain	n (axons)	significance test	significance loss (p value)	significance gain (p value)
control	1.01	1.52	0.18	0.23	32			
Anisomycin	1.15	1.86	0.14	0.24	33	control / ani	0.53	0.31
Lactacystin	1.17	1.55	0.17	0.33	31	control / lacta	0.54	0.92

New varicosities formed and existing ones disappeared at the same rates as under control, drug-free conditions indicating that new varicosities can emerge during acute blocade of protein synthesis. Moreover, the relative contributions by the distinct types of structural plasticity also did not change significantly, indicating that both processes, relocalization and *de novo* gain, remain unimpaired. (Figure 4-6 and Table 3). Likewise, baseline synaptic responses were unaltered under these conditions, which is consistent with previous reports about the effects of protein synthesis blockade on synaptic transmission (Figure 4-6. fEPSP amplitudes after two hours as % of baseline: 92.77 ± 1.86% for control, n=4, 102.49 ± 5.2 % for anisomycin, n=4. P (control/anisomycin): p = 0.17) (Frey, M et al. 1988; Sajikumar and Frey 2003; Fonseca, Vabulas et al. 2006).

Table 3: Baseline structural dynamics were independent of protein synthesis and degradation

Varicosity turnover / 100 µm	total varicosity loss	total varicosity gain	loss by relocalization	gain by relocalization	straight loss	*de novo* gain	n (axons)
control	0.34	0.47	1.01	1.52	0.67	1.05	32
Anisomycin	0.33	0.41	1.15	1.86	0.82	1.45	33
Lactacystin	0.34	0.46	1.17	1.55	0.84	1.09	31
standard errors							
Control	0.09	0.12	0.18	0.23	0.19	0.24	
Anisomycin control	0.11	0.09	0.14	0.24	0.14	0.20	
Lactacystin control	0.11	0.13	0.17	0.33	0.16	0.28	
Significances (p values)	total varicosity loss	total varicosity gain	loss by relocalization	gain by relocalization	straight loss	*de novo* gain	
Control / Anisomycin	0.53	0.31	0.95	0.70	0.52	0.21	
Control / Lactacystin	0.54	0.92	0.99	0.95	0.55	0.90	

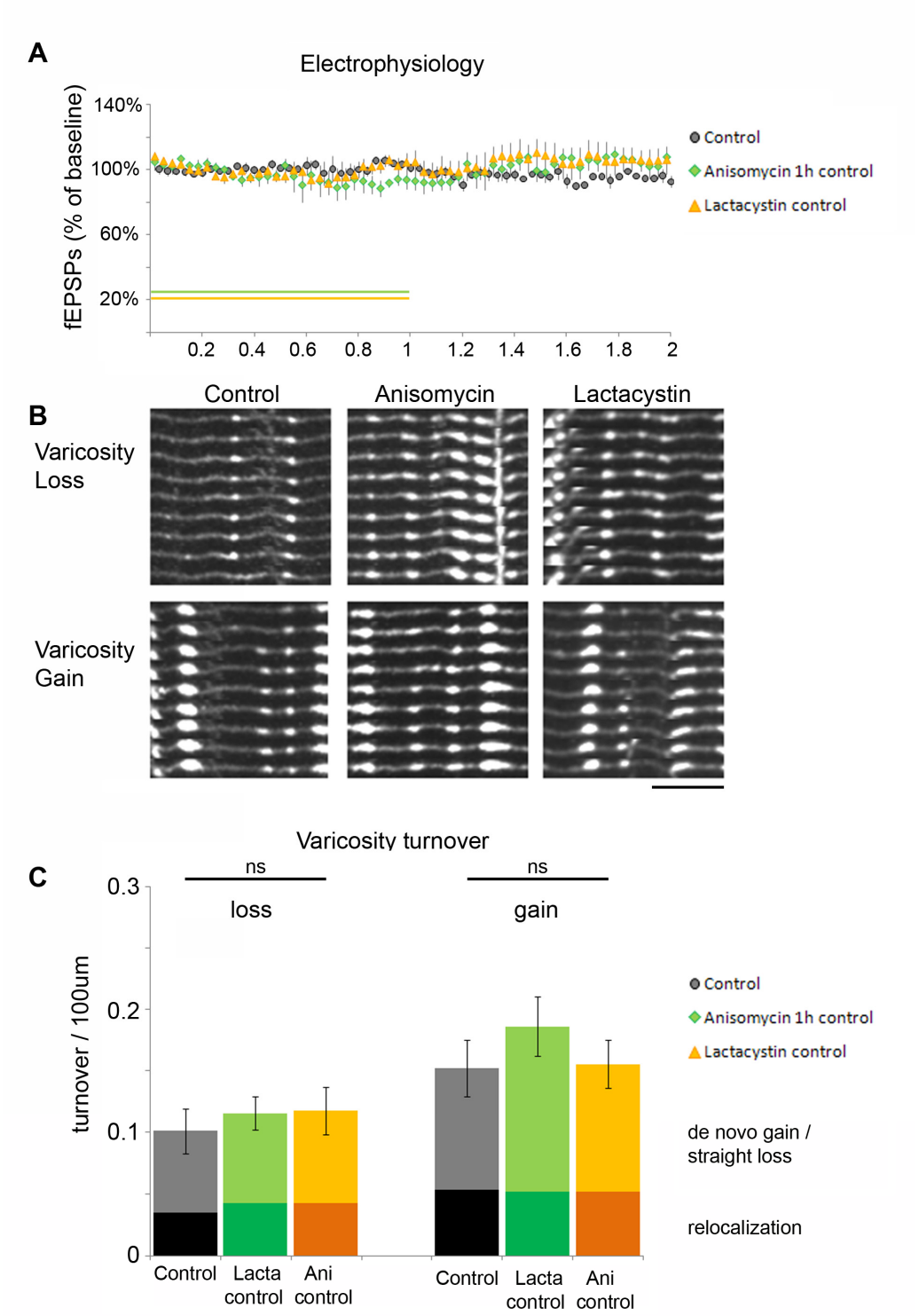

Figure 4-6: Baseline structural dynamics were independent of protein synthesis and degradation

Application of anisomycin (ani) or lactacystin (lacta) for one hour did not alter synaptic transmission, and turnover of axonal varicosities remained unchanged. (A) Electrophysiological recordings. Application of pharmacological inhibitors is depicted by the horizontal bars. (B) Illustrative examples of varicosity turnover. (C) Loss and gain of presynaptic varicosities. Dark colored fraction within each bar represents the relocalization-mediated turnover. ns: not significant. Scale bar: 5 µm.

To test the role of protein degradation via the ubiquitin-proteasome system for the structural plasticity of axonal varicosities, I repeated the experiments in the presence of lactacystin, a specific and irreversible blocker of the proteasome. I counted the numbers of varicosities gained and lost and quantified the contributions by the different structural mechanisms. As for anisomycin, I did not detect any appreciable changes in the structural dynamics of axonal varicosities, neither with regards to the numbers of gained or lost varicosities, nor with regards to the types of structural plasticity (Figure 4-6 and Table 3). Moreover, I did not observe any changes in baseline synaptic responses under conditions of blockade of protein degradation (Figure 4-6. fEPSP amplitudes after two hours as % of baseline: 92.77 ± 1.86% for control conditions, n=4, 102.5 ± 8.5 % for lacta, n=6. P (control/lacta): $p = 0.23$) which is also consistent with previous reports (Colledge, Snyder et al. 2003; Sajikumar and Frey 2003; Fonseca, Vabulas et al. 2006).

Taken together, these data demonstrate that synaptic structures can be built without the need for new proteins and suggest the use of preexisting proteins for the formation of new varicosities. Conversely, the results show that axonal varicosities can be disassembled in the absence of protein degradation, which indicates that the varicosity constituents remain within the axon. Moreover, the fraction of the distinct mechanisms that contribute to varicosity turnover remained by large unaltered, indicating that all types of morphological plasticity are independent of protein synthesis and protein degradation under baseline conditions.

4.1.4 LTD-induced presynaptic structural plasticity

A recent study in this laboratory has demonstrated that the structural dynamics of axonal varicosities is upregulated by the induction of LTD in CA3 - CA1 synapses in organotypic hippocampal slice cultures (Becker, Wierenga et al. 2008). I wanted to confirm these data and to determine by which structural mechanisms the elevation of varicosity turnover is achieved. To this end, I stimulated CA3 pyramidal neurons by extracellular stimulation using a low-frequency stimulation protocol (LFS: 900 pulses at 1 Hz). As expected, LFS induced robust LTD, which I monitored in parallel with the time lapse experiments (Figure 4-7. fEPSPs after two hours as % of baseline: LTD: 68.2 ± 4.6. P (LTD/baseline): $p < 0.001$, $n = 4$ control; p (LTD/control): $p < 0.01$, $n = 4$).

Structural dynamics were analyzed by counting the numbers of gained and lost axonal varicosities before and after LTD induction. LTD induction significantly enhanced the rate at which varicosities were gained and lost over a four hour period (Figure 4-7 and Table 4), which is consistent with the previous study from Becker et al. (Becker, Wierenga et al. 2008).

In addition, I determined the relative contributions by the distinct types of structural plasticity to the varicosity turnover. Notably, whereas LTD induction significantly increased the turnover of axonal varicosities by *de novo* gain and straight loss, the varicosity turnover by volume relocalization did not increase significantly after the induction of LTD (Table 4). Accordingly, the fraction of varicosities that were assembled by volume relocalization declined from 30% to 26%, while the fraction of varicosities that disappeared as a result of volume relocalization dropped from 33% to 22%.

Table 4: LTD enhanced the turnover of axonal varicosities

Varicosity turnover / 100 µm	total varicosity loss	total varicosity gain	loss by relocalization	gain by relocalization	straight loss	*de novo* gain	n (axons)
Control	1.01	1.52	0.34	0.47	0.67	1.05	32
LFS	2.30	2.51	0.50	0.66	1.80	1.85	35
standard errors							
Control	0.18	0.23	0.09	0.12	0.19	0.24	
LFS	0.26	0.29	0.13	0.15	0.32	0.34	
Significances	total varicosity loss	total varicosity gain	loss by relocalization	gain by relocalization	straight loss	*de novo* gain	
Control / LFS	< 0.001	0.005	0.16	0.17	0.002	0.003	

Taken together, the data confirm that LTD enhances both gain and loss of axonal varicosities. They indicate that the LTD-induced increase in turnover can mostly be accounted for by *de novo* gain and straight loss of axonal varicosities. Moreover, it suggests that relocalization processes are differently regulated and not affected by synaptic plasticity.

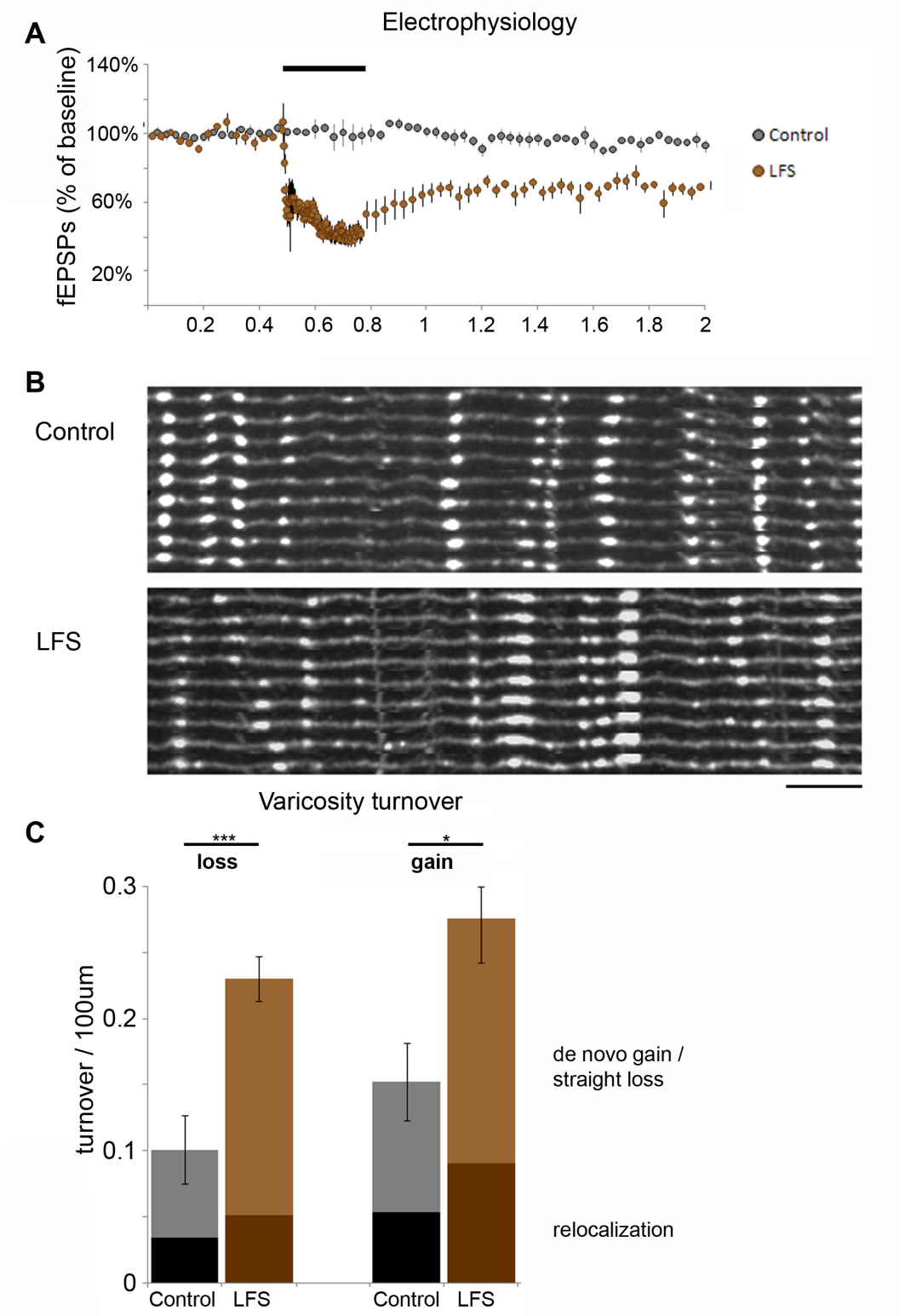

Figure 4-7: LTD induction enhanced plasticity of presynaptic axonal varicosities

Low frequency stimulation resulted in robust LTD and elevated both gain and loss of presynaptic axonal varicosities. (A) Electrophysiological recordings. LTD induction is indicated by the black bar. (B) Illustrative examples of varicosity turnover. (C) Loss and gain of presynaptic varicosities. Dark colors within each bar represent the relocalization mediated turnover. Scale bar: 5 µm.

4.1.5 Effect of protein synthesis and degradation on activity-dependent turnover of axonal varicosities

Blocking protein synthesis and degradation has been reported to impair functional synaptic plasticity, but it remained unknown if protein synthesis and degradation also influence LTD-associated structural plasticity. To examine this question, I started out by verifying the functional effects of protein synthesis or blockade inhibition under blind experimental conditions, and then proceeded to test the effect of protein synthesis and degradation blockade in further experiments.

Effects of blocking protein synthesis and protein degradation on the functional expression of LTD

It has been well established that blocking protein synthesis and degradation at the time of plasticity induction blocks expression of the late phase of LTP, and that protein synthesis blockade impairs the expression of LTD (Frey, M et al. 1988; Sajikumar and Frey 2003; Fonseca, Vabulas et al. 2006; Karpova, Mikhaylova et al. 2006). Intriguingly only one study thus far has evidenced that blocking protein degradation impairs LTD (Colledge, Snyder et al. 2003). To verify the reported effects, I performed a set of blind experiments under my experimental conditions. I stimulated CA3 pyramidal neurons by LFS and applied either anisomycin or lactacystin for one hour during LFS at the beginning of the experiment. The results confirm that application of anisomycin blocked the expression of LTD, as consistent with the literature. In contrast, application of lactacystin did not alter the level or the time course of LTD expression as compared to LTD without drug application (Figure 4-8).

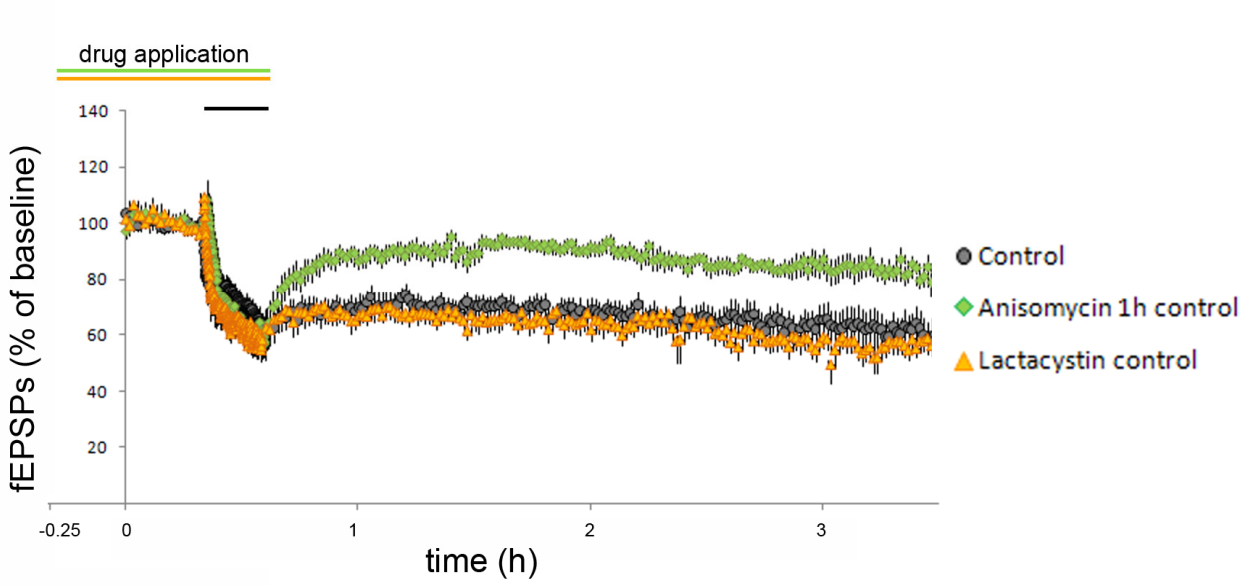

Figure 4-8: Functional expression of LTD was blocked by anisomycin, but not by lactacystin

Application of anisomycin for one hour blocked the expression of LTD, while application of lactacystin did not. In the graph, amplitudes of fEPSPs are plotted over time. LTD induction is indicated by the black bar, the duration of drug application is indicated by the yellow and green bars.

Effects of blocking protein synthesis and protein degradation on the LTD-dependent structural plasticity

To test whether proteins must be produced for the expression of LTD-induced structural plasticity of axonal varicosities, I examined the effects of blocking protein synthesis on LTD-induced structural plasticity. Therefore, I repeated the LTD experiments described in 4.1.4 in the presence of anisomycin and analyzed the structural dynamics by counting the numbers of gained and lost axonal varicosities before and after LTD induction. My electrophysiological recordings confirmed that anisomycin blocked the expression of LTD in the analyzed experiments. In contrast to the case of baseline turnover of axonal varicosities, where anisomycin affected neither their gain nor their loss, I observed that anisomycin substantially impacted the LTD-induced increase in turnover of axonal varicosities (Figure 4-9 and Table 5).

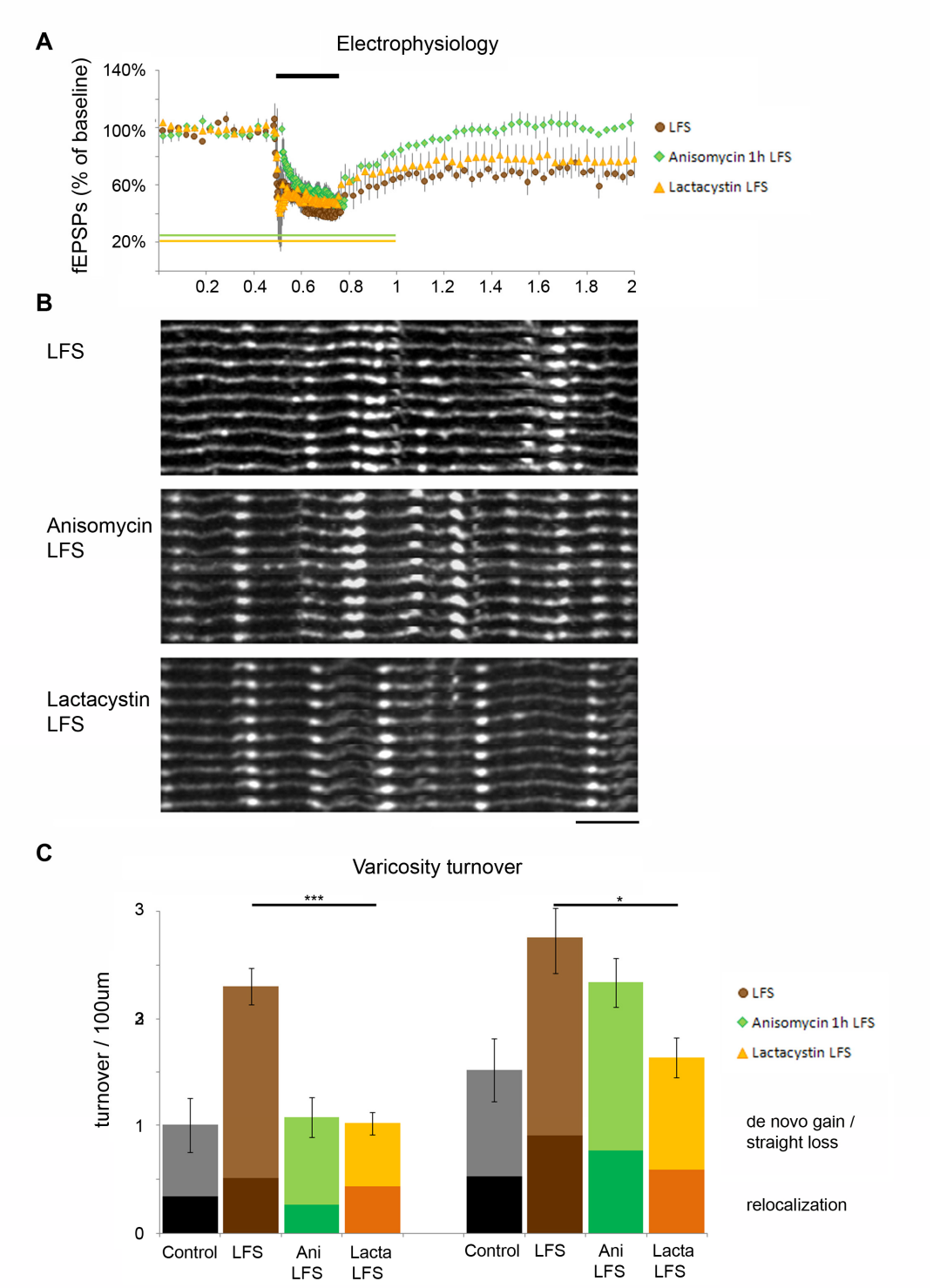

Figure 4-9: LTD-induced presynaptic structural plasticity depended on protein synthesis and degradation

The application of anisomycin or lactacystin blocked the LTD-specific varicosity turnover. (A) Electrophysiological recordings. Application of pharmacological inhibitors are depicted as colored bars, LTD induction as black bar. (B) Examples of varicosity turnover. (C) Loss and gain of varicosities. Dark colors within each bar represent the relocalization mediated turnover. Scale bar: 5 µm.

Table 5 Effects of protein synthesis and degradation blockade on LTD-induced presynaptic structural plasticity

Varicosity turnover / 100 µm	total varicosity loss	total varicosity gain	loss by relocalization	gain by relocalization	straight loss	*de novo* gain	n (axons)
Control	1.01	1.52	0.34	0.47	0.67	1.05	32
LFS	2.30	2.51	0.50	0.66	1.80	1.85	35
LFS + Anisomycin 1h	1.08	2.17	0.26	0.55	0.82	1.61	30
LFS + Anisomycin 4h	1.03	2.06	0.28	0.56	0.75	1.51	31
LFS + Lactacystin	1.02	1.66	0.38	0.51	0.64	1.16	41
standard error							
Control	0.18	0.23	0.09	0.12	0.19	0.24	
LFS	0.26	0.29	0.13	0.15	0.32	0.34	
LFS + Anisomycin 1h	0.17	0.33	0.11	0.13	0.16	0.29	
LFS + Anisomycin 4h	0.19	0.22	0.08	0.14	0.19	0.21	
Lactacystin LFS	0.11	0.19	0.08	0.08	0.12	0.18	
Significances (p values)	total varicosity loss	total varicosity gain	loss by relocalization	gain by relocalization	straight loss	*de novo* gain	
LFS / LFS + Anisomycin	< 0.001	0.22	0.08	0.30	0.00479	0.30	
LFS / LFS + Lactacystin	< 0.001	0.00887	0.22	0.18	< 0.001	0.03885	

Interestingly, blocking protein synthesis around the time of LTD-induction significantly reduced the LTD-associated loss of axonal varicosities to a level which was indistinguishable from the loss observed under baseline conditions. While anisomycin effectively prevented the LTD-induced increase in the loss of varicosities, its effect on the gain of axonal varicosities was less pronounced, reducing it slightly (by 13.6%) if anisomycin was present for one hour, and by 17.7% if anisomycin was present for the whole duration of the experiment (4h). However, neither effect reached significance. In summary, the presented data reports that anisomycin blocked the functional expression of LTD and impaired the LTD-induced enhancement of varicosity loss.

To check whether anisomycin treatment affects structural changes other than *de novo* formation and straight loss of axonal varicosities, I counted the number of varicosities that were assembled or lost due to relocalization of axonal volumes (Figure 4-9 and Table 5). Interestingly, the number of axonal varicosities that disappeared in this manner was remained by large unchanged, indicating that blocking protein synthesis did not primarily affect relocalization processes.

I then used the drug lactacystin to examine the role of protein degradation for LTD-associated structural plasticity. In marked contrast to the finding that lactacystin did not impair functional plasticity, lactacystin completely blocked the LTD-induced increase in turnover of axonal varicosities, indicating that LTD levels can be maintained even though LTD-induced increases in the turnover of axonal varicosities are blocked. (Figure 4-9 and Table 5).

Next, I analyzed whether inhibiting protein degradation affected the number of varicosities that were created or lost due to volume relocalization (Figure 4-9 and Table 5). In contrast to the LTD-induced increase in the turnover of axonal varicosities involving *de novo* formation and straight loss, this type of structural plasticity was not affected by lactacystin treatment. These results suggest that volume-mediated structural plasticity reflect distinct cell biological mechanisms that are not affected by functional plasticity.

Taken together, these data report that baseline structural plasticity was independent from protein synthesis and degradation, whereas the LTD-specific turnover depended on the two processes. Furthermore, they reveal that about a third of all varicosity turnover was mediated by relocalization processes that were not altered by synaptic plasticity or pharmacological treatments. The presented data suggests that preexisting material can be used to form axonal varicosities, and that varicosity material can be retained after varicosity disassembly. Furthermore, they report the induction and maintenance of functional LTD without elevated loss of axonal varicosities during blockade of protein degradation.

4.2 Detection of the synaptic marker VGluT-1-Venus at static and dynamic presynaptic axonal varicosities

While it has recently been investigated how the formation of postsynaptic dendritic spines leads to synaptogenesis in synaptic plasticity, little is known about the formation of presynaptic terminals in dynamic axonal varicosities. To this end, I set out to characterize the functional status of morphologically identified stable and dynamic axonal varicosities. I monitored the presynaptic marker content of presynaptic axonal varicosities in CA3 hippocampal neurons using time-lapse microscopy.

To examine if axonal varicosities comprise the potential of forming functional synapses, I monitored their content of an essential and specifically localized presynaptic protein. I used VGluT-1 (Vesicular Glutamate transporters) as synaptic marker, as Vesicular Glutamate Transporters are specifically localized to presynaptic terminals and essential for synaptic transmission of glutamatergic neurons (Bellocchio, Reimer et al. 2000; Wojcik, Rhee et al. 2004). VGluT proteins colocalize with glutamate-filled synaptic vesicles at presynaptic terminals, and they reliably label sites of neurotransmitter release. Moreover, their amount scales with synaptic strength (Wilson, Kang et al. 2005; Takamori 2006). VGluT-1 is the predominant isoform of glutamate transporters in mature CA3 and CA1 hippocampal neurons. For this reasons, VGluT-1 strongly implies the potential of axonal varicosities to release neurotransmitter and serves as synaptic marker in my experiments. Importantly, the localization of VGluT-1 to axonal varicosities cannot provide prove of presynaptic function or of the presence of a postsynaptic partner.

In this chapter of my thesis, I examined the localization of VGluT-1-Venus to morphologically identified axonal varicosities. To this end, I used time-lapse two-photon imaging of knock-in VGluT-1-Venus mice - a courtesy from Etienne Herzog, Max Planck Institute for Experimental Medicine in Göttingen, Germany - in combination with acute labeling of CA3 neurons with a morphological marker. In addition to monitoring stable axonal varicosities, I investigated the VGluT-1-Venus content of dynamic axonal varicosities during structural plasticity.

4.2.1 VGluT-1-Venus localizes to presynaptic varicosities

The VGluT-1-Venus knock-in mouse line I employed in my experiments expresses VGluT-1 fused to the fluorophor Venus under the endogenous promoter, resembling the endogenous conditions of VGluT-1 expression. However, the mouse line was recently generated and the expression of VGluT-1-Venus had not been fully characterized so far. Therefore, I set out to characterize the VGluT-1-Venus expression and to test if the mouse line provides a suitable tool for investigating VGluT-1 dynamics.

To this end, I characterized the localization and specificity of VGluT-1-Venus expression in hippocampal Gähwiler cultures using two-photon microscopy of knock-in VGluT-1-Venus mice in combination with acute labeling of CA3 neurons with morphological volume markers. In the first set of experiments, I performed bolus loading of Calcein red-orange AM in VGluT-1-Venus hippocampal cultures. I sequentially excited and monitored the two fluorophors Calcein-red orange AM and Venus to detect the morphology of CA3 neurons and VGluT-1-Venus, respectively.

I observed that not all Gähwiler cultures from the homozygous VGluT-1-Venus mice displayed a detectable VGluT-1-Venus fluorescence at 15-20 days in vitro. The expression levels in all VGluT-1-Venus expressing cultures were high over all areas of the slices (n = 16 slices, Figure 4-10). Only these cultures were used to perform the experiments.

The non-overlapping two-photon excitation spectra of Calcein and Venus required a sequential excitation of the fluorophors, which resulted in a small lateral displacement of the two single-color image stacks (~2-5 µm). This displacement was caused by chromatic aberrations, specific properties of the laser beam alignments as well as by a slow drift of the slice in the imaging chamber that occurred between the imaging sessions. It was manually corrected by aligning the image stacks to prominent structures in the slice that were visible in both emission channels (Figure 4-10).

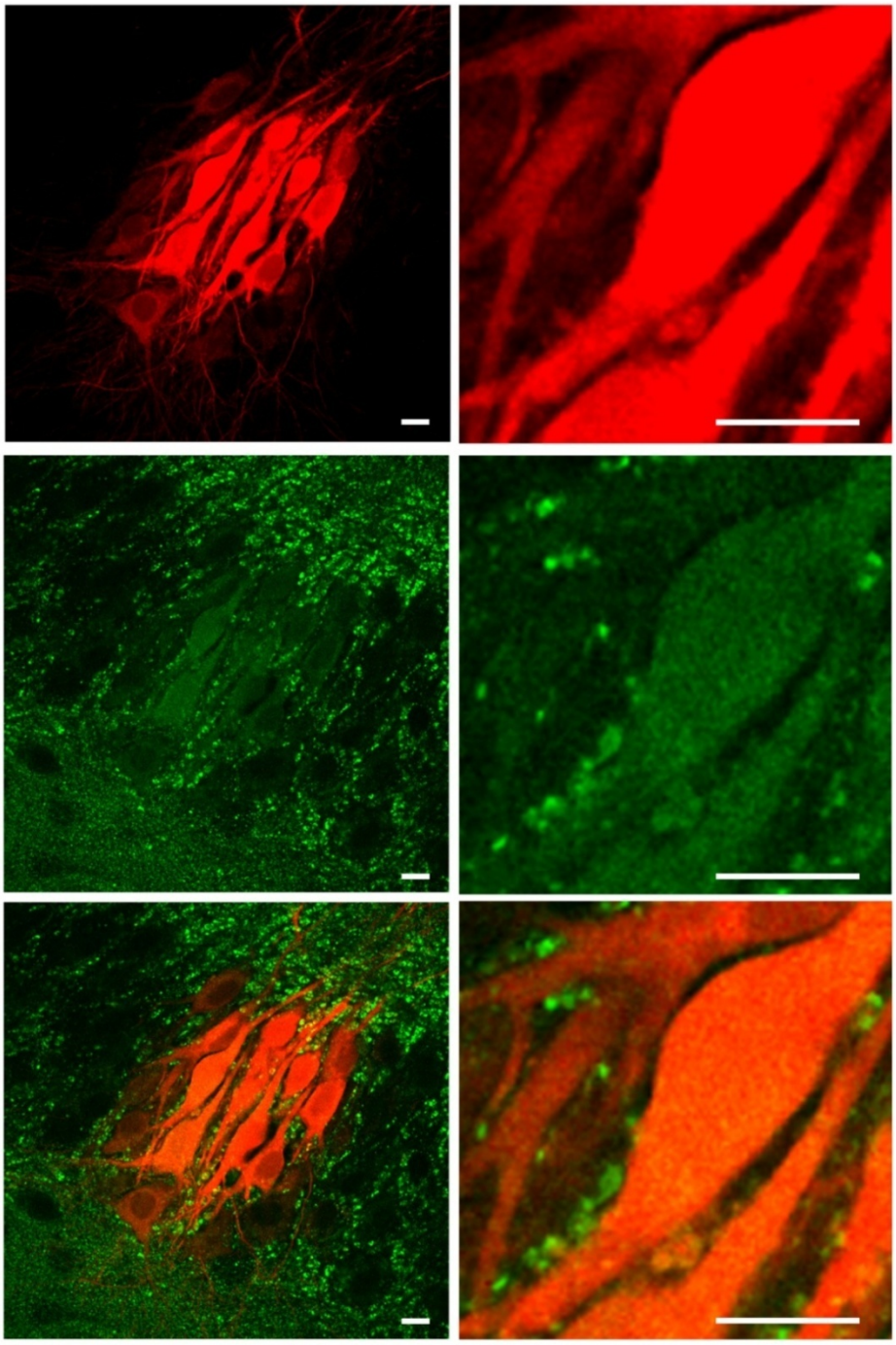

Figure 4-10: VGluT-1-Venus exhibited a dense and punctate expression in knock-in VGluT-1-Venus mice

Imaging Calcein red-orange AM labeled hippocampal CA3 neurons in VGluT-1-Venus mice Gähwiler cultures. The two fluorophors Calcein and Venus were sequentially excited, and the displacement of the two channels was corrected manually. VGluT-1-Venus exhibited a punctate and dense staining that did not overlap with labeled CA3 cell somata. Left panel: Overview. Right panel: close up. Top: CA3 population. Middle: VGlut-1-Venus. Bottom: Overlay. Scale bars: 10 µm.

In all experiments, the VGluT-1-Venus fluorescence (green channel) was detectable throughout CA3 and CA1 in a punctated fashion. Individual puncta appeared either as small oval shapes with a major diameter of 0.61 ± 0.02 µm, a minor diameter of 0.51 ± 0.01 µm and a volume of 0.13 μm^3 ± 0.07 μm^3 (n = 66), or as larger, complex shaped formations. The larger formations were suggestive of two or more adjacent structures localized in close vicinity, beyond the optical resolution of the microscope.

The density of the VGluT-1-Venus puncta was high, on average 42.2 ± 3.05 VGluT-1 puncta per 100 μm^3 (n = 10 slices), which is in the range of the density of presynaptic terminals in rat cortex (Own experiments: 4.2 ± 0.3 * 10^8 puncta / mm^3, n = 10 slices. Literature: 2 – 40 * 10^8 synapses / mm^3). In contrast, some oval-shaped areas of a major diameter of 28.83 ± 0.69 µm and a minor diameter of 12.58 ± 0.34 µm (n = 16) exhibited no staining at all. A subset of theses structures exhibited Calcein red-orange AM fluorescence (red channel), clearly indicating morphologically labeled CA3 cell bodies. Given the similarity of shape, orientation and parallel arrangement of the oval structures, the data strongly suggest that also most other unlabeled oval structures represent cell bodies. The data is in agreement with the reported absence of presynaptic specializations and VGluT-1 aggregations in cell somata (Takamori, Rhee et al. 2001). In summary, the visualized VGluT-1-Venus distribution of hippocampal Gähwiler cultures of VGluT-1-Venus knock-in mice was in agreement with the expected endogenous VGluT-1 distribution.

Next, I examined the VGluT-1-Venus expression in axons and axonal varicosities of Schaffer collaterals. In the previous experiments, the displacement of the different fluorescence channels could be sufficiently aligned manually, as only larger structures were colocalized, but this post-hoc alignment was not accurate enough for correlations of axonal varicosities and VGluT-1-Venus on the sub-micrometer level. I therefore replaced Calcein red-orange AM with Alexa 568, a morphological marker that is excitable at the same wavelength as Venus. For each experiment, one or two CA3 neurons were patch-filled with Alexa 568 and gave rise to multiple stained axons in CA1 (red channel,Figure 4-11).

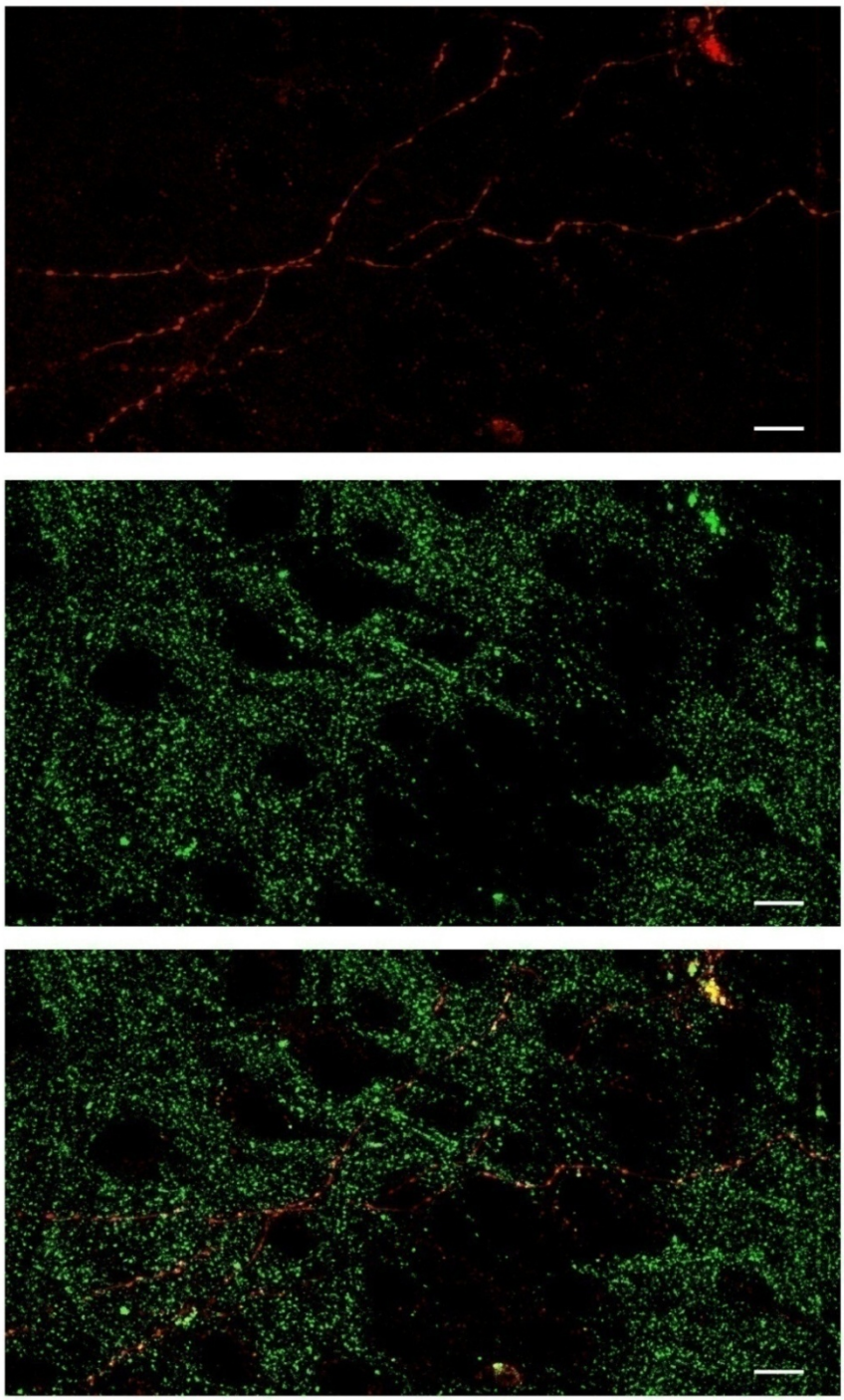

Figure 4-11: Alexa 568 labeled axons and endogenous VGluT-1-Venus were simultaneously detectable

A single CA3 neuron was patch-filled with Alexa 568 which can be excited simultaneously to VGluT-1-Venus, and its multiple axons were detectable in CA1. VGluT-1-Venus label colocalized with axons and spared out oval structures indicative of CA1 cell bodies. Top: Alexa 568 labeled axons. Middle: VGluT-1 Venus. Bottom: Overlay. Scale bars: 10 µm.

VGluT-1-Venus (green channel) was expressed in the same punctuate pattern in CA1 as in CA3, again sparing out oval structures (major diameter: 17.68 ± 0.64 µm, minor diameter: 11.7 ± 0.44 µm n=16). Colocalization of VGluT-1-Venus and the morphological marker Alexa 568 (red channel) revealed that 90.4% of all morphologically detected axonal varicosities contained VGluT-1-Venus fluorescence (n= 337 varicosities, 6 experiments, Figure 4-12).

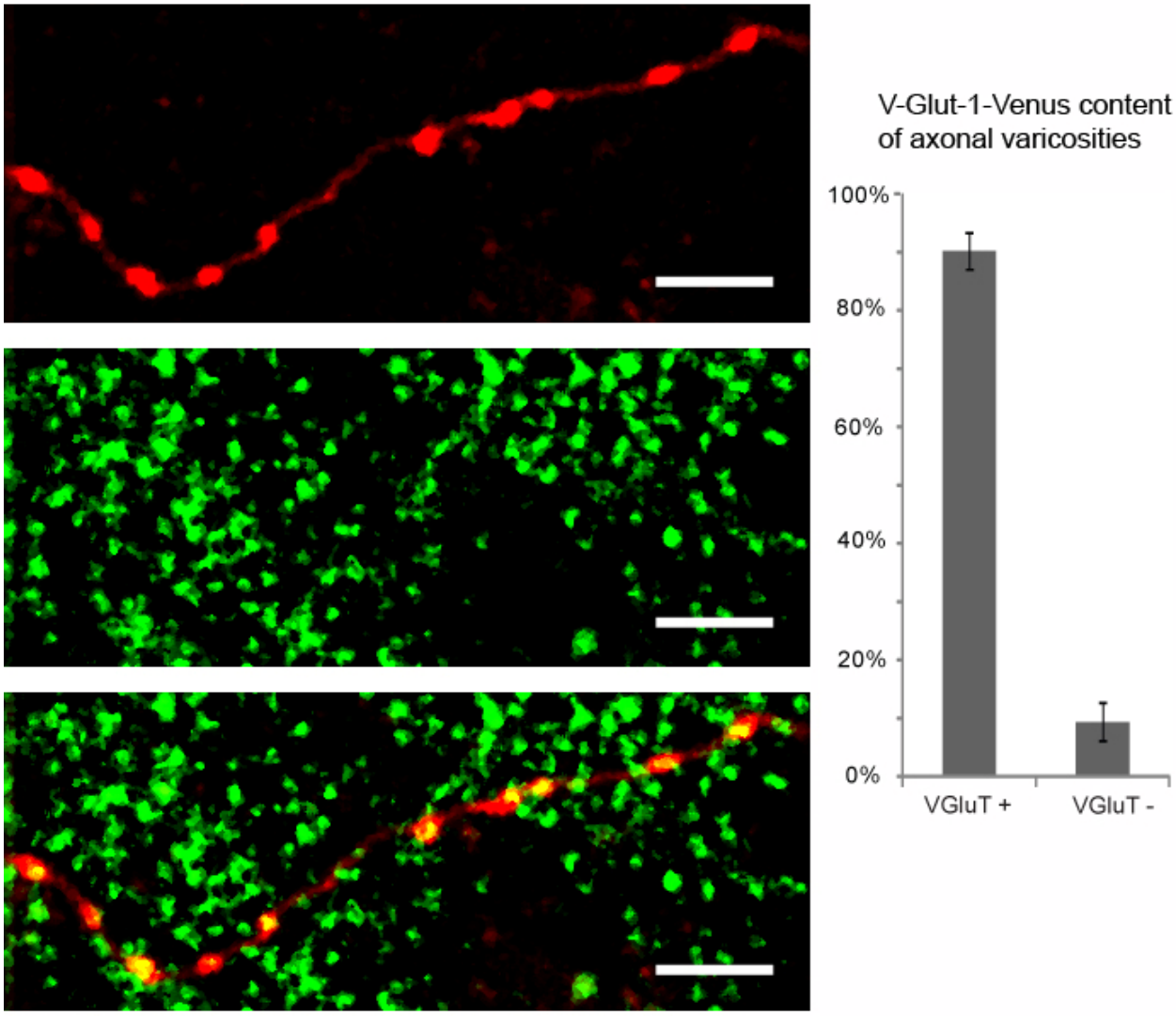

Figure 4-12: Morphologically identified axonal varicosities colocalized with VGluT-1-Venus

CA3 cells were patch-filled with Alexa 568 and imaged on the VGluT-1-Venus mice background. Colocalization of axonal varicosities and VGluT-1-Venus was performed on single sections, revealing that 90.4 % of all identified axonal varicosities were VGluT-1-Venus positive. Top left: Alexa 568 labeled axon and axonal varicosities. Middle left: VGluT-1-Venus. Bottom left: Overlay. Right: Summary, fraction of VGluT-1-Venus positive and negative axonal varicosities. Scale bars: 5 µm.

Given the high density of the VGluT-1-Venus label, it was expected that a fraction of axonal varicosities colocalized with VGluT-1-Venus due to random fluorescence overlap. To test for this number of varicosities that were VGlut-1-Venus positive by chance, one color channel of a data set was rotated by 180°. This control was analyzed in parallel with the non-rotated data in a blind fashion (Figure 4-13).

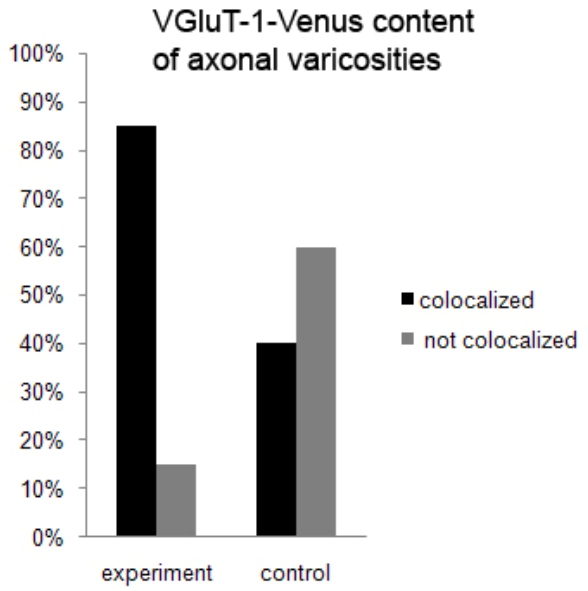

Figure 4-13: Comparison of specific versus by-chance colocalization confirmed the specificity of the VGluT-1-Venus label

To control for unspecific colocalization, a single data set was analyzed after correct overlay of the two fluorescent channels (“experiment”, left bars) and after rotation of one channel (“control”, right bars) In the experiment, 85.0% of axonal varicosities colocalized, while in the control, only 40.0% colocalized. The colocalization of the control conditions was non-specific and caused by a high density of the VGluT-1-Venus label that overlaped with varicosities by chance. Experiments were analyzed blind regarding the condition.

In the original data set, 85.0% of all axonal varicosities colocalized with VGluT-1, while the colocalization in the rotated data set was 40.0%. The results indicate the specificity of the label, yet also a relatively high by-chance colocalization, likely caused by the high density of the VGluT-1-Venus label.

In some areas of the neuropil the punctuate VGluT-1-Venus labeling was enriched, assembling to tubes with an inner diameter ranging from 0.52 µm to 1.35 µm (average:

0.94 µm ± 0.06 µm, n=16.Figure 4-14). The steric organization of VGluT-1-Venus suggests the sheathing of a round, branched structure in the center of the tube. Speculatively, the presynaptic structures possibly target postsynaptic excitatory dendrites or inhibitory neuronal processes. However, I never detected a labeled CA3 dendrite within the tubular structure, and the postsynaptic partners remained unknown.

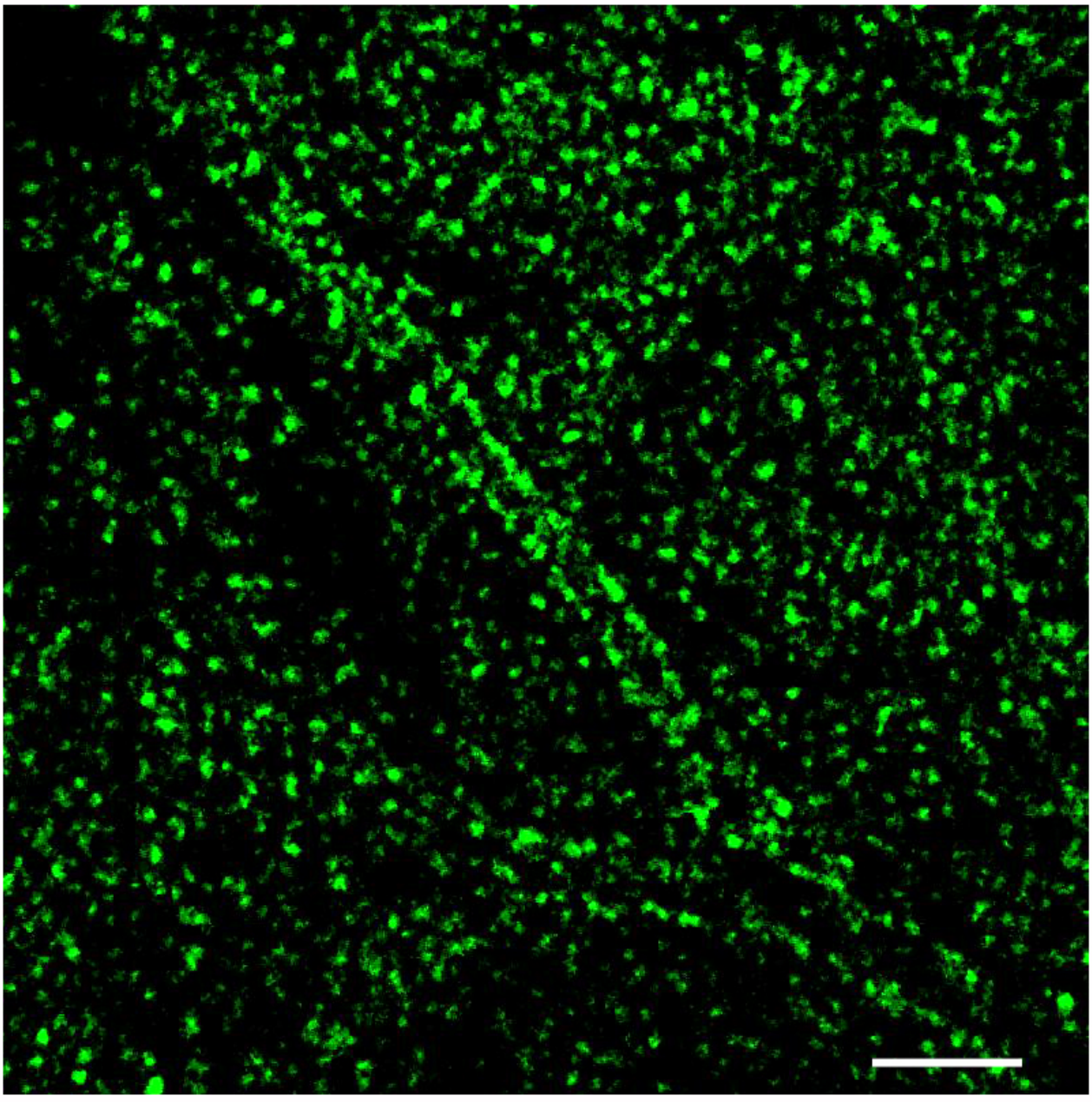

Figure 4-14: VGluT-1-Venus was occasionally enriched in tubular structures

VGluT-1-Venus puncta formed tubular structures of 0.94 ± 0.06 µm diameter, indicative of surrounding a neuronal process. Scale bar: 10 µm.

In summary, the experiments thus far document that VGluT-1-Venus fluorescence is easily detectable, specifically localizes to axonal varicosities and corresponds to the expression pattern of the endogenous VGluT-1 protein.

4.2.2 VGluT-1-Venus accumulates at newly formed axonal varicosities

To functionally contribute to network activity, the formation of an axonal varicositiy must be completed by proteins that enable their proper functionality. Recent experiments such as Calcium imaging provided first evidence that new axonal varicosities can rapidly gain the potential for Calcium influx, a crucial signaling step for mediating the release of glutamate filled synaptic vesicles (Becker, Wierenga et al. 2008). However, these experiments lacked information about the synaptic vesicle content itself. To date, the functional status of dynamic axonal varicosity remains mostly unknown. To expand the current knowledge, I set out to investigate the content of a synaptic marker protein at dynamic axonal varicosities.

To this end, I monitored the presence of VGluT-1-Venus at newly generated varicosities, and tested for VGluT-1-Venus localization prior to varicosity disassembly. Using time-lapse two-photon microscopy of VGluT-1-Venus knock in Gähwiler cultures and morphologically labeled CA3 axons, I followed the structural plasticity of individual axonal varicosities in combination with VGluT-1-Venus over the time course of four hours. The approach allowed to investigate if dynamic varicosities acquire VGluT-1-Venus up to four hours after their morphological assembly, or if they remain without the synaptic marker protein.

I observed that a fraction of newly formed varicosities colocalized with VGluT-1-Venus while other new axonal varicosities did not (Figure 4-15). Out of 38 dynamic varicosities, 55.3% (n=21) were VGluT-1-Venus positive at the end of the four hour imaging period, while 44.7% (n = 17) were VGluT-1-Venus negative.

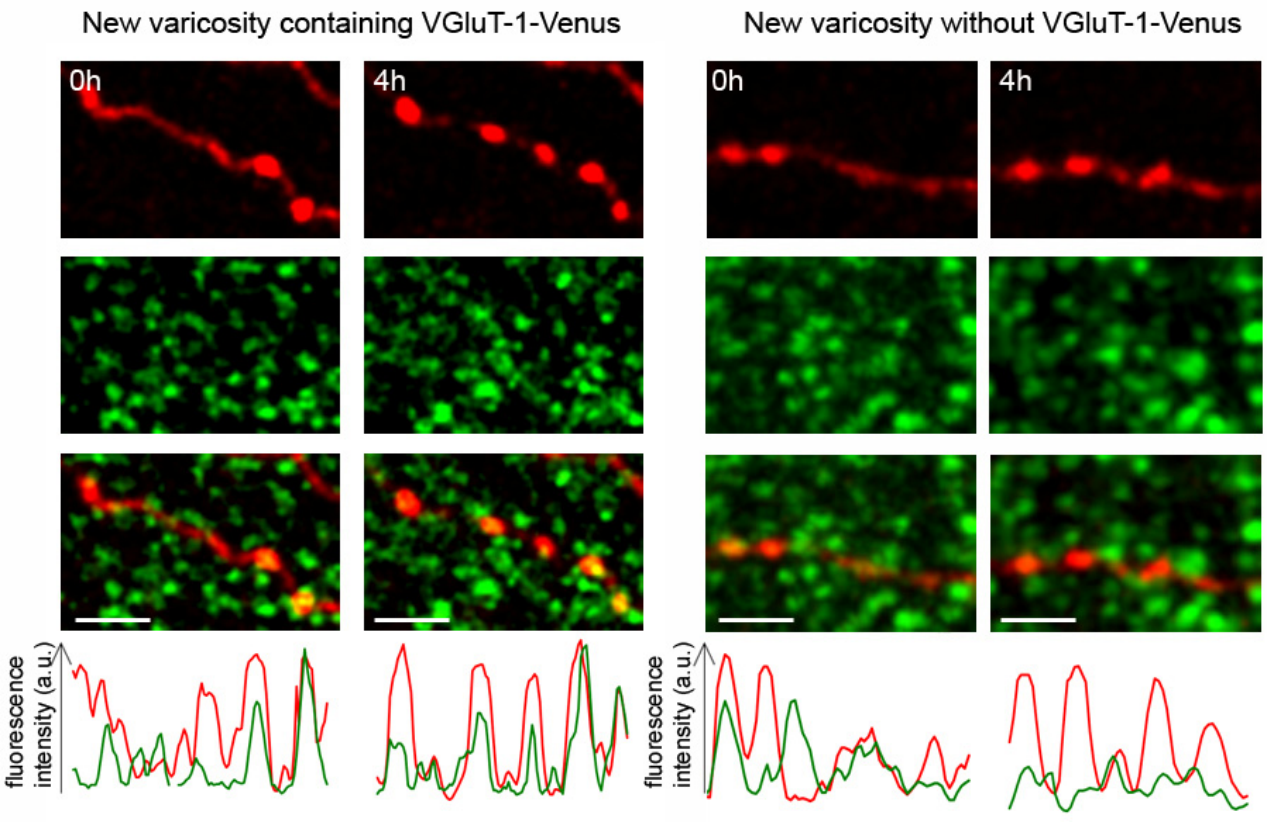

Figure 4-15: A fraction of new axonal varicosities contained VGluT-1-Venus after four hours

Time lapse imaging of VGluT-1-Venus and morphology of axonal varicosities. While a fraction of new axonal varicosities had acquired VGluT-1-Venus at the end of the imaging period, others did not. Left two panels: Example new varicosity where VGluT-1-Venus had accumulated after four hours. Right two Panels: Example new varicosity where no VGlut-1-Venus was detectable after four hours. Rows from top to bottom: Morphological stain of varicosities (Alexa 568); VGluT-1-Venus; Overlap; Intensity plot: Fluorescence intensity of morphology (red) and VGluT-1 (green) along the axon. Scale bars: 5 µm.

Importantly, both values represent only estimates that include a systematic level of uncertainty: The number of VGluT-1-Venus negative varicosities may be an overestimation as they can contain VGluT-1 on sub-threshold levels. On the other hand, a fraction of VGlut-1-Venus positive axonal varicosities may be caused by non-specific colocalization with the VGluT-1-Venus fluorescence. However, given the ratio of specific vs. unspecific staining that was obtained in 4.2.1 and the low numbers of VGluT-1-Venus prior to varicosity formation (see next paragraph), the high numbers of

varicosities that colocalized with VGluT-1-Venus clearly indicate that a remarkable fraction of the newly generated varicosities contain synaptic vesicles.

Next, I tested for the fraction of axonal varicosities where VGluT-1-Venus fluorescence was present in the axonal area prior to varicosity formation. In 8 % off all cases (3 of 38 observations) VGluT-1-Venus was detectable before and after varicosity formation. This small fraction of observations was, speculatively, caused by by-chance correlation of VGluT-1 and axonal varicosities, which appeared to be much lower than in 4.2.1. By demonstrating the absence of VGluT-1-Venus fluorescence in the majority of all observations (92%, 35 of 38 observations), my results strongly indicate that the formation of axonal varicosities was most often not preceded by VGluT-1 Venus accumulation. However, obtaining more overall events and comparing the colocalization numbers to the by-chance colocalization of the present data set would be required to further validate this hypothesis.

The presented data indicates that newly formed axonal varicosities can rapidly (< 4h) acquire the synaptic marker VGluT-1-Venus. It moreover suggests that VGluT-1-Venus typically accumulates not prior, but predominantly during or after the morphological varicosity formation. However, the experiments so far could not clarify the time course of VGluT-1-Venus accumulation. To further temporally resolve the VGluT-1-Venus acquisition, I performed time lapse experiments with 30 minutes imaging intervals and characterized the time course of two example axonal varicosities. In one case, the newly formed varicosity was VGluT-1-Venus positive, and in a second case the newly formed varicosity was VGluT-1-Venus negative (Figure 4-16).

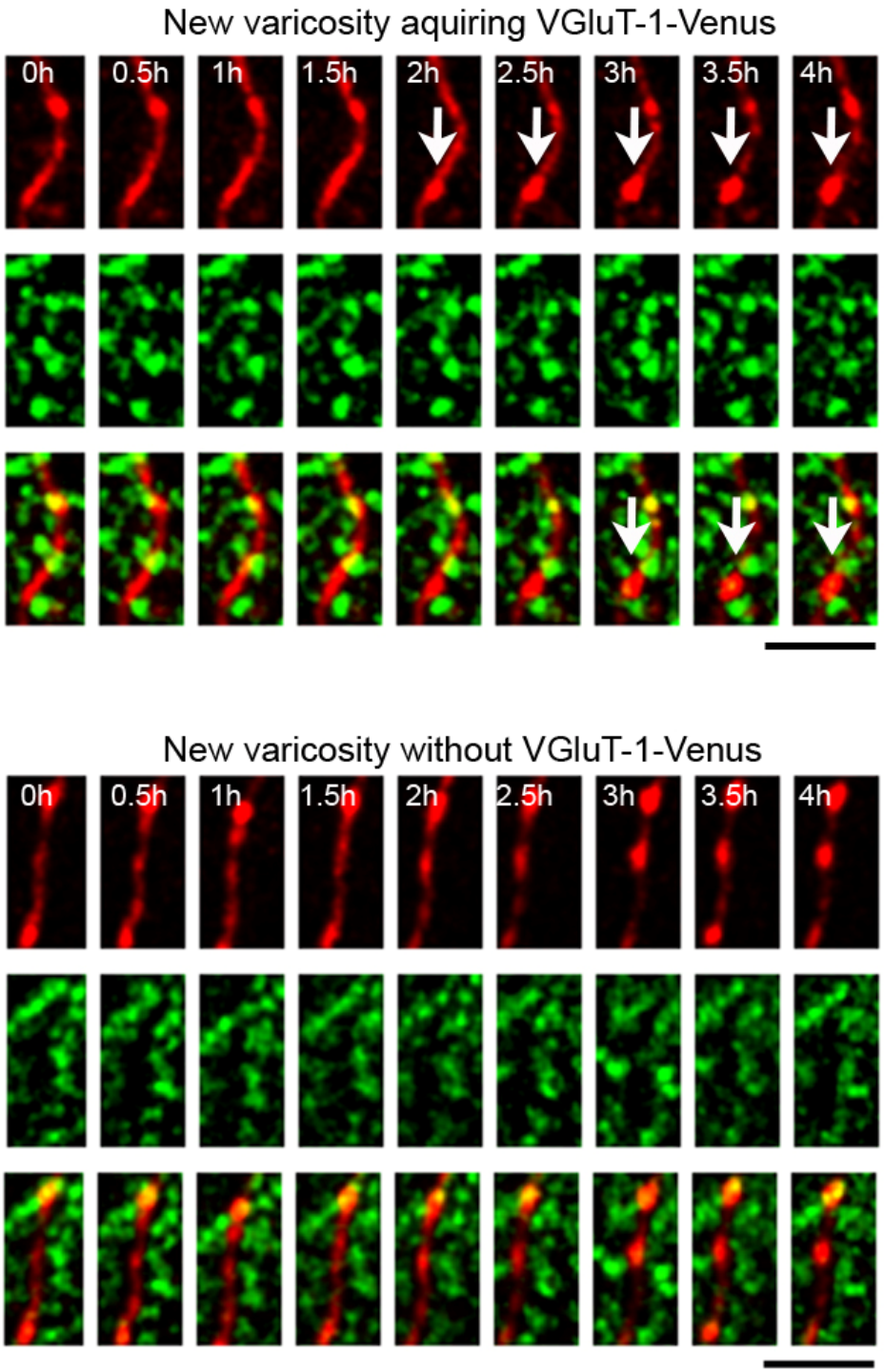

Figure 4-16: VGluT-1-Venus accumulated at new varicosities after their initial volume formation

Time-lapse experiments of newly assembled axonal varicosities with 30 min imaging intervals. Upper panels: Example axonal varicosity. VGluT-1-Venus accumulated with a one hour delay to the appearance of the varicosity volume. Lower panel: Example varicosity.VGluT-1-Venus did not accumulate at the new varicosity after 1.5 hours. Per panel: Top: morphological marker Alexa 568. Middle: VGluT-1-Venus. Bottom: Overlay. Arrows indicate the accumulation of VGluT-1-Venus at varicosities. Scale bars: 5 µm.

In the first example, the axonal area where the varicosity formed did not contain detectable VGluT-1-Venus levels at the beginning of the experiment. After two hours of experiment, the varicosity volume appeared in the red fluorescence channel as displayed by the morphological volume marker Alexa 568. After three hours, VGluT-1-Venus became detectable at the newly formed axonal varicosity. Thus, the increase in volume marker fluorescence preceded the accumulation of VGluT-1-Venus by one hour (Figure 4-16), suggesting that volume formation preceded the accumulation of glutamate-filled synaptic vesicles. The data is in agreement with the observation described earlier in this chapter, where after four hours a fraction of varicosities contained VGluT-1-Venus, and it suggests that the formation of morphological volume can precede the accumulation of the synaptic marker protein.

In the second example, the new axonal varicosity was detectable by the volume marker after three hours of imaging. Again, the respective axonal area did not contain detectable levels of VGluT-1-Venus before varicosity formation. In contrast to the first example, this axonal varicosity neither comprised VGluT-1-Venus at the end of the four hour imaging session nor at any time point in between, suggesting that synaptic vesicles did not accumulate at the varicosity until the end of the experiment. Interestingly, the examined varicosity appeared later than the varicosity before, and it remained possible that it acquired VGluT-1-Venus yet with similar kinetics as the first example. Notebly, both axonal varicosities did not comprise VGluT-1-Venus fluorescence before varicosity formation, which is consistent with the previous experiments in this chapter and suggests that the volume formation precedes the accumulation of the synaptic marker.

In summary, the data infer that a fraction of newly formed axonal varicosities can rapidly acquire synaptic vesicles after their formation. Despite low numbers of observations, the findings favor the hypothesis that varicosity formation precedes the accumulation of the synaptic marker VGluT-1-Venus, and it disfavors the hypothesis that morphological varicosity formation typically follows the accumulation of VGluT-1-Venus.

4.2.3 VGluT-1-Venus content of instable axonal varicosities

While to date few knowledge is available about the functional status of newly generated axonal varicosities, even less is known about the functional capacity of axonal varicosities shortly before their disassembly. A former study had reported functional calcium transients in new varicosities, but the technical properties of calcium imaging had thus far not allowed to investigate calcium transients in disassembling varicosities (Becker, Wierenga et al. 2008).

I aimed to test if instable axonal varicosities contain a synaptic marker protein prior to their disassembly. To address this question, I analyzed the previous VGluT-1-Venus time lapse experiments for axonal varicosities that were disassembled, benefiting from the ability to retrospectively identify instable axonal varicosities and to sample relatively high varicosity numbers within the experiments. Most imaging data was obtained in two-timepoint experiments (Figure 4-17).

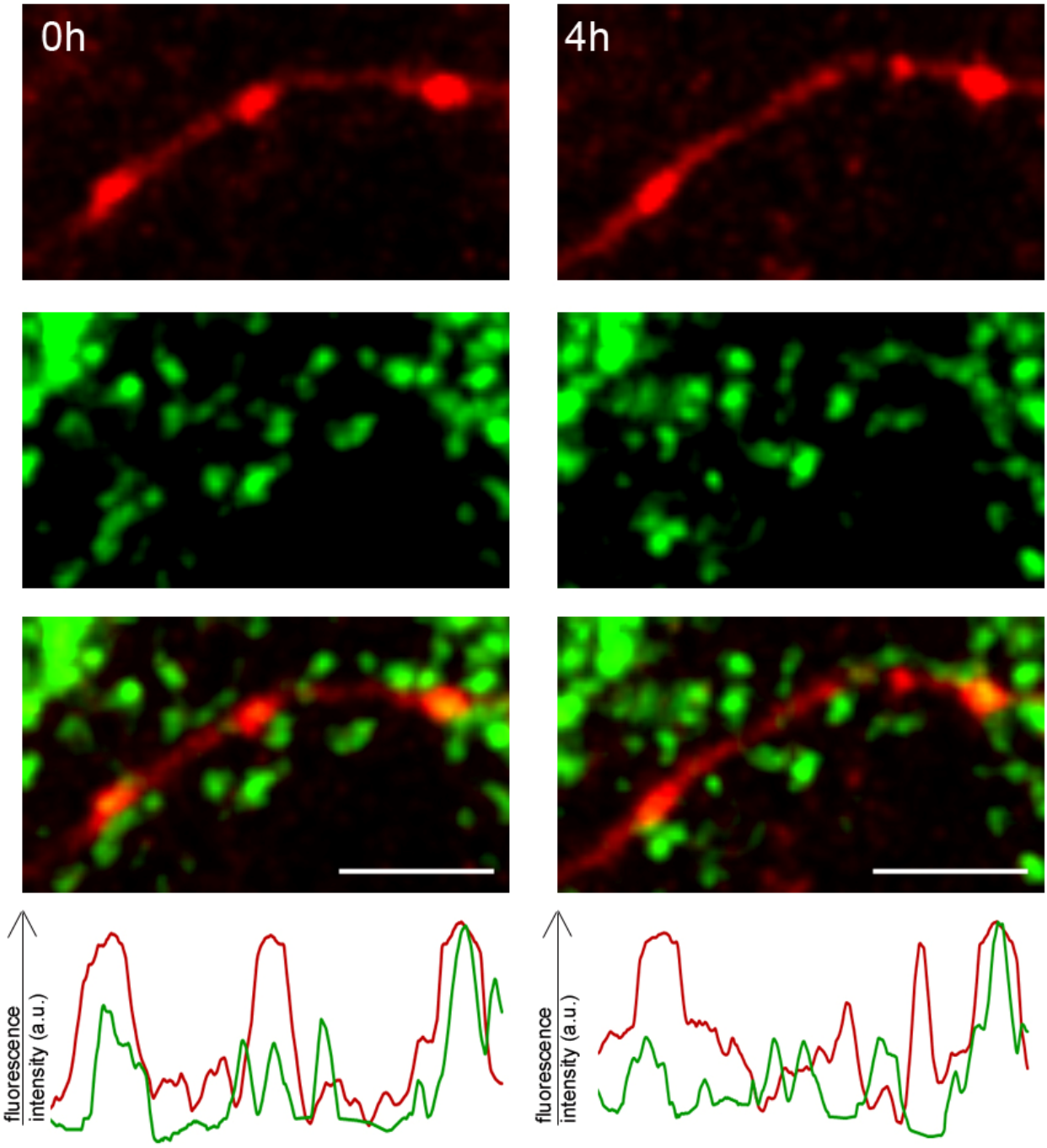

Figure 4-17: VGluT-1-Venus content of disassembling axonal varicosities

Time lapse imaging of VGluT-1-Venus and axonal varicosities that were disassembled within the four hour imaging period. Six out of seven varicosities did not contain detectable VGluT-1-Venus levels within 4 hours before their disassembly. Left column: varicosity at the beginning of the experiment (0h). Right column: varicosity at the end of the experiment (4h). Rows from top to bottom: morphological varicosity fluorescence, VGluT-1-Venus fluorescence; Overlay; Intensity plot: fluorescence intensity of morphology (red) and VGluT-1-Venus fluorescence (green) along the axon. Scale bar: 5 µm.

I detected seven varicosity loss events, and in six out of seven cases the axonal varicosity did not contain detectable amounts of VGluT-1-Venus at the beginning of the experiment. Despite low numbers, the data report that synaptic markers were not always maintained until the disassembly of the varicosity volume. It would be interesting to clarify in further time-lapse experiments if the axonal marker disappeared prior to assembly, or if it was never present at the disassembled varicosity. However, I did not yet obtain a detailed time course of a varicosity disassembly.

In my experiments, I observed the dynamics of a VGlut-1-Venus negative axonal varicosity in a detailed time course experiment with 30 minutes imaging intervals (Figure 4-18).

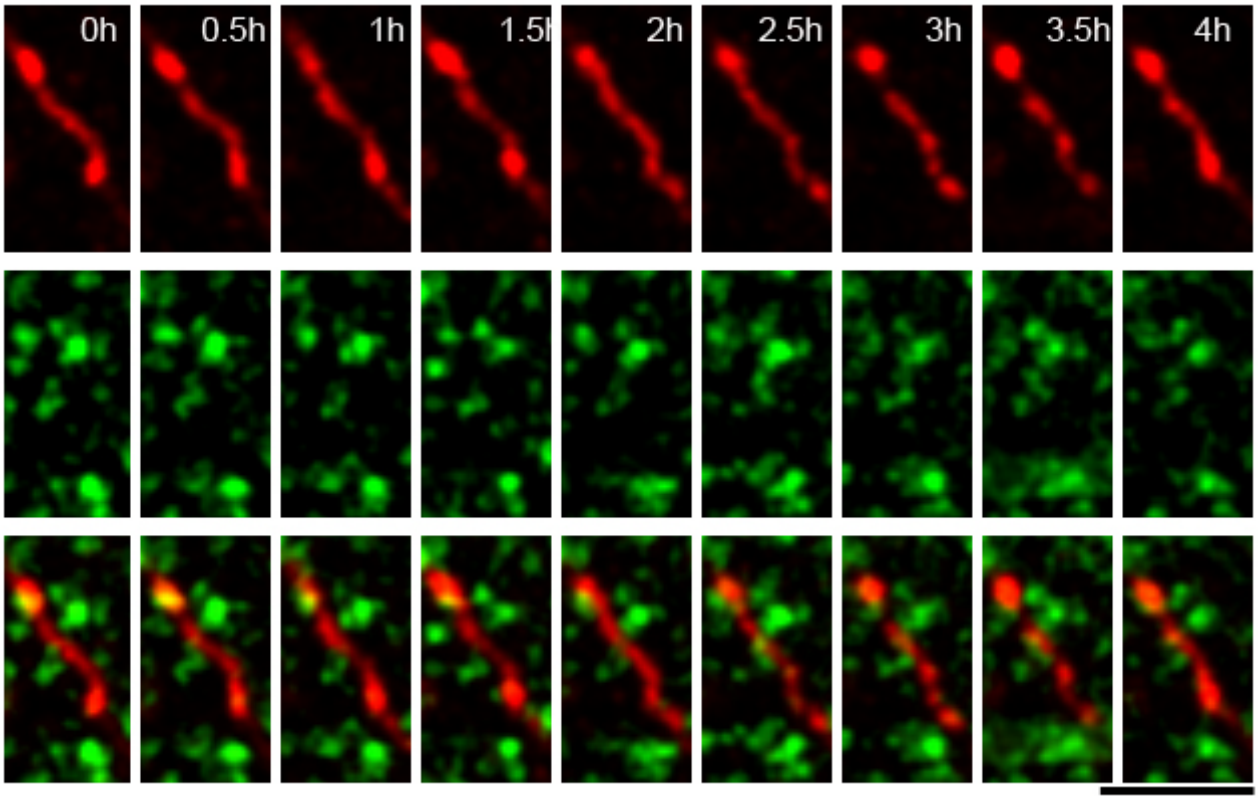

Figure 4-18:A VGluT-1 negative axonal varicosity exhibited unstable properties in detailed time-lapse

Time lapse experiment of VGluT-1-Venus and the morphology of an example axonal varicosity. The respective varicosity was selected as an example of a VGluT-1-Venus negative varicosity, and was then analyzed in a detailed time course. The varicosity temporarily disappeared between 2h and 3.5h. Rows from top to bottom: morphological marker fluorescence, VGluT-1-Venus fluorescence; Overlay. Scale bar: 5 µm.

In the example, the morphological maker (red channel) showed the presence of the axonal varicosity simultaneous with a lack of VGluT-1-Venus (green channel) at the beginning and the end of the experiment. Surprisingly, the morphological structure was not detectable in intermediate time points (2h - 3.5h), revealing that the regarded morphological volume of the varicosity was transient. In parallel, VGluT-1-Venus was not detectable at any time point of the experiment. The data provide an example that a transient varicosity did not contain detectable levels of VGluT-1-Venus. It would be of interest to investigate if the same finding also applies for other transient varicosities. The data is in agreement with the hypothesis that the accumulation of a synaptic marker protein requires the stable presence of the morphological varicosity volume.

In summary, despite the few numbers of observations (n=7), the results suggest that axonal varicosities that undergo disassembly typically did not contain an accumulation of synaptic markers. They either lost the synaptic markers before the morphological shrinkage or they never contained them.

4.2.4 VGluT-1-Venus content of merging axonal varicosities

In the first part of my thesis I reported the reorganization of preexisting axonal material as important mechanism for axonal varicosity turnover. It was therefore of interest to investigate if merging and dividing axonal varicosities contain the synaptic marker VGluT-1-Venus, and if VGluT-1-Venus relocalizes in parallel to the volume relocalization. I obtained one example of two merging axonal varicosities in a detailed time course experiment (Figure 4-19).

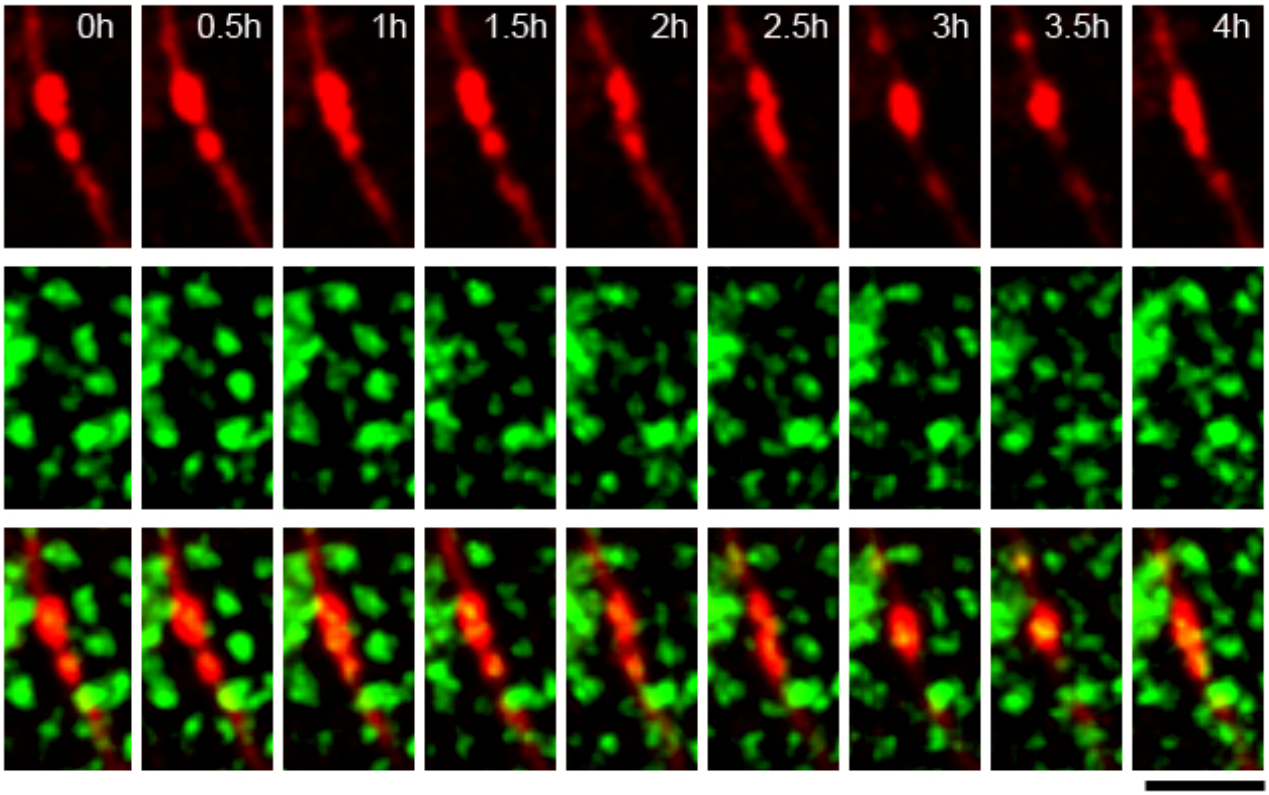

Figure 4-19: Merging of two varicosities was accompanied by merging of individual VGluT-1-Venus puncta

Time lapse experiment of VGluT-1-Venus and morphology of an axonal varicosity. Two varicosities, each containing a single VGluT-1-Venus punctum, merged into a single varicosity after three hours. In parallel, the VGluT-1-Venus puncta also reorganized via an intermediate stage into a single VGluT-1-Venus accumulation. Rows from top to bottom: morphological varicosity fluorescence, VGluT-1-Venus; Overlay. Scale bar: 5 µm.

In the figure, the morphological marker Alexa 568 depicts the existence of two individual varicosities at the beginning of the experiment. After one and 1.5 hours, the two varicosities had reached an intermediate stage where they were inseparably close to each other, yet still formed two individual fluorescent peaks. After three and 3.5 hours the varicosities had merged into one morphological structure displaying only one center of maximal fluorescence, which spread out again at the end of the experiment (4 h).

The morphological relocalization was paralleled by the relocalization of the VGluT-1-Venus accumulations. At the first time point, each varicosity colocalized individually with VGluT-1-Venus. At the intermediate stage of 1 and 1.5 hours, VGluT-1-Venus was

detectable in three close but distinct accumulations within this intermediate structure, while no VGluT-1-Venus resided in the axonal area where the axonal varicosities had previously been localized. Paralleling this, the VGluT-1-Venus fluorescence had concentrated in one larger accumulation at the center of the merged varicosities. Finally, after 4 hours the fluorescence was detectable again as multiple puncta. The data describe that the merging of two varicosities can occur in parallel to simultaneous relocalizations of synaptic vesicles. It suggests that merging of two varicosities can be accompanied by the merging of the individual synaptic vesicle pools, recycling preexisting synaptic constituents. However, if this example represents a general mechanism and if varicosities that relocalize over larger distances also contain synaptic markers during traffic requires further testing.

5 Discussion

Activity-dependent changes of synaptic connectivity provide an important cellular mechanism for learning and memory. Since the advent of new imaging techniques in recent years allowed to investigate morphological dynamics in physiological networks, structural plasticity and its link to synaptic functionality have been intensively studied. While most studies concentrated on morphological changes of postsynaptic spines, little is known about presynaptic structural plasticity of axonal varicosities, the morphological specializations of presynaptic terminals. The underlying cellular mechanisms of baseline and activity-dependent presynaptic structural plasticity remain mostly unknown to date, and the potential of dynamic axonal varicosities to form functional synapses remains by large elusive. To this end, I set out to characterize structural and cellular mechanisms of presynaptic axonal varicosities and to investigate the synaptic marker content of stable and dynamic axonal varicosities.

In my thesis, I used a combination of time-lapse two photon imaging, electrophysiological recordings and pharmacology in organotypic hippocampal cultures to observe different types of structural dynamics and to investigate the effect of protein synthesis and degradation blockade on baseline and plasticity-associated varicosity turnover. By time-lapse imaging VGluT-1-Venus mouse line hippocampal cultures I observed the synaptic marker protein VGluT-1-Venus content of stable and dynamic axonal varicosities.

I report that distinct types of morphological dynamics contributed side by side to the turnover of axonal varicosities. D*e novo* formation and straight loss provide means to gradually adapt presynaptic size, and relocalization processes allow to step-wise change presynaptic structures. The data showed that new varicosities could emerge without acute protein synthesis, and that varicosities could disassemble without protein degradation, implying the use and recycling of varicosity proteins from a preexisting pool of varicosity constituents. In contrast to baseline turnover, plasticity-induced turnover of axonal varicosities required the synthesis and degradation of proteins, indicating the need for additional presynaptic factors. 90 % of all stable varicosities

colocalized with a synaptic marker protein, and a considerable fraction of dynamic varicosities acquired the synaptic marker within four hours after their appearance. The results suggest the functionality of axonal varicosities and that a subset of dynamic varicosities can rapidly acquire the potential to exert presynaptic function. Moreover, the reported data indicate that structural volume dynamics typically precede the accumulation of the synaptic marker protein. In summary, my thesis provides novel insights into cellular mechanisms and functional consequences of structural plasticity.

5.1 Dependence of presynaptic structural plasticity on protein synthesis and degradation

5.1.1 Distinct types of morphological dynamics of axonal varicosities

The experiments in this thesis describe that formation of new axonal varicosities can be mediated by distinct types of structural dynamics. *De novo* formation and straight loss referred to the growth and shrinkage of bulbus-shaped axonal varicosities on a much dimmer stretch of axon, whereas relocalization of axonal volumes referred to the rearrangements of visible clusters that fulfilled the size criteria of axonal varicosities but displayed a higher mobility. De novo formation and straight loss enable the gradual adaptation of the size of axonal varicosities, and relocalization processes can provide an intriguing mean to change presynaptic size in defined steps. Axonal volumes divided and coalesced with other motile volumes or presynaptic stable varicosities, rapidly altering their size, or stabilized as whole entity to form a presynaptic varicosity. It has been reported that the size of axonal varicosities strongly correlates with the size of the synaptic vesicle reserve pool and synaptic strength (M. B. L. Yeow 1991; Pierce and Mendell 1993), and thus fusion and fission of volumes possibly provide mechanisms to rapidly modulating the functional strength of presynaptic terminals. The relocalization processes also included the slow merging or dividing of neighboring axonal varicosities, indicative of merging their individual synaptic protein content. It would be interesting to monitor if structural relocalizations are accompanied by the relocalizations of presynaptic marker proteins to further answer this questions. While I provide an

intriguing example in 4.2.4 that indeed a functional marker relocalizes together with morphology, this question awaits further study.

In addition to rapidly altering presynaptic size of axonal varicosities, the motile volumes may themselves represent functional units. It has been reported that axonal volumes that split from pre-exisiting varicosities as well as motile transport vesicles can undergo depolarization-dependent synaptic vesicle release. (Kraszewski, Mundigl et al. 1995; Dai and Peng 1996; Krueger, Kolar et al. 2003). It would be interesting to experimentally test if the motile axonal volumes display functional properties, e.g. by synaptic vesicle proteins fused to pH-sensitive pHluorins, Calcium imaging or other synaptic markers.

It is currently subject of research if motile volumes stabilize randomly along the axon or if they preferentially stabilize at predefined sites. For developing neurons, the existence of predefined sites along axons has been reported, where STVs (synaptic vesicle protein transport vesicles) preferentially pause and form varicosities (Sabo, Gomes et al. 2006). A couple of observations within my data-set go in line with this hypothesis, as they are suggestive for predefined sites: For example, I detected the salutatory moving and pausing of axonal volumes, and the repeated stabilization of an axonal volume at the same axonal site. In some instances, the same axonal site was subject of varicosity loss, also suggesting a predefined site. (Figure 5-1). However, the examples remained rare occasions.

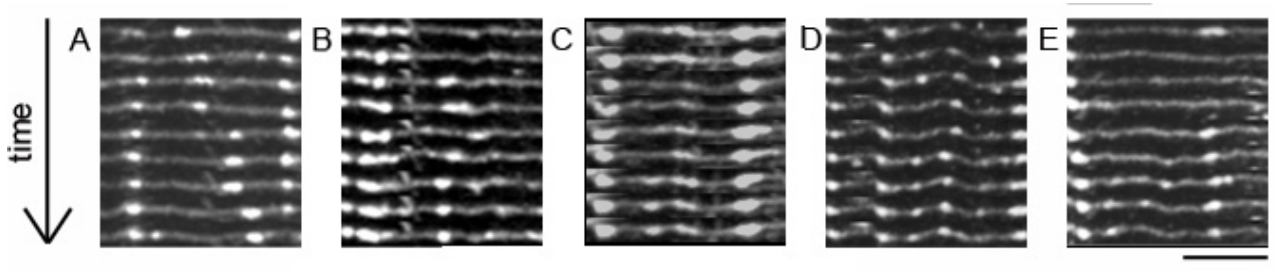

Figure 5-1: Suggestive examples of predefined pause sites along axons

Kymographs of time series of volume relocalizations and varicosity turnover. The axon stretches along the x- axis, time is represented on the y axis. (A) Saltatory movement. An axonal volume moves laterally and stabilized intermittently. (B) Repetitive visit of a varicosity at the same axonal site before stabilization. (C,D,E) The loss of a varicosity was followed by the gain of a varicosity at the same axonal site. Scale bars: 5 µm.

The bidirectional translocation, splitting and merging behavior of axonal volumes is in agreement with studies on synaptic vesicle protein transport vesicles (STVs) and piccolo-basson transport vesicles (PTVs) in developing neurons (Kraszewski, Mundigl et al. 1995; Dai and Peng 1996; Nakata, Terada et al. 1998; Ahmari, Buchanan et al. 2000; Zhai, Vardinon-Friedman et al. 2001; Shapira, Zhai et al. 2003; Sabo, Gomes et al. 2006). However, despite the similarity in behavior, it remains unknown if the detected volumes resemble particles similar to STVs or PTVs. Rearrangements of clustered volumes are reported to be predominant mechanism for assembling nascent synapses (Kraszewski, Mundigl et al. 1995; Shapira, Zhai et al. 2003; Ziv and Garner 2004), but their contribution to presynaptic formation has only rarely been described (Krueger, Kolar et al. 2003). Other reports proposed exchange processes on the single molecule level to regulate the presynaptic protein content (Tsuriel, Geva et al. 2006). My experiments extend the current knowledge by reporting the two processes side-by-side in mature networks and under physiological conditions.

It is possible that the volume rearrangements reported in my thesis underestimate the actual number of rearrangements and that the imaging rate of one frame per 30 minutes temporally undersamples. To assure the same identity of the volume between imaging sessions, I included only volumes with a maximum velocity of 4 µm / 30 min, a rate that is considerably slower than the velocity reported for mobile STV and PTVs between pause sites (0.1-1 µm/sec). (Kraszewski, Mundigl et al. 1995; Nakata, Terada et al. 1998; Ahmari, Buchanan et al. 2000; Kaether, Skehel et al. 2000; Ziv and Garner 2004). It is thus possible that I only observed a slow subpopulation of all motile axonal volumes and thus underestimate the contribution of relocalization processes. However, I had also performed experiments with faster imaging rates on VGluT-1-Venus Gähwiler cultures (1 image / 5 minutes) where I did not detect a higher amount or a faster rate of relocalizations (data not shown). The difference between my observed velocity and the velocity of PTVs and STVs can potentially be explained by their pausing behaviour. As the total velocity of varicosities is likely dominated by the number of pauses and pause duration, it is possible that changing the pausing behavior leads to an overall slower motility of axonal volumes.

5.1.2 Dependence of baseline turnover on protein synthesis and degradation

To investigate cellular mechanisms of axonal varicosity turnover, I tested the effect of acute protein synthesis and degradation blockade on the loss and gain of varicosities. My experiments reveal that turnover of axonal varicosities was independent from acute protein synthesis and degradation. As the rates of gain and loss remain unaltered during acute protein synthesis and degradation blockade, the data indicates that new varicosities can emerge without the generation of new proteins, and that axonal varicosities can disassembly without degrading proteins. Also, the relative fractions of the distinct turnover types remained unchanged, indicating that none of the required protein synthesis or degradation. The data infers that the constituents for axonal varicosities preexisted before their formation and that they can persist after their disassembly, implying a shared pool of presynaptic varicosity proteins. This hypothesis is substantiated by reports that single molecules and synaptic vesicles can be shared amongst synapses (Chi, Greengard et al. 2001; Darcy, Staras et al. 2006; Tsuriel, Geva et al. 2006). Alternatively, it would also be possible that presynaptic constituents directly recycle without contributing to a 'buffering' pool. However, the loss of an axonal varicosity was not always accompanied by the gain of another varicositiy in the same axon and vice versa, and varicosity turnover was not balanced within single axons, arguing against this hypothesis. Moreover, the presynaptic turnover rates were maintained during acute blockade of protein synthesis or degradation for one or four hours, suggesting that the available protein pool is large and stable enough to persist even when it is not replenished for longer periods. The hypothesis of a presynaptic protein pool leads to new hypotheses. For example, a shared pool of proteins may lead to competition amongst synapses for the same proteins - a similar mechanism has been reported on the postsynaptic side (Fonseca, Nägerl et al. 2004).

I document that gain and loss of axonal varicosities remains unchanged during protein synthesis and degradation blockade, arguing against the possibility that the constituents of varicosities are acutely synthesized or degraded at the site of the dynamic varicosity. The data is in agreement that local synaptic translation has been reported in many

cases for postsynaptic densities, but not for presynaptic axonal varicosities (Martin 2004; Martin and Zukin 2006; Schuman, Dynes et al. 2006; Bramham and Wells 2007). The presented data argue against the requirement of acute presynaptic local protein synthesis and degradation for baseline varicosity turnover.

Neuronal viability during blockade of protein synthesis and degradation

The aim of my baseline experiments was to observe a constitutive varicosity turnover that corresponds to a functionally stable neuronal population without net changes in synaptic transmission. I therefore subjected all experiments to strong quality criterions to assure neuronal healthiness and unchanged electrical properties. First, all experiments with unstable electrophysiological properties were discarded, and I included only those baseline experiments where synaptic transmission remained greater than 80% of the initial values. This decline in synaptic transmission has been frequently reported (Frey, M et al. 1988; Fonseca, Nägerl et al. 2004; Fonseca, Nägerl et al. 2006; Fonseca, Vabulas et al. 2006; Becker, Wierenga et al. 2008). Second, I excluded experiments that exhibited morphological signs of degradation, such as axon blebbing or axon disintegration. Third, the age of the slice cultures was chosen accordingly to monitor a functional mature network with stable transmission properties. At this age (10-20 days in vitro), the gross of synapse formation has already taken place and the neuronal population is intact and healthy. In my experiments, the turnover of synapses is nearly balanced, with a net increase of 0.5 synapses / 100 µm and corresponding to what has been reported in this culture system before (Becker, Wierenga et al. 2008). To ensure that neuronal viability was not impaired by the pharmacological treatments, I applied the pharmacological inhibitors for a duration of one hour, a treatment that has been frequently performed without affecting neuronal viability (Sajikumar and Frey 2003; Fonseca, Nägerl et al. 2004; Fonseca, Vabulas et al. 2006; Fioravante, Liu et al. 2008). Furthermore, the simultaneous field recordings within the same experiments reassured the healthiness of the neuronal population. Conversely, the duration of pharmacological inhibitor application has already been reported to be effective in hippocampal slices (Fonseca, Vabulas et al. 2006).

In summary, the data of this chapter strongly implies that baseline turnover is independent of protein synthesis and degradation and relies on the recycling of presynaptic material. It suggests the existence of a presynaptic protein pool for baseline presynaptic structural plasticity.

5.1.3 LTD-induced turnover of axonal varicosities

The presented data confirmed previous findings that LTD induction enhances varicosity turnover. Until recently, the effect of functional synaptic plasticity on presynaptic axonal varicosities had remained elusive, but it was intuitively hypothesized that LTD would lead to a loss of presynaptic varicosities, paralleling postsynaptic spine plasticity (Nägerl, Eberhorn et al. 2004). Surprisingly, a recent study from this laboratory reported that the induction of LTD enhanced both assembly and disassembly of varicosities without reducing their total numbers (Becker, Wierenga et al. 2008). The data of my thesis provide an important confirmation of the previous findings from Becker et al. and extend them by reporting that not all types of turnover are activity-dependent: While *de novo* gain and straight loss are significantly enhanced by LTD, relocalization processes remain largely unaffected. It is possible that *de novo* gain and straight loss are regulated differently than relocalization processes. However, it is also possible that the lower numbers of relocalizations in combination with a high variance of the data mask an effect, and further testing would be required to fully answer this question.

5.1.4 Dependence of LTD-induced turnover on protein synthesis and degradation

The data of this thesis reports that ubiquitin-dependent protein degradation was not required for LFS-induced LTD, which is in marked contrast to a report in the literature (Colledge, Snyder et al. 2003), where comparable concentrations of lactacystin in acute slices abolished LTD. However, while the effect of protein synthesis on LTD is widely established, the effect of protein degradation has not been reproduced in the literature thus far, and my experiments are in agreement with unpublished findings of Schuman et al. Colledge et al. used a different LTD-inducing protocol (intracellular pairing of pre- and postsynaptic potentials) as well as a similar yet different experimental system which

potentially explains the diverging results. However, my data put a general requirement of protein degradation for LTD into question.

5.1.5 Dependence of LTD-induced turnover on protein synthesis and degradation

It has previously been demonstrated that blocking protein synthesis or protein degradation impairs functional synaptic plasticity without affecting baseline transmission (Frey, M et al. 1988; Sajikumar and Frey 2003), but it remained unknown if the two processes are also important for plasticity-induced presynaptic structural dynamics. The presented data reports that protein synthesis and degradation critically regulate the LTD-induced turnover of presynaptic varicosities and imply that LTD-induced presynaptic structural plasticity involves the active regulation of cellular processes. While the constitutive turnover can rely on a pool of presynaptic proteins, the plasticity-induced turnover requires synthesis and degradation of novel factors, implying the need for presynaptic structural plasticity factors which distinguish plasticity-specific turnover from baseline turnover. A similar concept has already been established for postsynaptic neurons, where protein synthesis of plasticity factors is required for the stable expression of functional and structural synaptic plasticity (Frey and Morris 1997; Steward and Worley 2001; Bramham and Helen 2007). I here report that a similar requirement of protein synthesis and degradation exists for presynaptic structural plasticity. While it was elucidated for the postsynaptic side that plasticity factors must exert their functions directly at the postsynaptic spine (Bramham, Worley et al. 2008), it remains unknown if the presynaptic proteins are required also locally, or if they affect cell-wide mechanisms.

I report that the effects of protein synthesis and degradation blockade are complex and indirect, and blocking protein degradation abolished not only the loss, but also the gain of varicosities, documenting that blocking protein synthesis does not only acts on degrading but also on assembling varicosities. Conversely, blocking protein synthesis had a prominent effect on the disassembly of varicosities. Both findings indicate that the processes are involved in upstream mechanisms that affect both sides of turnover.

Notably, the blockade of protein synthesis only partially reduced the plasticity-dependent gain of presynaptic varicosities, and this incomplete effect was not caused by the duration of anisomycin application, as similar experiments with four hours of anisomycin application yielded identical results. Although the cause of the partial reduction remains elusive, it is not surprising with regards to the indirect role of protein synthesis and degradation on structural plasticity. Also, turnover levels do not completely break down but return to the constitutive turnover levels, indicating no interference between baseline and activity-dependent structural plasticity. This independence may enable the selective modulation of presynaptic structures in synaptic plasticity without altering the baseline properties of the hippocampal network.

This thesis provides data about the functional expression of LTD without an elevated loss of axonal varicosities. In my experiments, functional LTD was induced and maintained for two hours while the structural turnover was abolished. However, I detected an increase of fEPSP sizes at the end of some experiments, possibly caused by the intrinsic experimental variability. Further experiments would be required to clarify this instability. However, I here report that LTD is induced and maintained for two hours without the corresponding loss of presynaptic axonal varicosities. The data highlights the question if structural plasticity contributes to the functional expression of the ongoing synaptic plasticity or if it rather contributes to the potential of the network for future plasticity. The results indicate that the loss of synaptic structures is not required for the current expression of LTD.

5.1.6 Functionality of axonal varicosities

In my thesis, I visualized the assembly and disassembly of presynaptic varicosities along Schaffer collateral axons that are indicative of presynaptic terminals. Several lines of evidence suggest that the majority of axonal varicosities represent functional presynaptic specializations: First, presynaptic specializations of Schaffer collaterals are localized in axonal varicosities (Palay 1956), and I carefully opted for structures that resembled the reported morphology (Ziv and Garner 2004). Second, a recent study within this laboratory that engaged the same labeling and detection techniques demonstrated that 89% and 80% of all axonal varicosities colocalized with the

presynaptic marker proteins Synapsin and VGluT-1, respectively (Becker, Wierenga et al. 2008). This study also confirmed the presence of the presynaptic constituents on the ultrastructural level and confirmed a Ca^{2+} concentration rise associated with action potential firing in presynaptic varicosities, an important functional criterion for presynaptic function. Finally, in the second chapter of the results, I report that 90.4% of all morphologically identified varicosities colocalized with VGluT-1-Venus fluorescence, a strong indicator for potentially functional synapses.

While the given data strongly imply the synaptic identity for the total population of axonal varicosities, the synaptic nature of dynamic varicosities remains more elusive, and I addressed this aspect in further detail in the second part of the results. To select potentially functional varicosities, I only subjected those varicosities to analysis that were stably localized (movements less than 1 µm / h) for at least 30 minutes (two consecutive time points). I excluded the more mobile structures as stable localization is an essential prerequisite for synaptic transmission, because synapse formation requires a stable physical contact between presynaptic varicosity and postsynaptic spine. This contact must be established and requires the alignment of presynaptic active zone and the postsynaptic density before proper functioning. The formation of a postsynaptic density has been described to be completed within 30 minutes (Nikonenko, Jourdain et al. 2003), and Becker et al. have shown that the majority of new varicosities exhibit Ca^{2+} transients 30 minutes after their initial appearance (Becker, Wierenga et al. 2008). Finally, it has also been reported that newly formed varicosities can become release-competent within this time (Friedman, Bresler et al. 2000), and an even faster time course was indicated by Krueger et al. (Krueger, Kolar et al. 2003).

In summary, different lines of experimental evidence indicate that my analysis considers axonal varicosities that are likely to represent functional presynaptic specializations. Nevertheless, it remains an open and important question if dynamic varicosities are functional and contribute to a functional synapse with a postsynaptic partner.

5.2 Functional status of static and dynamic axonal varicosities investigated by VGluT-1-Venus time-lapse imaging

The contribution of morphological plasticity of postsynaptic spines to the formation of functional synapses has recently been investigated (Knott, Holtmaat et al. 2006; Nagerl, Kostinger et al. 2007), but the functional status of dynamic presynaptic varicosities remained largely unknown to date. As the development of novel microscopy and labeling techniques since recently allows to study presynaptic structural turnover in combination with functional indicators of presynaptic terminals in physiological networks, I set out to better understand the functional status of static and dynamic axonal varicosities. In the second part of my thesis, I performed time lapse two photon microscopy of a novel knock-in mouse line in combination with acute labeling of morphological structures to simultaneously monitor presynaptic morphology and the dynamics of VGluT-1-Venus (Vesicular glutamate transporter 1 - Venus) which served as synaptic marker in my experiments. The presented data contribute to the current knowledge by describing that a considerable fraction of new varicosities rapidly (< 4h) acquired the synaptic marker protein that indicates the potential for presynaptic function. The observations strongly suggest that formation of morphological varicosity volumes in the axon typically precedes accumulation of VGluT-1-Venus, and conversely, that VGluT-1-Venus accumulation is lost prior to varicosity disassembly. These data provide novel insights into the potential of dynamic axonal varicosities to become functional, and document the time course and sequence of presynaptic assembly by time-lapse imaging in physiological networks. I report that VGluT-1-Venus accumulations can coalesced in parallel with the fusion of morphological volumes, indicating that functional constituents of synaptic varicosities can undergo recycling and that fusion of axonal varicosity potentially alters synaptic function. The presented data links structural dynamics to functional changes and enhances the understanding of functional consequences of structural plasticity. Finally, the results describe the VGluT-1-Venus

knock-in mouse as a well-suited system for the simultaneous investigation of presynaptic structure and a presynaptic marker protein with single synapse resolution.

5.2.1 VGluT-1-Venus as synaptic marker

To investigate the synaptic protein content of axonal varicosities and to better understand their functional status, I combined morphological volume labeling of CA3 axons with monitoring a synaptic marker protein. As the physiological significance of this approach critically relies on the employed synaptic marker, it was initially tested to what extent VGluT-1-Venus was adequate for the intended use. Several lines of evidence confirm that VGluT-1-Venus specifically and reliably labeles presynaptic sites and serves as a strong indicator for presynaptic functionality.

First, endogenous VGluT-1 protein has been reported as a synaptic maker: VGluT-1confers glutamate uptake into synaptic vesicles in presynaptic terminals (Takamori, Rhee et al. 2000) and is specifically localized to glutamate-filled synaptic vesicles in presynaptic terminals. VGluT-1 is the prevalent isoform of vesicular glutamate transporters in the hippocampus (Fremeau, Troyer et al. 2001; Takamori, Rhee et al. 2001) and essential for synaptic transmission of CA3 neurons. The total amount of VGluT-1 is thought to determine the amount of glutamate release from a single synaptic vesicle, determining the quantal size of synaptic neurotransmitter release (Wilson, Kang et al. 2005). Vesicular glutamate transporters are thought to be the most reliable way of visualizing sites of glutamate release (Takamori 2006). Therefore, VGlut-1 is a strong indicator for the presence of glutamate-filled synaptic vesicles at presynaptic sites. However, as a small fraction of hippocampal neurons partially or exclusively expresses VGluT-2 (Wojcik, Rhee et al. 2004; Etienne Herzog 2006), it remains possible that a small fraction of functional varicosities within my experiments do not contain VGluT-1. Although VGluT-1 strongly indicates the presence of synaptic vesicles at presynaptic sites, it cannot prove neurotransmitter release or presynaptic functionality, and it does not convey information about the presence of an opposed postsynaptic partner.

Next, the presented two-photon data strongly suggest that the VGluT-1-Venus expression resembled the expression of the endogenous VGluT-1 protein. VGluT-1-

Venus was expressed throughout all areas of the Gähwiler cultures in a punctate manner. The density of VGluT-1-Venus puncta corresponds with reported synaptic density in rat cortex, and the size of individual puncta is slightly smaller than the size of individual synaptic terminals within my experimental system (Figure 4-12). The observation of larger and complex shaped structures is likely due to the resolution limit of the microscope (0.37 µm in x / y dimension and 1.38 µm in z dimension at the present setup). The detection of complex structures is therefore in agreement with the narrow distribution of presynaptic terminals in the tissue. The expression pattern is devoid of oval structures that are strongly suggestive of pyramidal cell bodies by their size, shape and parallel arrangement, and Calcein-AM clearly identified a subset of oval structures as CA3 somata. Thus, the expression pattern is in line with the reported absence of VGluT-1 in the cell soma (Wojcik, Rhee et al. 2004).

Finally, I report that 90.4 % of all morphologically labeled presynaptic terminals colocalized with VGluT-1-fluorescence, indicating its specific localization to presynaptic varicosities. In summary, the detected VGluT-1-Venus expression is consistent with the expression pattern of the endogenous protein. Nevertheless, a thorough comparison of VGluT-1-Venus expression and the endogenous protein by biochemical means will be essential to further test the applicability of the experimental system to mark functional presynaptic sites.

Taken together, the function and localization of endogenous VGluT-1 in combination with the physiological expression pattern of VGluT-1-Venus strongly indicates that VGluT-1-Venus serves as synaptic marker. VGluT-1 primarily reports on the glutamate content of synaptic vesicles and the quantal strength of synaptic transmission. Moreover, it strongly indicates the size of the present synaptic vesicle pool, correlates to the strength of the synapse and documents the potential for synaptic function without proving functionality or conveying information about the apposed postsynaptic partner.

5.2.2 VGluT-1-Venus content of stable axonal varicosities

I report that a high fraction (90.4%) of all morphologically identified axonal varicosities colocalized with VGluT-1-Venus fluorescence, suggesting their potential for

functionality. The data is in line with previous reports that almost all varicosities of mature neurons comprise presynaptic molecular specializations and are functional (Palay 1956; Yao, Qi et al. 2006). Importantly, the reported numbers of the analysis are estimates that are influenced by intrinsic and methodological characteristics and possibly include false positive or false negative counts. First, the detection thresholds during image analysis may not detect weakly labeled structures or include non-specifically overlapping structures. To estimate the number of unspecific colocalization, a comparison of specific versus rotated data was performed. The results clearly indicate that the majority of puncta are specifically localized as opposed to the rotated data that yielded a much lower colocalization. Still, a comparison of rotated versus non-rotated data will be required to validate the obtained data for each separate image analysis. Next, intrinsic properties may also lead to false negative counts, for example if only few synaptic vesicles are present as presumed for nascent synapses during development or for small synapses (Palay 1956; Ahmari, Buchanan et al. 2000). Their emitted fluorescence may remain below the sensitivity of the microscopy system. It thus cannot be excluded if the VGluT-1-Venus negative fraction contains subthreshold amounts of VGluT-1-Venus. It would furthermore be interesting to test to what extend the VGluT-1-Venus containing presynaptic vesicles represent release competent presynaptic sites or functional synapses. Technically challenging experiments such as FM staining within Gähwiler cultures or simultaneous presynaptic stimulation and postsynaptic Calcium-imaging would be required to address this question. Finally, I report the accumulation of VGluT-1 as tube-like structures, suggesting the dense accumulation around postsynaptic dendrites. It would be interesting to clarify if the postsynaptic partner is excitatory or inhibitory.

In summary, the experiments report that the vast majority of morphologically identified presynaptic varicosities contain the synaptic marker VGluT-1-Venus, indicating their potential for functionality. This finding is in line with multiple previous reports that the vast majority of axonal varicosities represent presynaptic terminals (Palay 1956; M. B. L. Yeow 1991; Tsuriel, Geva et al. 2006; Yao, Qi et al. 2006; Becker, Wierenga et al. 2008). More importantly, it strongly indicates that the morphologically identified axonal

varicosities in the first part of my thesis indeed represent potentionally functional presynaptic specializations.

5.2.3 VGluT-1-Venus content of newly assembled axonal varicosities

To date, it remains largely unknown how generation of axonal varicosities relates to synaptogenesis or to the formation of functional presynaptic terminals in mature networks (for review see (Ziv and Garner 2004)). The results of this thesis contribute to the current knowledge by documenting that a substantial fraction of axonal varicosities can rapidly acquire synaptic markers and by suggesting that the acquisition of this marker is delayed to the formation of the morphological volume.

In my experiments, 55% of all newly formed varicosities rapidly (<4h) acquired detectable levels of VGluT-1-Venus fluorescence (21 of 38 observations). The observed VGluT-1-Venus puncta were typically smaller than the VGluT-1 fluorescence of neighboring varicosities. As the amount of VGluT-1 scales with the amount of neurotransmitter per synaptic vesicle release (Takamori, Rhee et al. 2000; Wilson, Kang et al. 2005), the results are in agreement with the hypothesis that newly formed presynaptic terminals were weaker than stable synapses. The data corresponds with reports that newly formed varicosities are substantially smaller than stable varicosities (Becker, Wierenga et al. 2008) and that the size of presynaptic varicosities positively correlates with the size of the synaptic vesicle pool (Pierce and Mendell 1993). Further experiments over longer imaging periods may be helpful to determine if the low VGluT-1-Venus expression reflects a transient and growing state. Along the same lines, it remains to be tested if the VGluT-1-Venus negative varicosities proceed to acquire VGluT-1-Venus or if they remain VGluT-1 negative and thus probably non-functional. The majority of experiments sampled only two time points, and thus it is possible that the 55% of VGluT-1-Venus containing varicosities represent an older and more mature population than the remaining 45%. Moreover, I also document that 92% of all new varicosities initially did not contain detectable levels of VGluT-1-Venus. This data strongly imply that VGluT-1 is typically not accumulated at the axonal site prior to varicosity formation. It suggest a model where the formation of the morphological volume precedes the accumulation of synaptic markers, consistent with reports that the

building of well-formed active zones and the accumulation of synaptic vesicles at nascent presynaptic sites is typically delayed by 2 to 3 hours (Ahmari, Buchanan et al. 2000; Friedman, Bresler et al. 2000). I present two detailed time-lapse examples of assembling varicosities that further support this hypothesis. In one example, VGluT-1-Venus accumulated at the newly formed varicosity with a delay of 1 to 1.5 hours. In the other example, VGluT-1-Venus did not accumulate until the end of the imaging period. Interestingly, it remains possible that the second varicosity would have acquired VGluT-1-Venus with a similar time course, later than the last imaging time point. Further extending the time-lapse experiments and obtaining more detailed time courses would allow to clarify the time course of synaptic marker accumulation, as well as the fraction of potentially functional new varicosities.

In summary, the data strongly infer that a subset of dynamic varicosities can acquire the potential for functionality. This finding intimately links structural and functional plasticity and highlights the importance of my previous findings on dynamic varicosities in the first part of my thesis. Also, it suggests a model that accumulation of functional markers follows the morphological dynamics.

5.2.4 VGluT-1-Venus content of instable axonal varicosities

To date, the knowledge about the functional status of axonal varicosities destined for disassembly remains very limited. This gap of knowledge is possibly due to experimental difficulties that prohibited to identify in advance the presynaptic structures that will be disassembled. The VGluT-1-Venus mouse system allows to simultaneously monitor high numbers of axonal varicosities and to retrospectively identify instable varicosities, and thus overcomes this difficulty.

In this thesis, I provide preliminary data about the functional status of instable axonal varicosities prior to their disassembly by documenting a few examples of axonal varicositiy loss (n=7). I report that in six out of seven observations the axonal varicosity did not comprise VGluT-1 accumulation before disassembly, and in no observation VGluT-1 was retained in the axonal area at detectable amounts. The data are in agreement with reports that synaptic vesicle accumulations are typically found outside

of bulbus-shaped axonal varicosities, not along the axon itself (Pierce and Mendell 1993; Kraszewski, Mundigl et al. 1995). Likewise, I observed a VGluT-1 negative varicosity that revealed instable properties in the detailed time lapse analysis whereas I never observed an instable axonal varicosity that instantly obtained VGluT-1-Venus. Despite low numbers, the observations of varicosity loss are in agreement with the hypothesis that VGluT-1-Venus is typically lost prior to varicosity disassembly and that a stable localization of morphological volume is required for VGluT-1 accumulation. Further increasing the number of observations and obtaining further time-lapse data will be required to strengthen this hypothesis. Furthermore, it would be interesting to resolve if the loss of VGluT-1-Venus is correlated to a loss of capability for neurotransmitter release. While it is possible that synaptic function is lost before morphological disintegration, it remains also possible that the axonal area of varicosity loss retains few but sufficient synaptic vesicles for neurotransmitter release. (Ahmari, Buchanan et al. 2000; Krueger, Kolar et al. 2003).

Finally, the observation of the parallel merging processes of two axonal varicosities and two individual VGluT-1-Venus puncta is a suggestive example that relocalization of morphological volumes can be accompanied by relocalization of synaptic markers. If this hypothesis held true, it would highlight the importance of merging and dividing processes for rapidly regulating presynaptic strength. Moreover, it supports the hypothesis of sharing and recycling of presynaptic constituents amongst synapses (Chi, Greengard et al. 2001; Tsuriel, Geva et al. 2006; Levitan 2008).

In summary, the results support a model that morphological volume assembly of presynaptic varicosity precedes the acquisition of functional synaptic markers, and likewise that the disassembly of the morphological volume is delayed to the loss of functional markers. Further experiments along the same lines will be required to test if the observed examples represent general mechanisms.

6 Conclusion and outlook

Activity-dependent changes of neuronal circuitry are thought to provide the key to understanding the cellular mechanisms of learning and memory. Advances in live-cell imaging techniques, notably two-photon microscopy, have permitted the study of the structural correlates of synaptic plasticity under physiological conditions at single synapse resolution. In my thesis, I provide novel insights into the phenomenology and mechanisms of structural plasticity of presynaptic axonal varicosities in CA3 hippocampal neurons.

I report that distinct types of structural dynamics contribute to the plasticity of axonal varicosities, indicating the potential to gradually or step-wise change the size of presynaptic structures.

My thesis provides novel data that the baseline turnover of axonal varicosities is independent of protein synthesis and degradation, implying that preexisting plasticity proteins are used and recycled during presynaptic structural plasticity. This finding is in agreement with the existence of a common pool of preexisting proteins that may be shared amongst presynaptic terminals. Moreover, the dependence of LTD-induced presynaptic structural plasticity on protein synthesis and degradation suggests the need for additional factors that mediate activity-dependent presynaptic structural plasticity. The finding strikingly parallels the existence of postsynaptic plasticity factors and may lead to formulating a new concept of yet unknown presynaptic rules of synaptic plasticity. Future experiments will be required for testing the functional consequence of presynaptic structural plasticity factors.

Next, my experiments demonstrate that functional plasticity can be induced without an elevated loss of presynaptic varicosities, meaning that structural plasticity of axonal varicosities is not required for LTD expression. The data raise the question if dynamic axonal varicosities contribute to the expression of present LTD or if they become functionally relevant in future synaptic plasticity.

By providing evidence for the functional status of dynamic axonal varicosities, the results of this thesis amend the current knowledge about the functional relevance of structural plasticity. My observations show that new axonal varicosities can rapidly acquire a synaptic marker, strongly suggesting their potential for presynaptic functionality. The data indicates that morphological assembly of axonal varicosities typically precedes the accumulation of synaptic markers and, conversely, that synaptic markers are lost prior to varicosity disassembly. In other words, the presence of the morphological structure temporally encloses the existence of synaptic markers and well-formed presynaptic terminals, possibly providing the basis for their formation.

My experiments highlight the potential of the genetically engineered VGluT-1-Venus mouse model to investigate the molecular assembly of dynamic synaptic structures on the level of single synapses. Combining this model with other methods such as pH-sensitive fluorescent molecules, split-GFP and calcium imaging is bound to shine new light on the link between morphological and functional synaptic plasticity.

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Seit 10/2005 **International Max Planck Research School, München, Deutschland**
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- Untersuchung zellulärer Mechanismen von Lernen und Gedächtnis mit Hilfe von 2-Photonen Mikroskopie.
- Internationale Kollaboration mit jährlichen Meetings.
- Kurse und Konferenzen:
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  - Imaging Kurs, Cold Spring Harbour Laboratories, NY, USA.
- Stipendium der Studienstiftung des deutschen Volkes.

9/2004 – 8/2005 **Ludwig Institute for Cancer Research, Melbourne, Australien**

- **Diplomarbeit.** Titel: Prostaglandin E2 is a Potent Regulator of Multiple functions in Human Dendritic Cells.
- Immunologie. Dendritische Zellen und der Prostaglandin Signalweg. Arbeit im Rahmen einer klinischen Studie.
- Mitautoren einer wissenschaftlichen Publikation.
- Auslandsstipendium der Studienstiftung des deutschen Volkes**.**

10/1999 – 7/2005 **Biologiestudium (Diplom), RWTH Aachen, Deutschland**
Diplom mit Auszeichnung.

- Diplomarbeit und alle Fächer: sehr gut / 1.0
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8/1990 – 7/1998 **Kaiser-Karls-Gymnasium, Aachen, Deutschland**

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- **Industriepraktikum.** Thema: Hypoxia inducible factor 1 transcriptional regulation in ischemic tissue.
- Molekularbiologie. Analyse und Modulation der Genexpression eines Onkogens.
- Einblick in die Forschung und Entwicklungsabteilung eines Pharmaunternehmens.
- Stipendium des Japan International Science and Technology Exchange Center: Research experience for European Graduate Students in Science and Technology. Das Programm finanziert jährlich europaweit zwölf Studenten.

9/2002 – 10/2002 Baylor College of Medicine, Houston, USA

- **Praktikum.** Thema: Molecular mechanisms of gene regulation during epidermal differentiation.
- Molekularbiologie. Klonierung und Genexpressionanalysen.
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5/2002 – 8/2003 Institut für Pflanzenphysiologie, Aachen, Deutschland

- **Forschungsprojekt.** Thema: Protein targeting in plant chloroplasts and analysis of protein interactions.
- Molekularbiologie. Proteinanalyse, Proteinvernetzung, Yeast-two-hybrid System.
- Zweitautor einer wissenschaftlichen Publikation im Biochemical Journal (2002).

10/2001 – 4/2002 Institut for Ökotoxikologie, Aachen, Deutschland

- **Forschungsprojekt.** Thema: Genexpressionsanalysen.
- Molekularbiologie.
- Einführung und Etablierung einer molekularbiologischen Methode am Institut für Ökotoxikologie.
- Erste praktische Forschungstätigkeit im Grundstudium.

Publikationen

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Robson, Neil C. et al., Activin-A: a novel dendritic cell-derived cytokine that potently attenuates CD40 ligand-specific cytokine and chemokine production. *Blood* **111** (5), 2733 (2008).

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2003	Japan International Science and Technology Exchange Center Stipendium des REES Program: Forschungserfahrung in einer Firma in Japan.
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1997	Förderung europäischer Forschungsprojekte der Bayer AG: Untersuchung und Bestandsaufnahme der Heilwirkung einheimischer Heilpflanzen im Pilion (Griechenland).

Weiteres Engagement

- Organisation eines Bewerbungsworkshops (4 / 2008).
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- Mitorganisation eines Doktorandensymposiums mit 500 Teilnehmern (2007, 2009).
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