The actin-binding protein profilin II in neuronal plasticity

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I. SUMMARY

Activity-dependent plasticity in neurons involves changes in synaptic transmission and connectivity. These changes lead to altered neuronal circuit properties and are thought to underlie learning and memory. Transcription and protein synthesis are indispensable in order to maintain changes in neural circuitry over periods of several hours or longer. Therefore signaling from the synapse to the nucleus is required to control activity-dependent expression of RNA and proteins which have to be transported back to the activated synaptic sites.

The small actin-binding protein profilin has been shown to accumulate in postsynaptic dendritic spines of pyramidal neurons as a necessary element in activity-dependent stabilization of synaptic morphology, a putative anatomical correlate of changes in transmission strength. In this work I show that profilin also enters the nucleus in an NMDA receptor and Ca^{2+} dependent manner. However, in contrast to spine targeting, nuclear enrichment is reversible within minutes after removal of the stimulus. Nuclear accumulation of profilin is likely coupled to activity-dependent actin polymerization at the cell cortex which also takes place in response to NMDA receptor stimulation.

Nuclear profilin has been implicated in different steps of gene expression including transcription and pre-mRNA splicing. Activity-dependent nuclear and synaptic accumulation suggests profilin to be involved in different aspects of neuronal plasticity. To this end, I introduce approaches to elucidate profilin function in experience-dependent plasticity and gene expression.

II. INTRODUCTION

II.1. Neuronal plasticity and memory

Neurons convey information by transmitting electrical signals. Any information reaching our nervous system via sensory organs and needed to be processed will therefore be translated into electrical signals. Processing of information, be it selecting necessary from unnecessary information, storage, or retrieval, requires changes in electrical circuits. Neurons as cells of our bodies contain all the necessary elements to mediate changes in neuronal circuitry: They can modify or replace transmitter molecules at cell-cell junctions or channel molecules necessary for transmission along the cell, or can even grow new connections or retract old ones. These diverse properties of its constituents provide the nervous system with the ability to modulate electrical circuitry and ultimately to adapt to input changes, a property referred to as plasticity. Cellular and molecular adaptive changes, i.e. plasticity at a cellular and molecular level, should therefore lead to changes in electrical circuitry and ultimately to behavioral plasticity. This is tested in contemporary neuroscience research on various models of learning and memory, partly because learning of a new task is a behavioral output which can be tested according to defined criteria in genetic model organisms. Importantly, this allows neuroscientists to relate higher cognitive functions to changes in electrical circuits and eventually to molecular properties of a cell.

II.1.1. Synaptic plasticity

Connection points between neurons, synapses, are likely candidates for modulating neuronal circuitry. One famous model put forward by Donald Hebb (Hebb, 1949) suggests that learning occurs when synaptic connections become more effective. In particular, Hebb postulated that synaptic connections become stronger when pre- and postsynaptic elements were stimulated simultaneously.

Almost 25 years after Hebb's hypothesis, neuroscientists came up with a physiological observation describing a phenomenon which shared many properties with Hebb's postulated mechanism for a learning-related synaptic change (Bliss and Lomo, 1973): Long-term potentiation (LTP) of synapses, which describes a long-lasting change in synaptic efficacy following strong stimulation patterns, and has initially been discovered in the hippocampal formation of the forebrain. "LTP" as referred to in the literature is sometimes used synonymously with hippocampal LTP and I shall focus on the hippocampus as the main model system in the following description, although basic properties may be similar in other brain regions, e.g. the neocortex. In fact, hippocampal LTP comprises three basic properties: Cooperativity, i.e. the need for strong stimulation to overcome a threshold for induction; associativity, meaning that even a weak input can be potentiated if it is active at the same time as a strong stimulus to a separate but convergent input; input-specificity, meaning that inputs which are not active at the time of the strong stimulus do not show potentiation (reviewed in Bliss and Collingridge, 1993). These properties, and associativity in particular, are present in Hebb's model of changes in neuronal circuitry : "The general idea is an old one, that any two cells or systems of cells that are repeatedly active at the same time will tend to become associated, so that activity in one facilitates activity in the other" (Hebb, 1949). LTP and its brother, long term depression (LTD) (Lynch et al., 1977), which describes a long-lasting decrease in synaptic efficiency following other stimuli, have therefore - due to common principles with postulates for synaptic plasticity - been proposed to form a neural basis for learning and memory (Braunewell and Manahan-Vaughan, 2001; Maren and Baudry, 1995). The molecular basis for LTP and LTD has been under investigation by molecular neurobiologists and a picture has emerged that glutamate receptors play a major role in establishing principles of cellular plasticity. LTP (and LTD) induction depends on activation of the NMDA type of glutamate receptors, which in its mode of activation bears some properties of LTP: Under resting membrane conditions, the NMDA receptor is blocked by magnesium ions (Mg^{2+}), which are released upon strong depolarization of the postsynaptic cell. Thereby only strong,

cooperatively acting stimuli lead to activation of NMDA receptors. Depolarization of the postsynaptic neuron is initiated by activation of AMPA type of glutamate receptors and subsequent influx of sodium ions (Na^+). This type of receptor has been implicated to play a role in LTP/LTD via its surface expression: If a synapse were only to express NMDA but no AMPA receptors the lack of depolarization would mean the failure of any postsynaptic response (silent synapse). However, these synapses increase the number of postsynaptic AMPA receptors in response to LTP stimuli, providing a simple model for the expression of LTP (Malinow and Malenka, 2002). LTD, on the other hand, can then be explained by removal of AMPA receptors from the synapse in response to LTD stimuli (Beattie et al., 2000; Luscher et al., 1999). Together these data implicate glutamate receptors as major factors underlying hippocampal LTP and LTD.

Are changes in receptor expression or properties the only synaptic events responsible for plasticity at the synapse? Synaptic growth is one mechanism implicated in experience-dependent plasticity, both as changes in synaptic morphology and changes in synapse numbers. However, synaptic morphology and receptor expression are no alternative concepts of plasticity, but are linked: Synapse size is one determinant of the number of synaptic glutamate receptors in the hippocampus (Matsuzaki et al., 2001; Nusser et al., 1998), and theoretical models of synaptic transmission identify the size of the synaptic zone itself as an important parameter of synaptic strength (Kruk et al., 1997). Growth of new connections has been observed in experience-dependent plasticity in the rat barrel cortex for inhibitory synapses (Knott et al., 2002). Moreover, a fraction of spine synapses on pyramidal neurons in the mouse barrel cortex and visual cortex was shown to undergo turnover in long-term *in vivo* imaging experiments (Grutzendler et al., 2002; Trachtenberg et al., 2002). Thus a change in wiring at the synaptic level emerges as a concept of experience-dependent plasticity, in addition to changes in synaptic “weight” as expressed by the phenomena of LTP and LTD.

How are changes in wiring patterns and synaptic transmission efficiency linked or do they describe alternative concepts of synaptic plasticity? This question remains unanswered, although it was shown that LTP stimuli can induce growth of new dendritic protrusions in a slice culture system (Engert and Bonhoeffer, 1999).

Morphological plasticity in postsynaptic dendritic spines is mediated by the actin cytoskeleton, which possesses the dynamic properties necessary to confer subsecond shape changes as well as growth and retraction during development (Fischer et al., 1998; Maletic-Savatic et al., 1999). Strikingly, drugs preventing proper actin assembly also interfere with formation of LTP (Kim and Lisman, 1999; Krucker et al., 2000). This together with data showing the actin cytoskeleton implicated in arrangement of synaptic signaling molecules including neurotransmitter receptors (Allison et al., 1998; Shen et al., 2000) implies actin as a mediator of synaptic plasticity and suggest it to be a necessary element in linking synaptic wiring and transmission strength. Given the importance of the actin cytoskeleton in synaptic plasticity and its central role of the work described in this thesis, I shall discuss it in more detail in a following chapter.

II.1.2. Neuronal plasticity: Pathways emerging from the synapse

Synaptic plasticity, as discussed in the previous chapter, involves molecular changes at the synapse, either by insertion or removal of proteins or by posttranslational modifications. In this regard, different molecular pathways may underlie short-term and long-term synaptic changes (McGaugh, 2000). In the long term, newly synthesized proteins may be needed to replenish stores of proteins which have been recruited to the synapse or also simply to make up for turnover of synaptically localized proteins. This is in line with observations that protein synthesis is essential for both the late phase of long-term changes in synaptic transmission and for long-term memory (Kelleher et al., 2004; McGaugh, 2000).

Proteins being used at the synapse could both be synthesized locally or in the soma and be targeted to synapses. There is evidence for both scenarios, as protein synthesis has been shown to occur in isolated dendrites and somatically synthesized proteins have been shown to localize to synaptic sites (Bresler et al., 2004; Kang and Schuman, 1996). In any case, novel protein synthesis depends on mRNA, implicating nuclear events like transcription and RNA processing in neuronal plasticity, which is in line with published data (Frey et al., 1996; Nguyen et al., 1994). Mature mRNA provides the template for somatic protein synthesis, but some mRNAs have also been shown to be transported to dendrites or even to synapses (Steward and Worley, 2001; Wang and Tiedge, 2004).

The dependence of long-term changes in synaptic strength on protein synthesis and transcription could either reflect the synthesis of specific proteins or RNAs necessary for modifying activated synapses in a way to establish them as potentiated or depressed units over a long timescale (several hours to days, weeks, or sometimes “forever”) or simply the need for exchange of these macromolecules which have a limited lifespan. Several lines of evidence argue for the former and against the latter possibility:

- Stimuli evoking long-term changes in synaptic transmission activate specific signaling pathways rather than enhancing neuronal transcription or translation globally (see below). Likewise, interference with specific pathways can block synaptic and behavioral plasticity.
- Activity-dependent gene expression comprises genes expressed at comparatively low levels in unstimulated neurons (Fagni et al., 2002).
- Genetic deletion of specific transcription factors influencing activity-dependent transcription has an impact on LTP, but not on neuronal development or basic synaptic transmission (Ramanan et al., 2005).

Different pathways have been implicated in activity dependent gene expression and long-term plasticity: (1) Ca^{2+}/calmodulin-dependent kinase pathways: Upon synaptic activity and postsynaptic influx of calcium either through NMDA or

voltage-gated calcium channels, members of the Ca^{2+}/calmodulin dependent protein kinase (CaMK) family are activated. The large holoenzyme CaMKII is recruited to synapses in an activity-dependent manner and this redistribution activates the kinase function. In its activated form, CaMKII is necessary and sufficient for the induction of LTP (Lledo et al., 1995; Otmakhov et al., 1997; Shen and Meyer, 1999). Mice with a genetic mutation in the CaMKII autophosphorylation loop, effectively inhibiting kinase activation, lack hippocampal LTP and fail to learn a spatial learning task (Giese et al., 1998). CamK IV, on the other hand, is involved in signal transduction of nuclear calcium waves and activation of transcription factors such as CREB (Hardingham et al., 2001).

(2) Ras/MAP kinase pathway: NMDA receptor-dependent Ca2+ influx activates the MAP kinase pathway, which is necessary for the late phase of LTP, expression of some immediate early genes and memory consolidation (Bozon et al., 2003).

(3) Protein kinase C (PKC): PKC isoforms are elevated in the hippocampus following induction of LTP, and inhibitors of this group of kinases specifically block persistence of LTP while leaving initial potentiation intact (Colley et al., 1990). Consistent with the effect on synaptic plasticity, infusion of PKC inhibitors into the hippocampus of rats after training induces retrograde amnesia (Jerusalinsky et al., 1994).

(4) Protein kinase A (PKA) pathway: Inhibitors of protein kinase A have been shown to disrupt the late, protein-synthesis dependent phase of LTP and impair memory when infused into the hippocampus several hours after training. PKA activity has been linked to phosphorylation of the transcription factor CREB, which likewise increases in the hippocampus after training and is implicated in memory consolidation (Bernabeu et al., 1997; Schafe et al., 1999).

Using the pathways described above, activation of postsynaptic sites leads to signaling to the nucleus, impacting upon transcription factors and activating gene expression. Some of these target genes are discussed in chapter III.2.3.

According to a hypothesis put forward by Frey and Morris, strong synaptic activation might set a molecular “tag” at the synapse. This tag would recruit target molecules which had been expressed in an activity-dependent manner, leading to a modification of the protein content of the synapse and long-term stabilization of a change in synaptic efficacy (Frey and Morris, 1998a). The model is explained in Fig. I1:

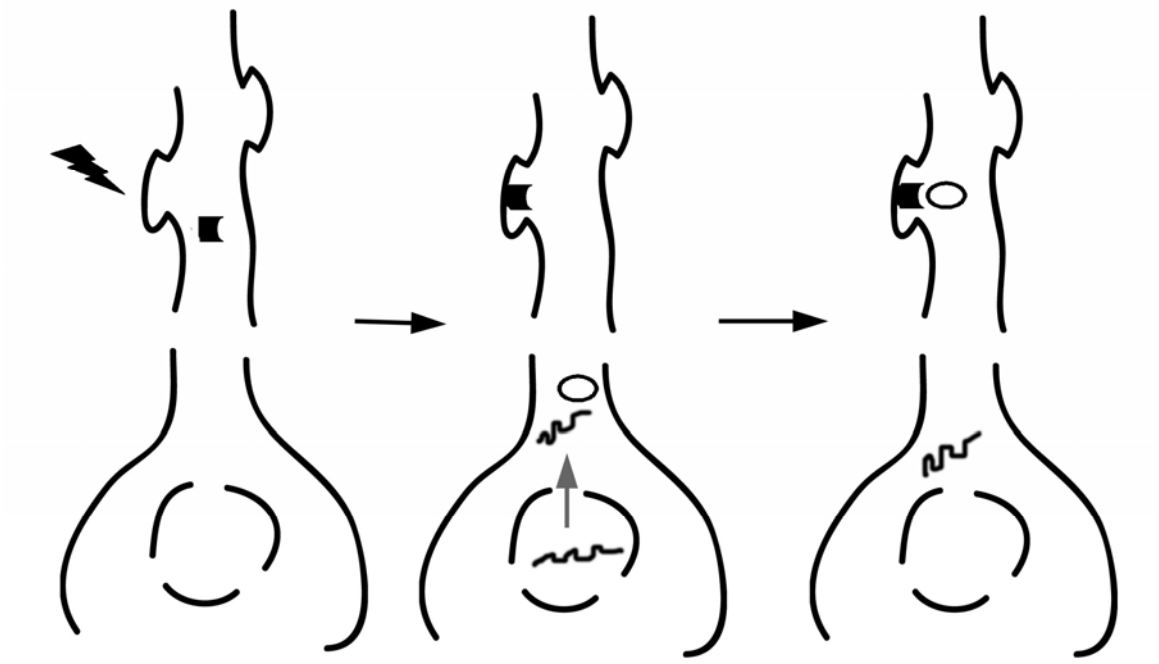

Fig. I1.: Synaptic tagging as a model for synapse-specific long-term plasticity. *Left*, a strongly activated synapse (lightning bolt) becomes rapidly enriched in a molecule (small filled symbol) present in the dendrite. *Middle*, Strong activation of the cell also leads to synthesis of RNA and proteins (empty ellipse) in the nucleus, soma or possibly dendrites. The molecule present as a molecular tag at the activated synapse then serves to recruit macromolecules expressed in an activity-dependent manner (*right*). Adapted from Frey and Morris, 1998.

II.2. The actin cytoskeleton

II.2.1. General properties

The actin cytoskeleton, equivalent to the microfilament system in mammalian cells, is one of three cytoskeletal elements, next to the microtubules and the intermediate filaments. A defining property of microfilaments, in addition to their small diameter, is their ability to change their arrangement rapidly, often within fractions of seconds (Pollard and Borisy, 2003). Electron microscopy of actin filaments in cells, pioneered by Svitkina and Borisy, shows a variety of microfilament structures ranging from finely woven meshes in lamellipodia to densely packed bundles in filopodia (Svitkina et al., 1995). These seemingly unrelated structures can be converted into one another by expression or downregulation of different kinds of actin-binding proteins, highlighting the central importance of these modulators of actin filament assembly (Mejillano et al., 2004; Svitkina et al., 2003; Vignjevic et al., 2003). Actin-binding proteins influence filament organization not by rebuilding a stiff framework, but by modulating the actin polymer assembly which is under constant renewal by exchange of subunits, a process called treadmilling.

The high degree of plasticity in actin filament assembly not only accounts for different shapes of cellular subdomains as mentioned above, but is also the reason for the variety of functions in which actin filaments are involved, including cell division, cell migration, endocytosis and muscle contraction. Moreover, globular actin does not only serve as a building block for filaments, but also has proposed nuclear functions as a monomer or in structures currently unknown (Pederson and Aebi, 2005).

II.2.2. Actin binding proteins

As mentioned above, actin filaments are under continuous turnover of their subunits even when they don't seem to grow, a process known as treadmilling. Thus one end of a particular filament shows a net loss of subunits and is therefore called the minus or shrinking end, whereas the other end shows a net gain of actin subunits and is referred to as the plus or growing end. Actin filaments can be decorated with myosin heads as a special preparation for electron microscopy, defining the plus and minus ends as barbed and pointed ends, respectively, with respect to their appearance on electron micrographs (Svitkina et al., 1995). Treadmilling is influenced by a number of actin binding proteins, leading to an enhanced treadmilling rate, which *in vivo* can be two orders of magnitude faster than for actin alone *in vitro* (Pollard and Borisy, 2003). Not surprising, whole sets of actin binding proteins are dedicated not only to treadmilling, but also to dendritic nucleation, bundling, crosslinking, capping or severing of actin filaments, and contribute to actin dynamics, as illustrated in a still simplified model for leading edge protrusion in Fig. I2.

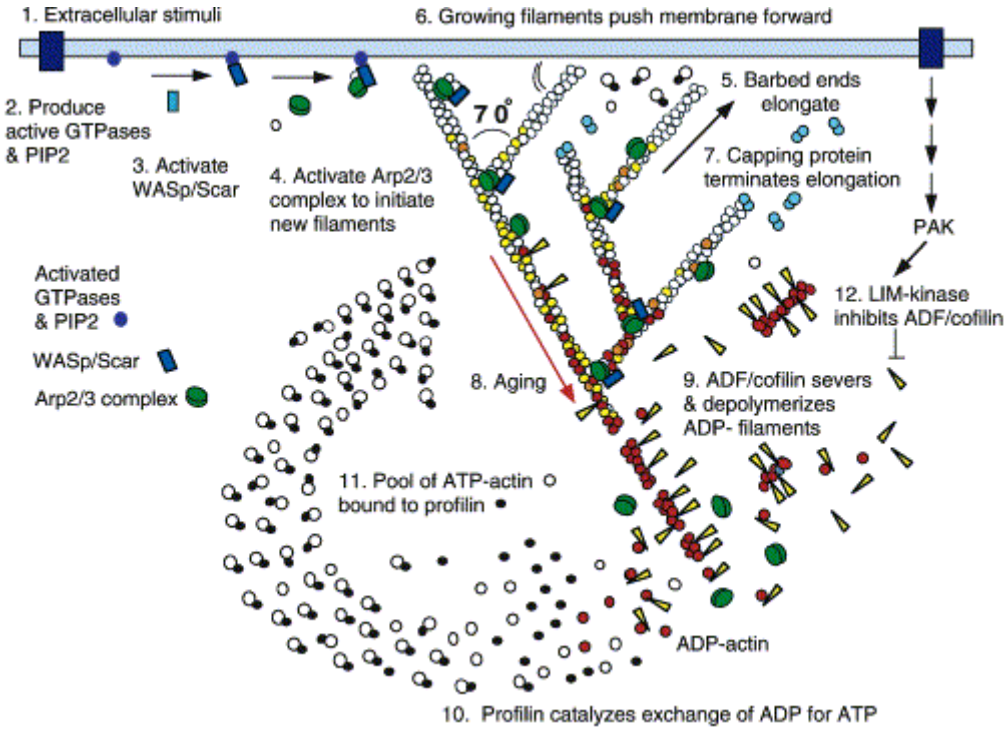

Fig. I2: A model for nucleation/array treadmilling for protrusion of the leading edge.
Growing filaments at the membrane are responsible for pushing the leading edge forward, and depolymerization at the shrinking edge of the actin cortex provides monomers for further growth. Regulatory steps are explained in the figure. From Pollard and Borisy, 2003.

Actin rearrangements at the cell cortex is performed with the cytoplasmic actin isoforms β and γ, which are expressed in all non-muscle cells, while skeletal, cardiac and smooth muscle cells use their respective α-actin isoforms (Rubenstein, 1990).
As outlined in Fig. I2, actin treadmilling is modulated by a number of actin monomer binding proteins which lead to local increase in polymerization-competent actin subunits, as the rate of actin filament elongation is proportional to the concentration of subunits (Pollard, 1986).
One factor in accelerating treadmilling is profilin, which binds actin monomers at the barbed end, catalyzes exchange of ADP in actin to ATP (Mockrin and Korn, 1980) and allows elongation of the barbed end of the filament. Profilin is regulated by a number of cellular factors via its poly-L-proline and phosphatidylinositol binding domains, as discussed in more detail in a separate chapter on profilin (II.2.5). Profilin competes for actin monomer binding with the actin-sequestering protein thymosin β4, but profilin's binding is tighter (Pantaloni and Carlier, 1993).
In the actin filament, ATP-bound actin hydrolyzes in an irreversible process to ADP-actin, marking the age of a subunit within the filament (Carlier and Pantaloni, 1986). At the pointed end, proteins of the ADF/cofilin family accelerate actin depolymerization and thus replenish the monomer pool. The concerted action of profilin, thymosin β4 and cofilin maintains a concentration of unpolymerized actin far from equilibrium, providing the cell with a monomer pool to sustain fast protrusion (cf. Fig.I2) (Pollard and Borisy, 2003).

New barbed ends for the formation of filament branches (or entirely new filaments) can be produced by three mechanisms: severing of existing filaments, uncapping of existing filaments, or *de novo* nucleation (i.e. the formation of a new filament from a nucleus of subunits) (Condeelis, 1993). For leading edge protrusion as depicted in Fig. I2, *de novo* nucleation seems to be the dominant process for which a complex of seven proteins termed Arp 2/3 complex is a central player, capping the pointed end and initiating new growth at a 70° angle (Mullins et al., 1998). As for other proteins modulating actin assembly, the importance of the Arp2/3 complex was established in Listeria motility assays which make use of the fact that certain bacteria exploit the cellular actin machinery for their intracellular movement (Welch et al., 1998). However, experiments in intact cells showed that Arp2/3 is not essential for leading edge motility, demonstrating that simplified models may not be accurate to display possibly redundant functions of a large number of actin binding molecules in a cell (Di Nardo et al., 2005).
As to severing functions, they are mainly exhibited by ADF/cofilin and by gelsolin, both of which have been shown to contribute to actin polymerization in vertebrate cells (Falet et al., 2002; Zebda et al., 2000).

Filaments grow until they are capped, hence capping limits the number of growing microfilaments and thereby funnels protrusive activity within a microdomain (Carlier and Pantaloni, 1997; Cooper and Schafer, 2000). Important molecules in this regard are capping protein/CapZ and gelsolin, which through its modular structure influences actin assembly in more than one way (Carlier and Pantaloni, 1994).

Further important functions for actin binding proteins are bundling and crosslinking, responsible for organization of individual filaments into higher order structures. Proteins falling into this category use their multiple (at least two) actin binding sites to direct the formation of either tight bundles (bundling proteins,

actin binding sites in close proximity) or loose assemblies (loose bundling, crosslinking proteins, actin binding sites further apart) (Puius et al., 1998).

II.2.3. The actin cytoskeleton in pyramidal neurons

Actin plays a major role in neuronal development regulating neurite formation and growth cone guidance (Dehmelt and Halpain, 2004; Dent and Gertler, 2003). Here I concentrate on the role of the actin cytoskeleton in mature pyramidal neurons as being relevant to the following experimental work.

Electron microscopy studies showed that actin in pyramidal neurons of the rat forebrain is mainly concentrated in postsynaptic dendritic spines, particularly at the postsynaptic density (PSD), but also in subsynaptic regions and the spine apparatus (Cohen et al., 1985; Matus et al., 1982). One study reported lower actin levels in axonal presynaptic sites associated with synaptic vesicles (Cohen et al., 1985). EM data did not report significant actin levels in the soma; however, light microscopy suggests that monomeric G-actin within the large volume of the cell body adds up detectable amounts (Friedman et al., 1998; Micheva et al., 1998), and filamentous (F-)actin accumulates in the soma upon calcium influx through synaptic receptors or upon anoxia (Friedman et al., 1998; Furuyashiki et al., 2002).

In dendritic spines, the actin filaments mediate seemingly contradictory functions: On the one hand, they exhibit resistance towards actin-depolymerizing drugs such as cytochalasins and latrunculins and stability over many hours (Allison et al., 1998), on the other hand subsecond changes in motility are also mediated by actin (Fischer et al., 1998). This has led to a model in which two types of actin filaments are present in spines: One stable pool of core actin filaments surrounded by a dynamic actin pool at the tip and cortex of the spine (Halpain, 2000).

Synaptic activity impacts upon the dynamic actin pool, stopping spine motility and imposing a round and morphologically stable spine structure (Fischer et al.,

2000). Actin-based motility is inversely correlated to mobility within the spine membrane, identifying one function of the spine microfilaments as regulating surface protein mobility (Richards et al., 2004). In line with these observations, the actin cytoskeleton has been shown to be a mediator of removal of AMPA receptors from the synapse in response to LTD stimuli (Allison et al., 1998; Shen et al., 2000).

LTP stimuli induce a volume increase of single postsynaptic spines mediated by the actin cytoskeleton (Matsuzaki et al., 2004). Moreover, hippocampal LTP depends on functional postsynaptic actin filaments as demonstrated by experiments involving blockers of filament assembly (Kim and Lisman, 1999; Krucker et al., 2000). However, it is not clear which function of the actin cytoskeleton is involved in induction of LTP. Morphological plasticity suggests an involvement of connective changes, but roles in synaptic signaling scaffolds as well as influences of receptor expression via endocytosis or exocytosis are equally possible (Lledo et al., 1998; Shirao and Sekino, 2001).

Activation of the NMDA receptor leads to long-term stability of the spine structure for at least several hours (Ackermann and Matus, 2003). This suggests that mechanisms mediating long-term stability of the actin cytoskeleton have to come into effect. Consistent with this, LTP in the perforant path (the connection between entorhinal cortex and dentate gyrus) induces F-actin accumulation in the dendritic layer of dentate gyrus neurons which lasts for weeks (Fukazawa et al., 2003).

II.2.4. Nuclear actin

Actin was reported to be present in nuclei as early as the 1970s, when Clark and Merriam discovered actin to dynamically distribute between the cytoplasm and the nucleus of Xenopus oocytes (Clark and Merriam, 1977). However, functions have only been ascribed to nuclear actin during the last few years, possibly owing to lack of recognition of nuclear actin in mammalian cells. Nuclear actin apparently takes on previously unknown structures that are not stained by

standard laboratory techniques such as dye-coupled phalloidin (Pederson and Aebi, 2002).

Actin in the nucleus has been linked to the following functions:

(1) RNA transcription: RNA is transcribed in the nucleus of eukaryotic cells by three polymerases (RNA polymerase I, II and III) synthesizing different RNAs. For messenger RNA transcription mediated by RNA polymerase II, actin was found to be associated with pre-mRNA binding proteins and stimulate transcription in insect cells (Percipalle et al., 2003; Percipalle et al., 2002; Percipalle et al., 2001). Soon thereafter, actin was identified to be a necessary cofactor for mRNA transcription in cultured mammalian cells (Hofmann et al., 2004). Actin and myosin I were identified to be involved in transcription by RNA polymerase I, which synthesizes ribosomal RNA in nucleoli (Fomproix and Percipalle, 2004; Philimonenko et al., 2004). Furthermore, actin associates with RNA polymerase III and was shown to localize to a gene transcribed by this polymerase *in vivo* (Hu et al., 2004). Of note, actin partially colocalizes with Cajal bodies, subnuclear structures suggested to be maturation or storage sites for transcriptional complexes (Gedge et al., 2005).
(2) chromatin remodeling: Chromatin remodeling is performed by huge protein complexes, and actin has been shown to be a constituent of various of these complexes in cells from different organisms (Olave et al., 2002). Interestingly, actin has a function in linking a protein involved in pre-mRNA transcription to a histone deacetylase, providing a connection between transcription and chromatin remodeling (Sjolinder et al., 2005).
(3) a function at the nuclear envelope: Field emission scanning electron microscopy identified “pore-linked filaments” (PLFs) attached to nuclear pores which are sensitive to latrunculin A and can be modified by jasplakinolide (Kiseleva et al., 2004). In this context, it is interesting to note that actin has been implicated in mRNA export from the nucleus (Hofmann et al., 2001; Kimura et al., 2000). Together with data showing an actin cortical network at the inner nuclear

membrane (Holaska et al., 2004), this raises the possibility that an intranuclear actin cortex dynamically interacts with the nuclear lamina and nuclear pore complexes to play a role in nuclear export of macromolecules (Pederson and Aebi, 2005).
(4) nuclear actin rods: Several stress signals induce the formation of large actin accumulations termed rods in cultured cells (Fukui and Katsumaru, 1979; Iida et al., 1986; Iida and Yahara, 1986). Interestingly, they often contain cofilin which is well known to produce rods in the cytoplasm upon overexpression (Aizawa et al., 1999; Nishida et al., 1987). The function of these rods has remained elusive, with the exception of nuclear and cytoplasmic rods in *Dictyostelium*, which have been implicated in the maintenance of dormancy and viability at the spore stage of the developmental cycle (Sameshima et al., 2001).

The structure(s) of actin in the nucleus are still unknown, although the purification of actin from chromatin-remodeling complexes and transcriptomes suggests that at least some of the actin performs a nuclear function as a monomer (Olave et al., 2002; Pederson and Aebi, 2002). A critical factor for the configuration of nuclear actin is the presence or absence of actin-binding proteins, some of which have been shown localize to the nucleus under different conditions (Pederson and Aebi, 2005). It remains to be seen which of these proteins exhibit a nuclear function of their own and which primarily impact upon nuclear function by influencing actin structure or binding properties.

II.2.5. Profilin

Profilin is a small, yet very versatile globular protein of only about 15 kDa. It was originally described as an actin binding protein from nonmuscle cells (Carlsson et al., 1977), and its principle role as a monomer binding protein in actin filament assembly has been described in chapter II.2.2. Since its discovery, the number of interaction partners ascribed to profilin in mammalian cells has grown to around 30 today, which in turn lead to novel functions attributed to profilin (Witke, 2004).

Here I try to introduce established or putative cellular functions of profilin isoforms in order to provide background knowledge for the results section of this thesis.

Proteins of the profilin family comprise at least four isoforms in mammals which show differential tissue distribution. The first profilin to be described, profilin I, is expressed in most tissues with the exception of skeletal muscle and therefore also often referred to as the "ubiquitous" isoform (Witke et al., 1998). Profilin II is almost exclusively expressed in the central nervous system and therefore also called the brain isoform. It can be alternatively spliced, although isoform profilin IIa makes up about 95% of brain profilin II and therefore is commonly used synonymously with profilin II (Di Nardo et al., 2000). I will follow this nomenclature and refer to profilin IIa as "profilin II" throughout the text. Profilins III and IV are recently discovered family members with testis-specific expression; knowledge about their properties and functions is very limited (Braun et al., 2002; Hu et al., 2001; Obermann et al., 2005). Although profilins I and II show limited sequence homology (65% sequence identity for mouse isoforms) their structures are almost superimposable (Nodelman et al., 1999).

Next to actin binding, profilin has two major binding sites: One for phosphoinositides (mainly PIP_2 and PIP_3) and one for poly-L-proline stretches (Lassing and Lindberg, 1985; Metzler et al., 1994). The poly-L-proline binding site and the actin binding site lie on opposite sides of the profilin protein and therefore profilin is still able to bind to actin when interacting with certain regulatory molecules, e.g. on the cell surface. The phosphoinositide binding region, however, overlaps with both the actin binding and the poly-L-proline binding site. Consequently, PIP_2 has been shown to regulate the binding of profilin to both actin and poly-L-proline (Lambrechts et al., 1997; Lassing and Lindberg, 1985).

The high number of profilin interacting molecules identified today is mainly due to poly-L-proline binding. In fact, next to actin, phosphoinositides and the neuronal scaffolding protein gephyrin (the binding site for the latter being still unknown), all

profilin binding proteins contain one or more poly-L-proline stretches (Witke, 2004). Figure I3 shows a schematic representation of profilin interactions in the cell leading to proposed functions as disussed below.

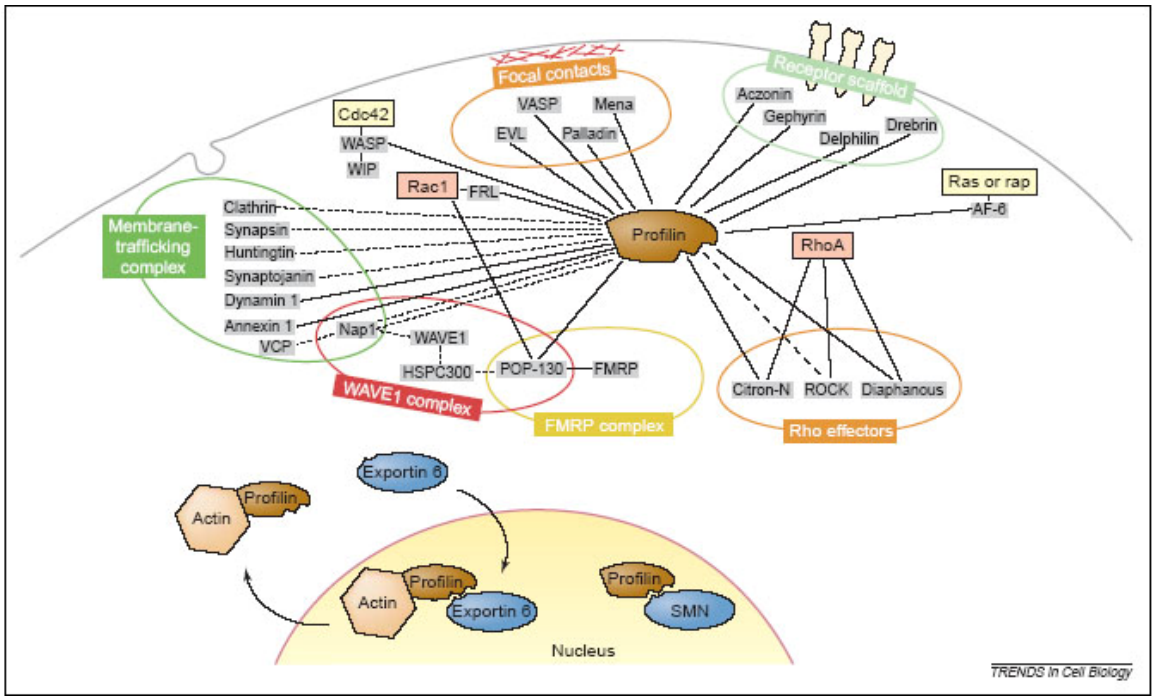

Fig. I3: Network of molecular interactions of profilin. Abbreviations used: AF-6, All-1 fusion partner from chromosome 6; EVL, Ena VASP like; FMRP, fragile X mental retardation protein; FRL, forming-related gene in leukocytes; HSP, heat shock protein; Mena, mouse homolog of Drosophila enabled; POP, partner of profilin; SMN, survival of motor neuron protein; VASP, vasodilator-stimulated phosphoprotein; VCP, valosine-containing protein; WASP, Wiskott-Aldrich syndrome protein; WAVE, WASP family verprolin-homologous protein; WIP, WASP interacting protein. From Witke, 2004.

Profilin can be recruited to sites of filament dynamics at the cell membrane via its interaction with surface-linker proteins of the Ena/VASP, WASP, ERM or formin-homology domain families (Holt and Koffer, 2001). VASP was the first protein identified in this respect and is thought to regulate actin polymerization at focal adhesions by antagonizing the capping of actin filaments and by nucleating actin polymerization (Bear et al., 2002; Walders-Harbeck et al., 2002).

A well-established function of profilin thanks to genetic experiments in yeast and *Drosophila* is its involvement in membrane trafficking and endocytosis (Pearson et al., 2003; Wolven et al., 2000). In mammalian cells, a proteomic approach on brain extracts demonstrated profilin binding to proteins of the secretory pathway, with some differences in binding affinities between profilin isoforms I and II (Witke et al., 1998). In further support of this function, profilin II can regulate dynamin 1, the central regulatory GTPase in vesicle budding, by competing with known dynamin ligands (Witke, 2004).

In neurons there is about three times more profilin II than profilin I (Witke et al., 2001). Recently, some publications indicated possible functions for profilins in both excitatory and inhibitory neurons. Profilin has been reported to play a role in the actin-dependent process of neurite outgrowth, regulated by signals activating the small GTPase RhoA and the subsequent activation of Rho-dependent kinase ROCK (Da Silva et al., 2003). However, this effect on early neurite growth in cultured hippocampal neurons was apparently compensated for in later stages of dendritic development.

A ROCK – profilin II pathway has also been shown to mediate organization of the Golgi apparatus regulated by the profilin binding protein Citron-N (Camera et al., 2003). Interestingly, Citron-N also localizes to postsynaptic densities of glutamatergic synapses onto GABAergic neurons in the hippocampus, suggesting a link between the secretory pathway and the postsynapse (Zhang et al., 1999). Interneurons also contain profilin at postsynaptic scaffolds of GABAergic synapses by means of profilin's interaction with gephyrin (Giesemann et al., 2003).

In hippocampal pyramidal neurons, profilin regulates actin-dependent morphological plasticity of postsynaptic dendritic spines (Ackermann and Matus, 2003). It is recruited to spines by activation of postsynaptic NMDA receptors with similar kinetics as the blockade of synaptic motility, and a peptide preventing binding of profilin to poly-L-proline interferes with redistribution. Profilin II targets more effectively to spine heads than profilin I, which may suggest an involvement

of Ena/VASP molecules as targeting sites which bind profilin II with higher affinities (Gertler et al., 1996; Reinhard et al., 1995). Interaction of profilin with the VASP family member Mena at postsynaptic sites has already been demonstrated in the aforementioned interaction of profilin with the scaffolding protein gephyrin (Giesemann et al., 2003).

Profilin has also been described as a nuclear protein, although its functions there are not yet fully understood. Some evidence points to a role in RNA processing: First, profilin interacts with SMN, the protein mutated in patients with a genetic form of spinal muscular atrophy (Giesemann et al., 1999). SMN complexes have been implicated in formation and maturation of ribonucleoprotein complexes, and could therefore act on transcription, pre-mRNA splicing and RNA transport (Gubitz et al., 2004). Second, a study using highly specific antibodies in fibroblasts showed profilin I to be present in Cajal bodies and splicing speckles, structures which have been postulated to be storage or maturation sites for transcriptional complexes and spliceosomes, respectively (Skare et al., 2003). In this work, Skare and colleagues showed that profilin accumulated in storage sites when transcription was blocked and antibodies against profilin inhibited transcription in an *in vitro* assay.

A recent report by Lederer and colleagues suggests that profilin acts as a transcriptional modulator: They identified a new profilin ligand, termed p42POP (partner of profilin) which is expressed in a variety of tissues, most heavily in brain (Lederer et al., 2005). Sequence homology to myb transcription factors suggested a role in transcription, and reporter gene assays showed that p42POP worked as a transcriptional repressor. Importantly, functionally binding profilin counteracted this effect, while profilin with a mutated poly-L-proline binding site had no influence. Interestingly, profilin has also been shown to be an essential co-factor for transcription of the RSV virus, supporting actin-dependent transcription (Bitko et al., 2003; Burke et al., 2000).

A putative nuclear function for profilin suggests that profilin nuclear localization is regulated so that profilin can influence gene expression in response to cellular

stimuli. In fact Stuven and colleagues found that regulation occurs at the level of nuclear export, with profilin being exported in a complex together with actin (Stuven et al., 2003). This is mediated by a novel nuclear export receptor termed exportin 6 that only has profilin and actin as known cargoes. Exportin 6 binds to actin which in turn is complexed with profilin and interference of profilin binding to actin prevents profilin export, but also hinders export of actin. The existence of a nuclear transport system specific for profilin and actin suggests that tight regulation of nuclear actin and profilin levels is important and further suggests that modulation of this export pathway specifically influences nuclear functions of profilin and actin, possibly gene expression.

II.3. Aim of this work

The aim of my thesis work was to investigate activity-dependent signaling from the actin cytoskeleton to the nucleus. A growing body of evidence implies actin-regulating proteins in influencing nuclear functions, particularly gene expression. Evidence for changes in actin dynamics impacting upon activity-dependent gene expression would describe a novel pathway of neuronal plasticity, linking stabilization of synaptic morphology to synthesis of macromolecules necessary for long-term plasticity. In particular, nuclear accumulation of profilin is of interest as profilin is a molecule necessary for blocking actin dynamics in activated postsynaptic dendritic spines. The aim of my work here was to describe the nuclear accumulation of profilin with respect to kinetics, signaling pathway, reversibility and function.

III. RESULTS

III.1. Reversible, activity-dependent targeting of profilin to neuronal nuclei

(as submitted to *Experimental Cell Research*)

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ABSTRACT

The actin cytoskeleton in pyramidal neurons plays a major role in activity-dependent processes underlying neuronal plasticity. The small actin-binding protein profilin shows NMDA receptor-dependent accumulation in dendritic spines, which leads to suppression of actin dynamics and long-term stabilization of synaptic morphology. Here we show that following NMDA receptor activation profilin also accumulates in the nucleus of hippocampal neurons via a process which involves rearrangement of the actin cytoskeleton. This bidirectional targeting suggests a novel mechanism of neuronal plasticity in which profilin both tags activated synapses and influences nuclear events.

Keywords: synaptic plasticity; pyramidal neuron; hippocampus; actin cytoskeleton

INTRODUCTION

Changes in connection strength between neurons have long been known to depend on protein synthesis and transcription (Kelleher et al., 2004; McGaugh, 2000) and several activity-dependent changes in signaling pathways leading to transcriptional activation have been implicated in learning and memory (Berman et al., 1998; Bourtchuladze et al., 1994; Silva et al., 1992). Nevertheless, knowledge about synapse-to-nucleus signaling in neurons is limited and few molecules entering the nucleus upon stimulation of neuronal activity have been identified.

Evidence implicates the postsynaptic actin cytoskeleton as a necessary element in NMDA receptor-dependent long-term potentiation (LTP) of synaptic transmission (Kim and Lisman, 1999; Krucker et al., 2000).

A putative anatomical correlate of this synaptic plasticity is found in dendritic spines, postsynaptic structures present at excitatory synapses which show prominent actin-based morphological plasticity (Dunaevsky et al., 1999; Fischer et al., 1998). Spine motility is modulated by activation of NMDA receptors, leading to suppression of actin dynamics and stabilization of synaptic structure that may last for several hours after the initiating stimulus (Brunig et al., 2004). Outstanding questions concern the signaling mechanism that mediates these changes in actin filament behavior and the identity of the molecules responsible for maintaining the stable state.

One candidate to have emerged recently is profilin, a small actin binding protein which is targeted to dendritic spines by stimulation patterns that block actin

dynamics and subsequently remains concentrated there for several hours (Ackermann and Matus, 2003). The implied relationship between profilin redistribution and long-duration stabilization of the spine cytoskeleton is further suggested by experiments showing that a small peptide which inhibits binding of profilin to cell surface proteins blocks NMDA receptor-induced actin cytoskeleton stabilization (Ackermann and Matus, 2003).

Despite its small size profilin binds a wide range of molecular partners in different cellular compartments (Witke, 2004). These include the nucleus where profilin isoforms are selectively associated with nuclear substructures including Cajal bodies (Skare et al., 2003) and are shuttled through the nucleus by a mechanism involving a defined export pathway (Stuven et al., 2003).

MATERIALS AND METHODS

Cell Culture, Transfection, and Microscopy. Neuronal cultures were prepared from either E19 rat or E17 mouse hippocampus as described (Goslin and Banker, 1991) and maintained in glia-conditioned, serum-free medium 21-30 days before imaging. Transfections were carried out using the Amaxa Nucleofector system according to the manufacturer's instructions. The expression plasmids for profilin II-GFP and GFP-actin fusion proteins have been described before (Ackermann and Matus, 2003; Kaech et al., 1997). Point mutations for the F59A and G120F variants were introduced into the profilin II cDNA using the Quik Change Mutagenesis Kit (Stratagene).

Imaging was performed in Tyrode's solution (119 mM NaCl, 5 mM KCl, 25 mM HEPES, 33 mM glucose, 2 mM $MgCl_2$, 2mM $CaCl_2$, 2 mM glycine) at 37°C. For stimulation experiments, either the stimulating agent was added to Tyrode's solution, or, for 0 Mg^{2+} activation, the solution was changed to Tyrode's without $MgCl_2$ supplemented with 5 μM glycine.

The rat embryonic fibroblast cell line REF52 was grown under standard conditions in DMEM with 10% fetal calf serum.

For organotypic slice cultures, slices were prepared from postnatal day 8 transgenic mice expressing profilin II-GFP from the chicken β–actin promoter (Ackermann and Matus, 2003) as described (Gahwiler et al., 1991). For microscopy, cultures were observed under continuous perfusion with artificial cerebrospinal fluid (ACSF: 124 mM NaCl, 2.5 mM KCl, 2 mM $MgSO_4$, 1.25 mM KH_2PO_4, 26 mM $NaHCO_3$, 10 mM glucose, 4 mM sucrose, 2.5 mM $CaCl_2$) saturated with 95% O_2/5% CO_2. ACSF (0 Mg^{2+}/glycine) was ACSF without $MgSO_4$ and supplemented with 5 μM glycine.

Imaging was carried out using a Leica DM-IRBE microscope, a Yokogawa microlens Nipkow confocal system, a cooled CCD camera (SensiCam, PCO computer optics) and MetaMorph imaging software.

Image analysis and quantification. Image analysis was carried out by quantification of confocal images of the same cells at different points in time using MetaMorph software. Levels of nuclear accumulation were measured by determining average fluorescence intensities in elliptic regions in the nucleus and the cytoplasm and calculating the ratio. Changes in nuclear/cytoplasmic

fluorescence ("Δ nuclear/cytoplasmic fluorescence") values were determined by subtracting the ratio at time point 0 (start of the experiment) from the ratios at respective time points. "Fold induction of nuclear fluorescence" was calculated by dividing nuclear fluorescence intensities after activation by intensities before the start of the experiment. For actin accumulation at the cell cortex, we performed a linescan around the edge of the soma of a confocal plane and divided the average intensity by the average fluorescence intensity of an elliptic region in the soma close to the nucleus ("cortical/perinuclear fluorescence"). For the relative cortical/perinuclear fluorescence, the above parameter was expressed as a fraction of its maximal value over time.

RESULTS

Neuronal activity induces reversible nuclear accumulation of profilin

To explore possible activity-dependent changes in distribution of profilin between cytoplasm and nucleus we examined dissociated cultures of pyramidal neurons from rat hippocampus and organotypic slice cultures of hippocampus from transgenic mice expressing profilin II-GFP under the control of the chicken β–actin promoter. In mature dissociated cultures (> 21 days *in vitro*) under resting conditions, the profilin II-GFP fusion protein was concentrated in the cytoplasm but largely excluded from the nucleus (Fig. 1a, left). However, after stimulating NMDA receptors by exposing the cultures to medium lacking the NMDA receptor blocker Mg^{2+} and containing the co-activator glycine, profilin II-GFP accumulated in the nucleus (Fig. 1a, middle). This effect was reversible since profilin II-GFP

returned to a predominantly cytoplasmic distribution when the cells were re-exposed to standard medium in which NMDA receptors are blocked (Fig. 1a, right). As previously reported (Ackermann and Matus, 2003) profilin II-GFP also accumulated in dendritic spines following NMDA receptor activation (Fig. 1b, compare left and middle panels). However in contrast to its reversible accumulation in the nucleus profilin II-GFP remained concentrated in dendritic spines after the receptor stimulating medium was removed. This difference is shown in Figs. 1a and 1b (right panels) which are taken from the same image stacks (see also Supplementary Material, Videos 1-3).

To determine the kinetics of nuclear accumulation, we performed confocal time-lapse microscopy on profilin II-GFP expressing neurons following NMDA receptor activation. Significant nuclear accumulation of profilin II-GFP, expressed as an increase in the ratio of nuclear to cytoplasmic fluorescence, was visible after two minutes (Fig. 1c, filled squares). Profilin continued to accumulate until the stimulus was withdrawn, and subsequently redistributed to the cytoplasm on a similar timescale. Some profilin II-GFP remained in the nucleus following stimulus withdrawal but this may be accounted for, at least in part, by a small non-specific increase in nuclear fluorescence apparent in control cultures subject to medium change without activation (Fig. 1c, empty squares). To assess whether cells would respond in the same way after having already been activated, we carried out an experiment in which the cycle of stimulation and recovery was repeated twice (Fig. 1d). As before, nuclear accumulation of profilin

II-GFP was fast and reversible in both rounds of activation. Taken together, these experiments establish profilin nuclear accumulation as a rapid and reversible process capable of repeated induction.

Nuclear accumulation of profilin depends on NMDA receptor stimulation and extracellular Ca^{2+}

The conditions of low Mg^{2+} and elevated glycine used above are designed to stimulate NMDA subtype glutamate receptors. To verify their involvement we carried out a pharmacological analysis of the nuclear accumulation of profilin and its reversal. Consistent with the involvement of NMDA receptors, reversible targeting of profilin II-GFP to the nucleus occurred when cultures were stimulated with either 0 Mg^{2+}/glycine, the endogenous neurotransmitter glutamate, or the receptor-specific agonist NMDA. Moreover in cells exposed to glutamate profilin nuclear accumulation could be blocked by the NMDA receptor antagonist APV, but not by NBQX, an antagonist of AMPA-type glutamate receptors (Fig. 2b). Nuclear targeting was also absent when cells were stimulated while in medium lacking Ca^{2+} indicating that influx of extracellular calcium is necessary for the effect to occur (Fig. 2b and c). Potential downstream signaling molecules involved in neuronal plasticity mechanisms include Ca^{2+}/calmodulin dependent enzymes, the MAP kinase cascade and protein kinase A (Curtis and Finkbeiner, 1999). However, neither the Ca^{2+}/calmodulin blocker W7, the MAP kinase blocker PD98059 nor the PKA activator forskolin had a significant effect on nuclear accumulation of profilin (Fig. 2a, b).

To determine whether nuclear targeting of profilin occurs in organized brain tissue we examined organotypic slice cultures from the hippocampus of transgenic mice expressing profilin II-GFP. Neurons in these cultures expressed the fusion protein to varying degrees but nevertheless showed reversible targeting of profilin II-GFP to the nucleus following transitory activation by exposure to 0 Mg^{2+}/glycine medium (Fig. 3a). As for dispersed cell cultures, profilin II-GFP accumulated in the nucleus within a few minutes and translocated back into the cytoplasm after removal of the stimulus (Fig. 3b).

Actin-binding is necessary for nuclear export of profilin

Recent evidence indicates that actin and profilin shuttle through the nucleus and are exported as a complex by means of a novel nuclear transport receptor, exportin 6 (Stuven et al., 2003). Consequently, the distribution of profilin between nucleus and cytoplasm should depend on its functional interaction with actin. To test whether this was the case in cultured hippocampal neurons, we examined the effects of two independent point mutations of profilin II, profilin IIF59A or profilin IIG120F, that have been demonstrated to reduce its binding to actin (Schluter et al., 1997). When expressed as fusion proteins with GFP each of these actin-binding mutants showed preferential accumulation in the nuclei of both neurons and fibroblasts in contrast to wild-type profilin II, which was excluded from the nucleus as expected (Fig. 4a). In hippocampal neurons the nuclear accumulation of these mutant proteins occurred in the absence of stimulation suggesting that the exclusion of profilin II from the nucleus under steady-state conditions depends on export of a profilin-actin complex, as already

suggested by data for exportin 6-mediated nuclear export(Stuven et al., 2003). If this were the case it might be expected that actin would distribute independently of profilin in stimulated neurons where profilin has accumulated in the nucleus. To examine this possibility we used time-lapse microscopy to follow the distribution of GFP-tagged β- and γ- cytoplasm actins in hippocampal neurons before and after stimulating NMDA receptors with 0 Mg^{2+}/glycine. Under conditions where profilin II-GFP showed strong accumulation in the nucleus (Fig. 4b) both N- and C- terminal fusions of β- and γ- actins remained outside the nucleus (Fig. 4b and c). Instead we observed an activity-induced increase in the concentration of γ-cytoplasm actin at discrete locations on the cell body surface accompanied by a corresponding decrease in the cytoplasm (Fig. 4b). These observations are consistent with previous evidence for activity-dependent accumulation of actin at postsynaptic sites on the cell body of hippocampal neurons(Furuyashiki et al., 2002).

Activity-dependent redistribution of actin to the cell cortex

Receptor-induced recruitment of actin to the cell cortex independently of profilin suggests a potential explanation for the accumulation of profilin in the nucleus where profilin export, which depends on its complex formation with actin, would be reduced (Stuven et al., 2003). Quantifying the ratio of GFP-γ-cytoplasmic actin at the cell cortex compared to the perinuclear region showed that actin translocation to the cell cortex is an activity-dependent and reversible phenomenon, similar to the nuclear accumulation of profilin (Fig. 5a and b). However, redistribution of actin to the cell cortex was more rapid than that of

profilin to the nucleus, being maximal after 2 minutes (Fig. 5c). By contrast, redistribution of actin from the cell cortex back to the cytoplasm following removal of the stimulus showed a similar time-course to that of profilin from the nucleus to the cytoplasm (Fig. 5c). When NMDA receptors were blocked by APV, no actin redistribution was observed (Fig. 5b).

Together these observations suggest a mechanism of activity-induced cytoskeletal changes in the pyramidal neuron cell body, initiated by the accumulation of filamentous actin at the cell cortex in response to a rise in somatic Ca^{2+} levels.

DISCUSSION

Recent work has begun to identify some cellular components involved in signaling from the actin cytoskeleton to the nucleus (Miralles et al., 2003; Ruegg et al., 2004), but the molecular mechanisms involved in this relationship are not fully understood. Large pyramidal neurons with their highly specialized cytoskeletal microdomains are involved in long-term morphological modifications dependent on transcription and protein synthesis. The accumulation of the actin-binding protein profilin II in the nucleus of hippocampal neurons upon stimulation of NMDA receptors coupled with the translocation of perinuclear actin to the somatic cell cortex, thus decreasing the amount of actin able to enter the nucleus, are of special interest in this regard. Dispersion of actin to the cell cortex might explain the nuclear accumulation of profilin, since its nuclear export depends on its binding to actin. In agreement with this model, our kinetic data

show a fast redistribution of actin and a slower response of profilin following NMDA receptor activation, but similar kinetics of relocation of the two proteins following removal of the stimuli.

NMDA receptor activation and Ca^{2+} influx are also necessary for long duration stabilization of the actin cytoskeleton in dendritic spines and for long term electrophysiological changes underlying synaptic plasticity (Bliss and Collingridge, 1993; Brunig et al., 2004). In this context it is interesting that recent work has identified a novel nuclear profilin-binding protein, p42POP (partner of profilin) which in reporter gene assays acts as a transcriptional repressor, whose activity is modulated by profilin binding (Lederer et al., 2005). A putative role for profilin in gene expression is also suggested by data showing profilin being necessary for RNA splicing *in vitro* (Skare et al., 2003), and interacting with SMN which is important for assembly of ribonucleoprotein particles (Giesemann et al., 1999; Gubitz et al., 2004).

It is striking that profilin accumulates in both postsynaptic dendritic spines and the nucleus in response to NMDA receptor signaling. The simultaneous targeting of profilin to these sites matches properties that have been hypothesized as necessary for synaptic tagging, a mechanism in which a molecular tag is set at individual activated synapses while at the same time the nuclear events required for long-term consolidation of activity-dependent changes are initiated (Frey and Morris, 1998a).

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FIGURE LEGENDS

Figure 1: Activity-dependent nuclear accumulation of profilin II, a rapid and reversible process.

(a), (b), confocal microscopy images of dispersed hippocampal neurons expressing a profilin II-GFP fusion protein. Cells were activated by an extracellular solution lacking the NMDA receptor blocker Mg^{2+} and supplemented with glycine (5μM). Confocal stacks were taken before and 25 minutes after the start of activation, the medium was then changed back to normal Tyrode's and another stack was taken after 25 minutes. Confocal nuclear (a) and dendritic (b) planes of the same stacks (cf. Supplementary Material, Videos 1-3) are shown. (c), cells were activated as described above, and image stacks were taken every 2 minutes. Medium was changed back to physiological Mg^{2+} concentrations at 15 minutes, as indicated. The change in nuclear versus cytoplasmic fluorescence (*Materials and Methods*) in confocal images was plotted against time (filled squares, error bars representing SEM; n=7). As a control, medium was replaced without changing its composition (empty squares; n=6). *difference between activity-induced profilin nuclear accumulation and a nonspecific increase in nuclear/cytoplasmic fluorescence was significant up to the value at 40 minutes (25 minutes after stimulus removal; t-tests, α=0.05, $p<0.05$) but not thereafter. (d), cells were activated as above for 5 minutes, allowed to recover for 10 minutes, stimulated again for 5 minutes and finally allowed to recover in regular

medium. Confocal images were analyzed as described above (n=5; error bars represent SEM).

Figure 2: Pharmacological experiments show NMDA receptor and Ca^{2+} dependence of profilin II nuclear accumulation.

(a), dispersed hippocampal neuronal cultures were activated by bath application of various stimuli in Tyrode's solution. Cells (n refers to number of cells observed) were followed by time-lapse microscopy and scored for showing visible nuclear accumulation after 20-30 minutes. Subsequently, in some experiments the medium was changed back to regular Tyrode's and cells were scored for partial reversibility of nuclear accumulation again 20-30 minutes later. For the forskolin experiments, a batch of cells showing high degree of nuclear accumulation (100%) with glutamate stimulation was used.

(b), batches of cells showing a high percentage of nuclear accumulation in control conditions (either glutamate bath application or 0 Mg^{2+} stimulation) were used to determine the influence of pharmacological agents on nuclear accumulation of profilin. Cultures were incubated in Tyrode's solution containing the indicated amount of blockers for 20-30 minutes prior to the beginning of the experiment, which was carried out as described in (a). Control experiments without the pharmacological agents were carried out in the same way using the same batches of cells.

(c), differences in response to activation by NMDA in the presence or absence of ectracellular Ca^{2+}. Cells were stimulated with 10 μM NMDA in Tyrode's either

containing (filled squares, error bars represent SEM; n=4) or lacking (empty squares; n=2) 2 mM Ca^{2+}. Medium was changed back to Tyrode's without NMDA at 15 minutes and data were analyzed as described in Figure 1.

Figure 3: Nuclear accumulation of profilin II in organotypic slice cultures. (a), an organotypic slice from a transgenic profilin II-GFP mouse cultured for 6 weeks was activated by replacing the imaging medium (ACSF) with ACSF (0 Mg^{2+}/glycine) (*Materials and Methods*). Stacks of confocal images were taken every 5 minutes and the stimulus was withdrawn at 30 minutes by changing back to ACSF. Images show a region of the slice at selected time points. Arrowheads highlight example cells responding to activation / recovery with different kinetics. (b), quantification of nuclear accumulation over time in 52 cells in 3 different organotypic slice cultures. Confocal images were used for quantification as described before (*Materials and Methods*). * difference in nuclear/cytoplasmic fluorescence after stimulation compared to before was significant (t-tests, α=0.05, p<0.01). ** difference in nuclear/cytoplasmic fluorescence after removal of the stimulus compared to before removal (29 minutes) was significant (t-tests, α=0.05, p<0.01).

Figure 4: Subcellular distribution of profilin depends on its binding to actin. (a), different profilin II-GFP fusion proteins – wildtype profilin II and actin-binding mutants F59A and G120F, respectively - were expressed in hippocampal

neurons (upper panel) and embryonic fibroblasts (lower panel). Images shown are epifluorescent images of neurons at 3 days *in vitro* and fibroblasts 24 hours after transfection. The distribution shown in these example images (cytoplasmic vs. nuclear+cytoplasmic) is representative for all cells observed under the described culture conditions (n=20 for each category).

(b), mature hippocampal neurons of the same batch transfected with either profilin II-GFP or two different actin-GFP fusions were stimulated with 0 Mg^{2+} solution as indicated and images taken before and 25 minutes after activation.

(c), nuclear accumulation of fusion proteins in cells followed by live microscopy as described in (b) was quantified by calculating the fold induction of nuclear fluorescence for each category (*Materials and Methods*). Abbreviations for categories represent: profilin II-GFP (PIIG), GFP-γ–actin (Gγ), β–actin-GFP (βG). Control stimulations were done by replacing the medium without changing its composition (n=7 in each category; error bars represent SEM; * difference between stimulation and control group is statistically significant for profilin II-GFP (t-test, α=0.05, $p<0.01$)).

Figure 5: Activity-dependent relocation of actin to the cell cortex.

(a), mature rat hippocampal neurons in dispersed culture transfected with GFP-γactin (Gγ) were stimulated with Tyrode's solution without Mg^{2+} and the stimulus removed after 25 minutes. Confocal stacks were taken before and at the end of the activation period and 25 minutes after recovery.

(b), quantification of the effect of actin accumulation at the cell cortex. Cortical/perinuclear fluorescence in comparable confocal planes was determined as described in *Methods*. Left, quantification of the experiments described in (a) (n=14; *differences between the values after activation compared to both before activation and after reversal are significant (ANOVA, α=0.05, p<0.01)). Right, quantification of similar experiments performed in the presence of 100 μM APV (n=20; error bars represent SEM).

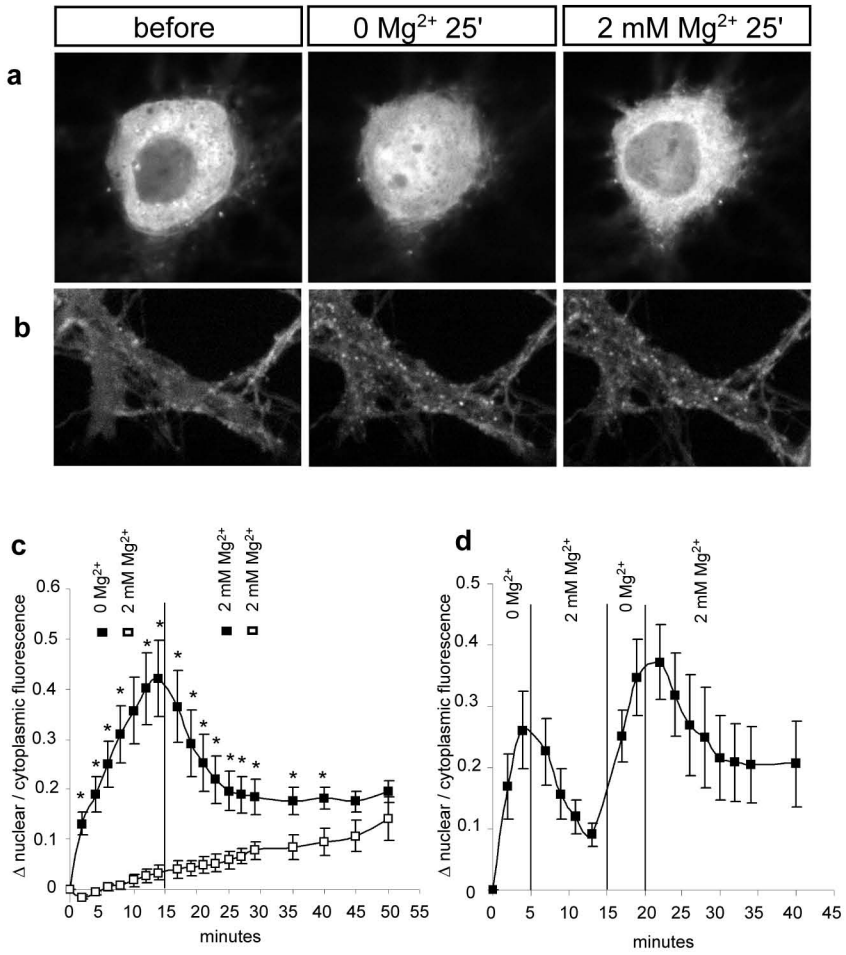
(c), single cells were followed over time with confocal stacks taken every 2 minutes. The stimulus (0 Mg^{2+}) was removed at 15 minutes, the relative cortical/perinuclear fluorescence for each time point was determined as described in *Materials and Methods* and plotted against time (n=4; error bars show SEM).

Supplementary Material, Video legends

Video 1: Z-stack of confocal fluorescent images of a hippocampal neuron in dispersed culture expressing the profilin II-GFP fusion protein. This stack was taken before activation, with the culture in regular medium (Tyrode's). Confocal planes are 0.5 μm apart.

Video 2: Z-stack of confocal fluorescent images of the same cell as in Video1, taken 25 minutes after activation with medium lacking Mg^{2+} and containing 5 μM glycine.

Video 3: Z-stack of confocal fluorescent images of the same cell as in Videos 1 and 2. Medium was changed back to regular Tyrode's after acquisition of Video 2 and Video 3 taken 25 minutes afterwards.



Birbach Figure 1

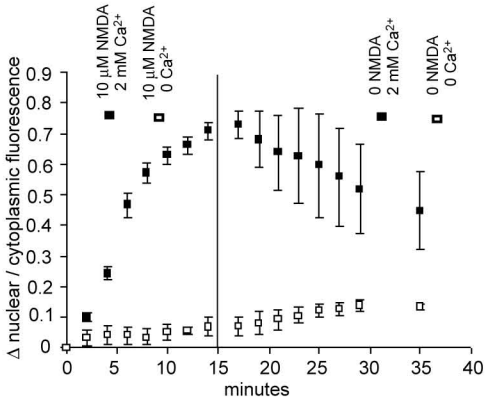
a

Stimulus	% cells showing nuclear accumulation	% cells showing reversal
0 Mg^{2+} / 5 μM glycine	65 (n=140)	72 (n=75)
2.5 - 7.5 μM Glutamate	80 (n=56)	50 (n=14)
5-10 μM NMDA	80 (n=20)	55 (n=11)
20 μM forskolin	0 (n=5)	n.a.

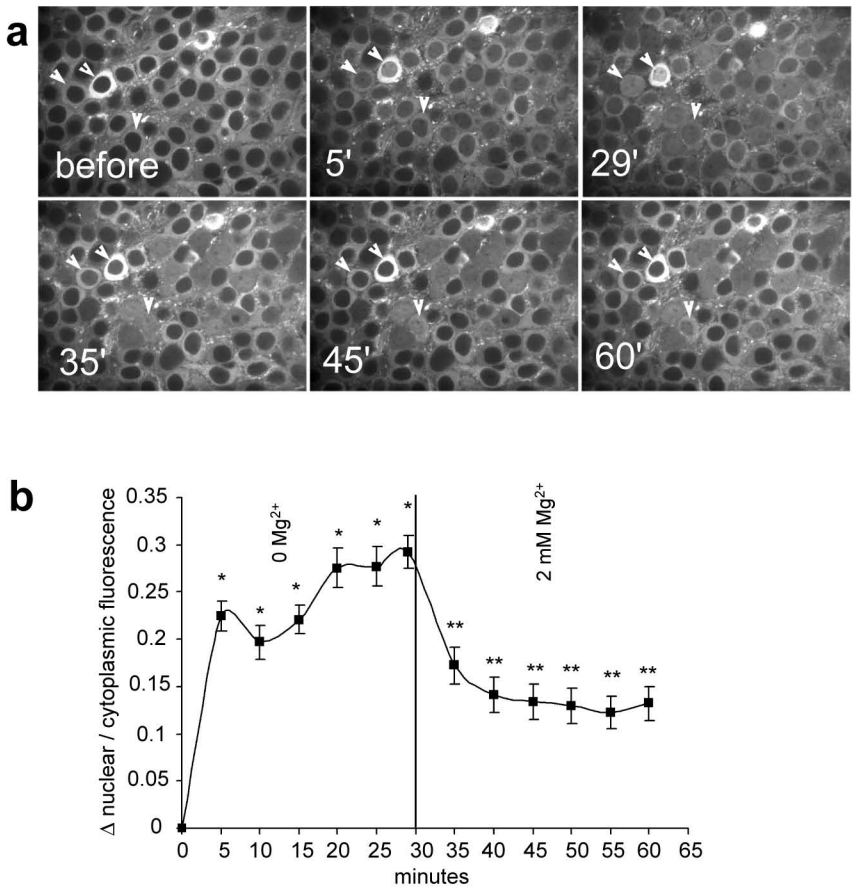
b

Blocking agent	% cells showing nuclear accumulation	% cells showing n. a. in control situation
100 μM APV	13 (n=15)	95 (n=19)
20 μM NBQX	80 (n=8)	88 (n=8)
$[Ca^{2+}]_o$=0	11 (n=27)	96 (n=23)
10 μM W7	100 (n=6)	100 (n=5)
10 μM PD98059	73 (n=11)	90 (n=10)

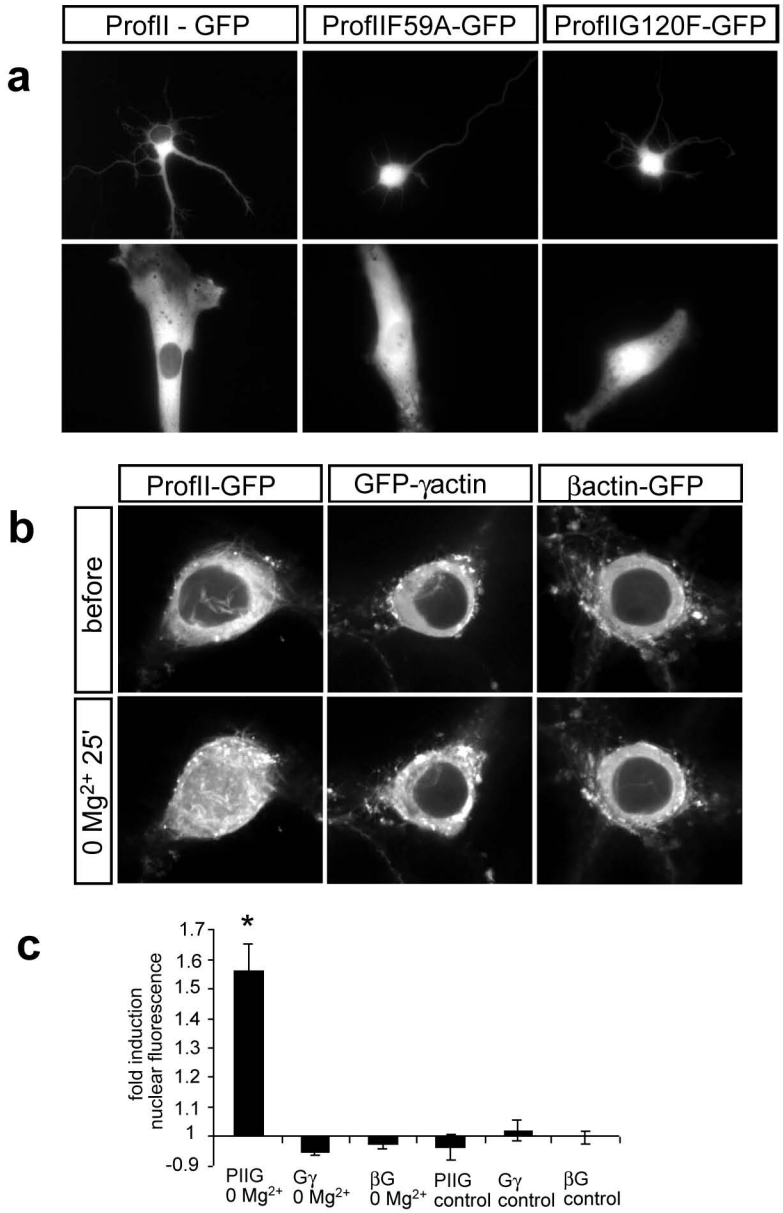
c



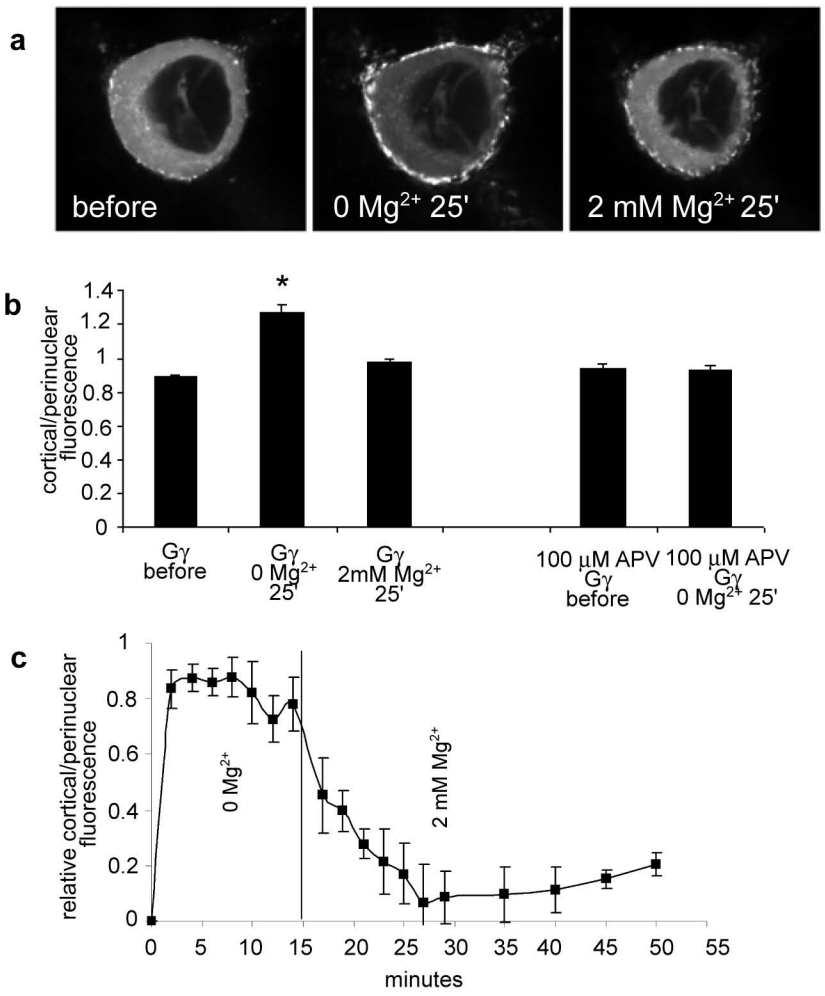
Birbach Figure 2



Birbach Figure 3



Birbach Figure 4



Birbach Figure 5

III.2. Supplementary data

The observation of activity-dependent profilin II accumulation implies many questions, most importantly functional ones. Among open questions, three important ones can were singled out to address the function of nuclear profilin II in brain neurons:

(1) Which brain regions/neurons show profilin II accumulation under which circumstances?
(2) What happens if we reduce profilin II levels in neurons?
(3) Can we make a link between profilin II and gene expression?

These questions were experimentally approached as follows:

(1) Generation of specific antibodies to profilin II in order to stain the endogenous protein in brain sections. This provides us with a tool to follow activity-dependent changes in profilin II distribution in different brain areas upon paradigms of *in vivo* activity.
(2) Small interfering RNAs (siRNAs) were designed to downregulate profilin II expression in neurons. The use of small hairpin RNAs in expression vectors allows the use of established transfection methods to interfere with gene expression in neuronal cultures. However, as transfection efficiency in neurons is limited, the use of gene-targeted profilin II -/- mice, which were obtained from the laboratory of Walter Witke (EMBL, Monterotondo), provides a more efficient way to study the consequences of lack of profilin II in neurons.
(3) Real-time RT-PCR of candidate genes was established in order to test for a possible involvement of profilin II in activity-dependent gene expression.

As the questions referred to above don't stand alone, but all converge on profilin II function, so do the tools created, being most effective when used in combination to perform experiments addressing profilin II function in neuronal nuclei. The following chapters concentrate on technical aspects of creation of these tools and their testing to prove applicability in experimental assays.

III.2.1 Generation of rabbit antibodies against profilin II

The use of green fluorescent protein (GFP) has revolutionized cell biology by allowing researchers to follow proteins in living cells, while in many cases not interfering with the functionality of proteins fused to GFP (Ludin and Matus, 1998; Tsien, 1998). However, expression of a fusion protein from a transgene does not give insight into the behavior of endogenous proteins as expression levels vary among tissues and cell types and artifacts of overexpression cannot be ruled out. Therefore investigation of the endogenous proteins is a valuable addition to live microscopy data of ectopically expressed proteins, and antibody staining is still the method of choice.

Polyclonal antibodies are commercially available from antisera of different species, most commonly rabbits. I decided to use a recombinant protein as antigen, which offers the advantage to obtain antibodies recognizing a folded version of the protein instead of a sequence that may be buried inside the native protein. A GST-profilin II protein was expressed in E. coli and purified using standard methods (see *Methods*). The expression system offered the possibility to cleave off the GST moiety by means of the protease thrombin, but cleavage was inefficient (Fig. S1a). Therefore, a mixture of profilin II and GST-profilin II was used to immunize two rabbits, and early and later bleeds were tested in immunoblots to determine their specificity. Antisera of rabbit #1947 recognized primarily profilin in early bleeds, but shifted specificity towards a cross-reactive protein in later bleeds (not shown). However, antisera of rabbit #1946 showed great specificity towards a single band of ca. 15 kDa in immunoblots of whole brain extracts, and could be shown to recognize a recombinant fusion protein of maltose binding protein to profilin II from E. coli (Fig. S1b). These experiments indicate that antisera from rabbit #1946 detect denatured profilin II protein on immunoblots.

The specificity of the antibody towards native profilin II in cells was tested on fibroblasts transfected with expression plasmids of either profilin II-GFP or profilin I-GFP (Fig. S1c). The antibody recognized cells transfected with profilin II-GFP,

but showed only background staining in cells transfected with profilin I-GFP, demonstrating isoform-specificity towards profilin II in cells.
As these results on ectopic profilin II were encouraging, the antibody was tested on dispersed hippocampal neurons to determine the distribution of the endogenous protein. However, despite testing several different fixation and permeabilization methods, the antibody always yielded a granular staining pattern throughout the cell which was never seen for profilin II-GFP. Moreover, expression of transgenic profilin II-GFP in neurons did neither alter staining pattern nor staining intensity, and siRNA constructs against profilin II did not decrease staining intensity (not shown). Therefore it has to be concluded that the antiserum #1946 cannot recognize profilin II in neurons using standard fixation/permeabilization methods. A possible explanation could be that the epitopes recognized by the antibody are masked in the presence of binding partners in neurons. Therefore another member of the Matus lab carried out experiments with tissue sections which had been treated with a heated citrate buffer to remove crosslinks and retrieve antigens (Pileri et al., 1997)(Urs Mueller, personal communication). This treatment gave rise to specific staining in tissue sections, demonstrating the usefulness of the antibody for visualizing endogenous profilin II levels.

III.2.2. A knock-down strategy to investigate profilin II function

Since their first description only a few years ago, small interfering RNAs have become a widely used tool to study gene function in a variety of cell systems (Tuschl and Borkhardt, 2002). siRNAs use an endogenous RNA decay pathway via the RNA induced silencing complex (RISC) to destroy mRNAs and thereby downregulate gene expression. More recently, plasmids expressing small doublestranded RNAs via a fold-back mechanism in a hairpin loop (therefore also termed small hairpin RNAs) have been found to be equally effective in mediating RNA decay (Brummelkamp et al., 2002). This method was employed in my work in order to establish a tool for downregulation of profilin II in neurons.

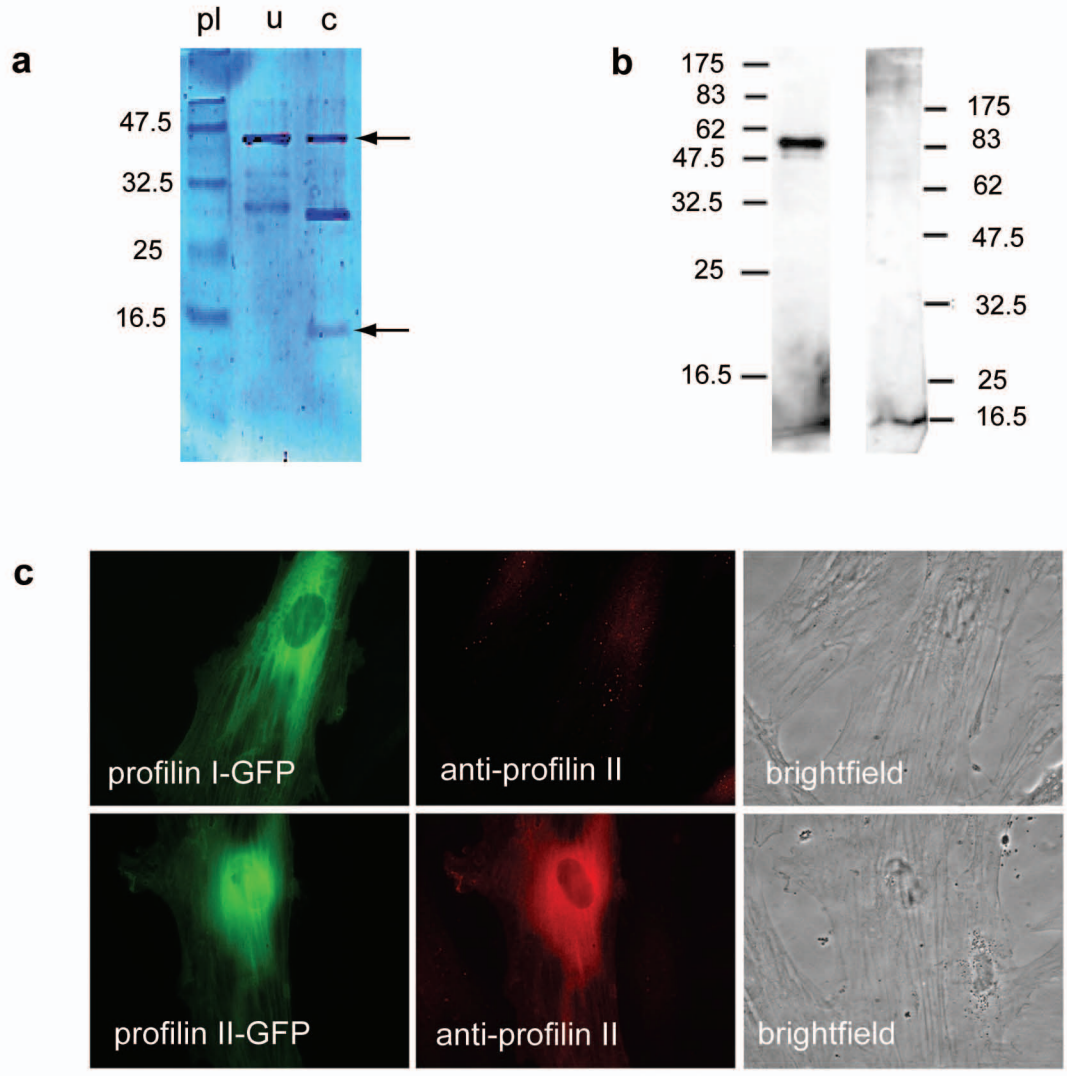


Figure S1: A rabbit antibody against profilin II recognizes profilin II in immunoblots and cells. (a) a GST-profilin II fusion protein was purified from Escherichia coli extracts, and either cleaved by thrombin or directly loaded on an SDS-polyacrylamide gel. band annotation: pl, protein ladder; u, uncleaved protein; c, thrombin-cleaved protein. upper arrow: GST-profilin II band, lower arrow: profilin II band. GST-profilinII fusion protein was used for injection of rabbits to induce production of specific antisera.
(b), antiserum GP1946 raised against GST-profilin II was used to probe immunoblots loaded with E. coli extract expressing MBP-profilin II (left) or whole mouse brain protein extract (right). The antibody recognized proteins of predicted sizes, 57 and 15 kDa, respectively. (c), antiserum GP1946 was used to stain rat embryonic fibroblsts transfected with profilin I- or profilin II-GFP, respectively. In this assay, GP1946 showed isoform-specificity.

Small interfering RNAs have been intensely studied during recent years, and the wealth of data on functional versus nonfunctional sequences led to the establishment of design rules (Reynolds et al., 2004). Employing these rules (see *Methods*) I selected two sequences termed siProfII20 and siProfII380 for the design of small hairpin RNAs. Constructs expressing these shRNAs were transfected into rat embryonic fibroblasts, together with an expression vector for profilin II-myc. Cell lysates were pepared 48 hours after transfection, separated by SDS-PAGE and the subsequent immunoblot probed with anti-myc antibody (Fig.S2a). The siRNA constructs proved to efficiently downregulate profilin II-myc expression compared to empty vector, with siProfII380 being more effective than siProfII20 (Fig.S2b). In order to test the usefulness in mouse neurons, dispersed hippocampal cultures from transgenic mice expressing profilin II-GFP were used, which express the transgene more homogenously than wildtype cultures transfected by one of the established transfection methods. SiProfII380 was co-transfected together with the fluorescent protein DsRed under the control of the CMV promoter in order to identify transfected neurons, and GFP signal intensity provided the readout for profilin II expression levels (FigS3c). Data analysis showed that the siRNA downregulated profilin II-GFP to 18±3 % (mean±SEM, Fig. S2d). Taken together, these experiments established the efficacy of small hairpin RNAs to downregulate exogenous profilin II in neurons.

III.2.3. Real-time PCR analysis of immediate early gene expression

Activity-dependent nuclear accumulation of profilin II and profilin's implication in transcriptional modulation (Burke et al., 2000; Lederer et al., 2005) and RNA processing (Giesemann et al., 1999; Skare et al., 2003) suggest a possible involvement of profilin II in activity-dependent gene expression. I decided to approach this question by real-time PCR of candidate genes which are known to be upregulated by neuronal activity. The following section gives an overview on these genes and their implication in activity-dependent gene expression, neuronal plasticity and memory:

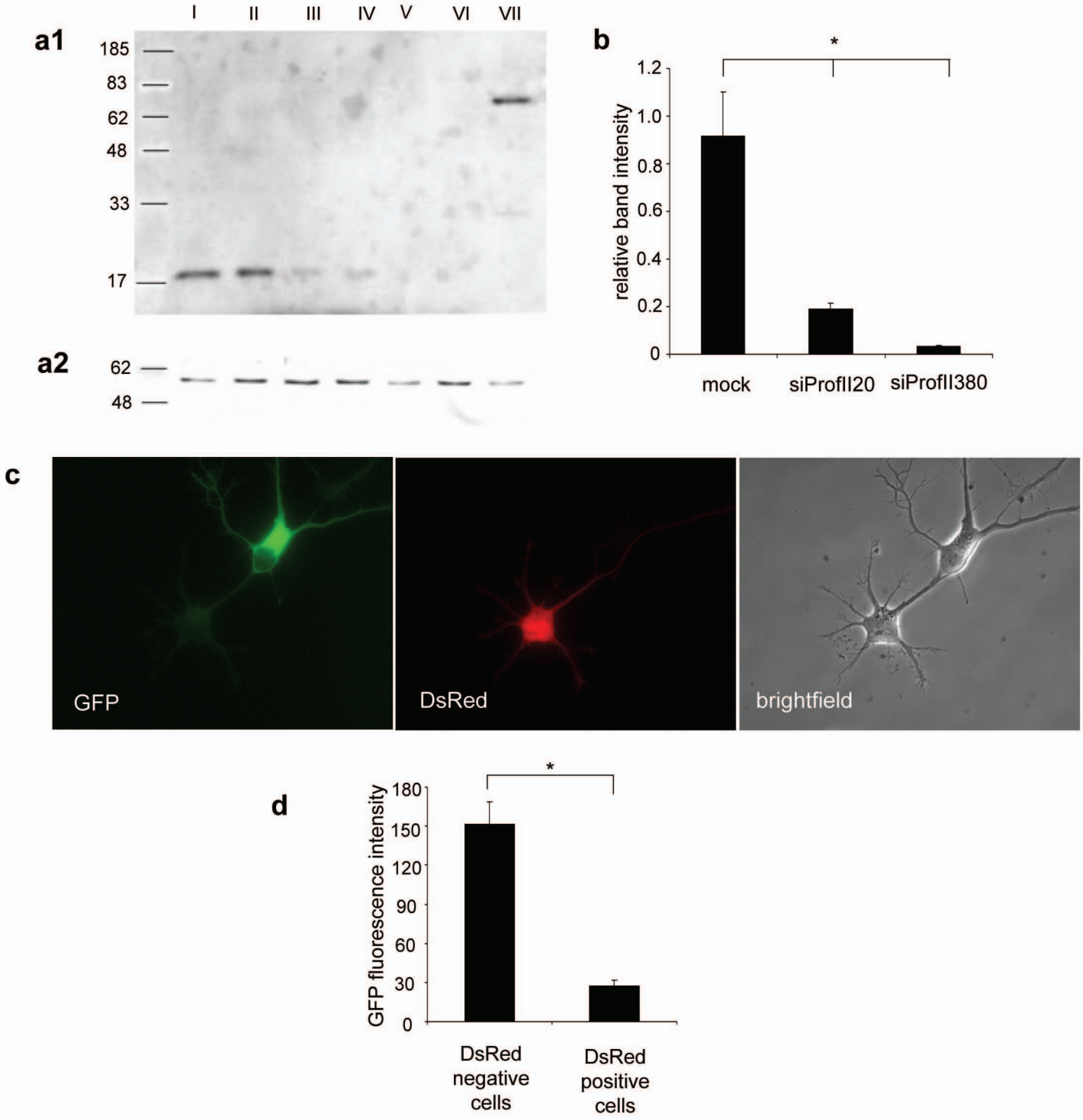


Figure S2: siRNA constructs downregulate ectopically expressed profilin II. (a), myc-tagged profilin II was expressed in HeLa cells with or without co-expression of small hairpin RNA constructs against profilin II. Cell extracts were resoved on SDS-PAGE and a subsequent immunoblot probed with anti-myc (a1) or anti-tubulin (loading control: a2) antibodies. Constructs expressed for each lane: I, II: myc-profilin II/ pTER, III, IV: myc-profilin II/pTER-siProfII20, V, VI: myc-profilin II/pTER-siProfII380, VII: myc-Pak3 (myc antibody control). (b), mean band intensity of myc-bands normalized to tubulin loading control (error bars: SEM; statistics: one-way ANOVA, α=0.05, p=0.02). (c), cultured hippocampal neurons (3 div) from transgenic profilin II-GFP mice transfected with pTER-siProfII380/pCMV-DsRed. (d), comparison of GFP fluorescence intensity of transfected and untransfected cells in the experiment described in (c), showing mean+SEM (n=25 and n=22 for left and right category, respectively; $p<0.01$ in t-test, α=0.05)

The activity-regulated cytoskeletal-associated protein (Arc) is regulated on the transcriptional level by rapid upregulation in response to diverse stimuli in neurons (Lyford et al., 1995), including growth factors, neurotransmitters, electrical stimulation patterns and drugs of abuse (Fosnaugh et al., 1995; Lyford et al., 1995; Yin et al., 2002). In a learning paradigm, it was noted that Arc mRNA, though elevated directly after a 30 minutes training period, already declined as training persisted and novelty was low at 60 minutes (Kelly and Deadwyler, 2003).

The transcription factor cFos has been known to be induced by neuronal activity for a long time (Dragunow and Faull, 1989). Therefore, staining for c-Fos has become the method of choice to demonstrate activity-induced gene expression in a variety of behavioral paradigms including several forms of learning and memory formation (Barth et al., 2004; Holahan and White, 2004; Puurunen et al., 2001). Induction of c-Fos depends on activation of NMDA receptors and on the other hand, absence of c-Fos causes impairments in NMDA-receptor dependent synaptic plasticity and hippocampus-dependent memory tasks (Fleischmann et al., 2003).

Homer1a is a variant of the Homer1 gene, with activity leading to upregulation of the gene and conversion of intronic to exonic sequences (Bottai et al., 2002). Homer proteins provide a scaffold for metabotropic glutamate receptors and TRP channels at the synapse which cannot be built by variant Homer1a (Fagni et al., 2002; Yuan et al., 2003). Consequently, activity-dependent Homer1a expression is implicated in functional and morphological synaptic plasticity (Sala et al., 2003).

The cytoplasmic β–actin is an immediate early gene in a variety of tissues, and is being upregulated in neurons upon activity (Ramanan et al., 2005). Moreover, β–actin mRNA levels are sensitive to changes in the actin cytoskeleton (Bershadsky et al., 1995), possibly by being one target gene of a feedback loop signaling changes in the F-actin/G-actin ratio to the nucleus (Sotiropoulos et al., 1999).

Together, these genes comprise NMDA-receptor dependent expression (all), neuron-specific induction (Arc) or widespread immediate early genes (c-Fos, β–actin), regulation on transcriptional and RNA-processing level (Homer1a), induction in response to changes in the actin cytoskeleton (β–actin) and signaling mechanisms back to the synapse (Homer1a, β–actin). Thus they constitute potential target genes for profilin in neuronal nuclei.

As control genes for which RNA levels should remain constant after NMDA-receptor activation, I selected 18S ribosomal RNA and α-tubulin.

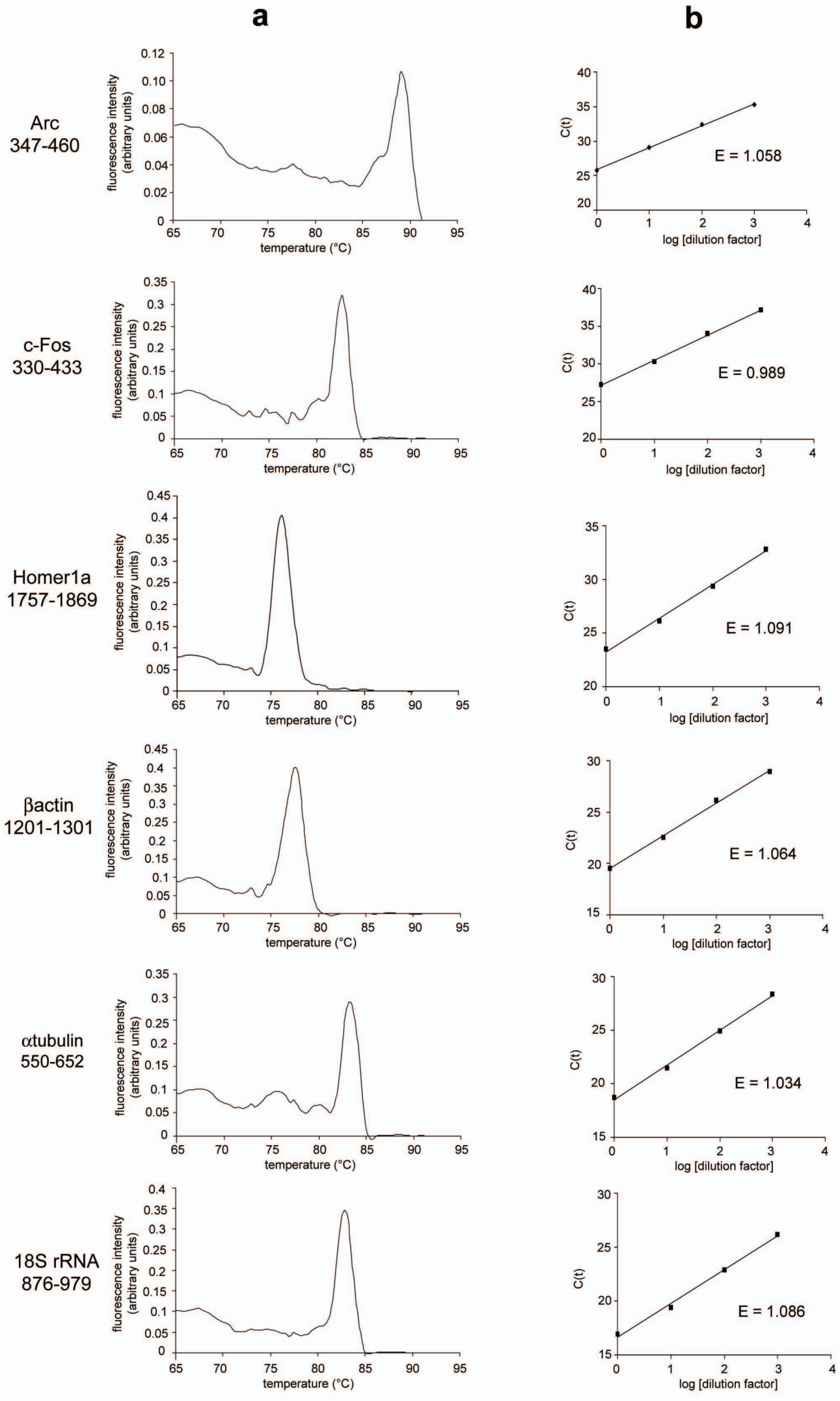
18S rRNA is a gene which has more recently emerged as the housekeeping gene of choice for researchers, as it has been shown to be superior in terms of stable expression to traditional housekeeping genes such as GAPDH or β-Actin in different contexts (Aerts et al., 2004; Al-Bader and Al-Sarraf, 2005; Bas et al., 2004).

A-tubulin as a component of microtubules is highly regulated in brain tissue during development when neurite outgrowth occurs (Bond and Farmer, 1983). However, in mature tissue, one would expect a low and constant expression level. In accordance with this, α-tubulin has been successfully used as an internal control gene for cortical tissue in a learning paradigm in adult monkeys (Tokuyama et al., 2002).

In order to set up a real-time PCR protocol for testing expression of candidate genes in neurons, I designed primer pairs along the RNA sequence of the respective six genes. These primer pairs were tested in regular RT-PCR on brain RNA/cDNA before being used in real-time assays. Following this testing, six primer pairs were selected which showed a single band in endpoint RT-PCR (not shown) and a single peak in dissociation temperature analysis in real-time assays, indicating the synthesis of a single PCR product without formation of primer dimers (Fig. S3a). The primer pairs were then screened for PCR efficiency in a dilution series experiment, and the C(t) values (indicating the number of cycles needed to reach the logarithmic phase) for each dilution plotted against the dilution factor (logarithmic) to obtain the efficiency curve (Fig. S3b). All PCR

reactions with primer pairs for the candidate genes / control genes showed PCR efficiencies between 90-110%, making them good tools to observe gene expression.
In conclusion, real-time RT-PCR was established for four candidate and two control genes to determine the influence of profilin II on activity-dependent gene expression.

Figure S3 (next page): Primer pairs for different genes were tested in real-time PCR. (a), representative dissociation curves of single measurements using the primer pairs noted on the left and cDNA from mouse brain RNA as a template. (b), a dilution series of cDNA from mouse brain RNA (1:1, 1:10, 1:100, 1:1000) was used in triplicates as templates for real time PCR, the C(t) values determined by the ABI software and the mean C(t) values plotted against the dilution factor (expressed as decade logarithms). The efficiency was determined as described in *Methods*


a
b
Arc
347-460
c-Fos
330-433
Homer1a
1757-1869
βactin
1201-1301
αtubulin
550-652
18S rRNA
876-979
fluorescence intensity
(arbitrary units)
temperature (°C)
C(t)
log [dilution factor]
E = 1.058
E = 0.989
E = 1.091
E = 1.064
E = 1.034
E = 1.086

IV. METHODS

Antibody generation. A GST-profilin II expression plasmid (pGEX4T1-profilin II) was cloned by excising the coding sequence of profilin II from pEGFPN2-profilin II (a gift from B. Jockusch, University of Braunschweig, Germany) using EcoRI/SalI restriction enzymes and inserting it into the same sites of pGEX4T-1 (Amersham Biosciences). The fusion protein was purified from E. coli strain BL21 using the following protocol: a 10 ml overnight culture was diluted to 100 ml in LB/ampicillin, grown for 1 hour at 37°C, fusion protein expression induced by addition of IPTG (1 mM), grown for 3 hours at 37°C and bacterial cells harvested by centrifugation (5000 g, 5'). The pellet was resuspended in 10 ml NETN buffer (20mM Tris/Cl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP 40, 1 mM DTT, protease inhibitors), sonicated 3 times for 10 seconds (70% power), the bacterial debris spinned down (SS34, 12 000 rpm, 10') and the supernatant incubated with pre-equilibrated glutathione-sepharose 4B (Pharmacia) at 4°C for one hour. The sepharose beads were gently pelleted (500 g), washed three times with NETN and once with 50 mM Tris pH 8, and finally the fusion protein was eluted by incubation of the beads with 15 mM glutathione in 50 mM Tris pH 8.
GST-profilin II was cleaved by the addition of thrombin, which cleaves after GST due to design of the pGEX4T-1 plasmid. GST was removed by incubating with glutathione-sepharose as described above, and profilin II protein and GST-profilin II were combined, dialysed against 50 mM Tris pH8 and used for injection of two rabbits (Eurogentec, Herstal, Belgium). Small (SZ) and large (GP) bleeds were precipitated by ammonium sulfate, and the protein precipitate dissolved in PBS and dialysed.
For the fusion of maltose binding protein (MBP) to profilin II, profilin II cDNA was excised from pEGFP-profilin II by EcoRI/SalI restriction digest and cloned into the same restriction sites of pMAL-c2x (New England Biolabs). MBP-profilin II protein was expressed in E. coli strain BL21 and purified using an amylose resin according to the manufacturer's instructions (New England Biolabs). In brief, bacteria were grown till they reached logarithmic growth phase in LB medium

containing 0.2% glucose to suppress the expression of amylase. MBP-fusion protein expression was induced by the addition of IPTG, bacteria incubated for ca. 3 hours, harvested and lysed by freeze-thawing and sonication. Extracts were cleared of bacterial debris by centrifugation and loaded on an amylose resin column; after washing steps, the fusion protein was eluted by a 10 mM maltose solution in column buffer.

siRNA design and constructs. Small interfering RNAs were designed using a web interface to select possible siRNA sequences in a given RNA (www.dharmacan.com/sidesign) . Further selection criteria were exposure on 2D structure (www.bioinfo.rpi.edu/applications/mfold/old/rna/form1.cgi) and lower internal stability at the 5'end of the antisense strand (Khvorova et al., 2003). Using these selection criteria, the two sequences siProfII20 (5'-ACGTGGATAACCTGATGTG-3') and siProfII380 (5'-AGGCATACTCAATGGCAAA-3') were chosen as stem sequences, and a loop sequence, transcriptional stop signal and restriction site overhangs added. This gave rise to oligo sequences 5'-CAACAAGATCTCACGTGGATAACCTGATGTGttcaagagaCACATCAGGTTATCCACGTTTTTTGGAAAAGCTTTGTTG-3' for siProfII20 and 5'-CAACAAGATCTCAGGCATACTCAATGGCAAAttcaagagaTTTGCCATTGAGTATGCCTTTTTTGGAAAAGCTTTGTTG-3' for siProfII380. Each of these oligos were mixed with their reverse homolog counterparts in ligation buffer (Roche), denatured by boiling in a water bath, and slowly cooled down by placing the water beaker on ice. The resulting DNA dimer was cut using BglII/HindIII, precipitated, dissolved and cloned into BamHI/HindIII sites of vector pTER which allows inducible small hairpin RNA expression in mammalian cells (van de Wetering et al., 2003).

Cell lysates, electrophoresis and immunoblot. For rat embryonic fibroblast extracts, cells grown in 6 well dishes were washed with PBS, scraped from the dish, spinned down in a microcentrifuge (1000 g) and the pellet dissolved in 100

μl cytoplasmic extraction (CE) buffer (10 mM HEPES pH7.6, 60 mM KCl, 1 mM EDTA, 0.075 % Nonidet-P40, 1 mM dithiothreitol, protease inhibitors), incubated on ice (5') with subsequent pelleting of nuclei (1500 g) and removal of the cytoplasm (cell extract) to add to SDS-loading buffer. Proteins were separated on SDS-polyacrylamide gels and blotted onto Immobilon-P PVDF membranes. Blots were probed with antibodies against c-myc (Santa Cruz sc-40 (9E10), 1:500), profilin II (GP1946, as described, 1:100) or β–tubulin (Santa Cruz sc-9104, 1:500).

Real-time PCR. RNA was extracted from mouse brain using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed using the Thermoscript RT-PCR system (Invitrogen). Real-time PCR reactions were performed in optical 96-well thermal cycling plates (Applied Biosystems). Reactions were set up employing the SYBR green method in which the dye intercalates DNA, leading to a correlation of fluorescence and doublestranded DNA produced. SYBR green PCR-super mix (Invitrogen) with ROX as reference dye was used to set up reactions in a total volume of 25 μl. The results were analyzed using the manufacturer's software (Applied Biosystems) and for further calculations data were transferred to Microsoft Excel. For dilution series, mean C(t) values were plotted against the decade logarithm of the dilution factor and the slope of the resulting fitted curve used to calculate the PCR efficiency by the formula E= 10exp(1/slope) -1.
Sequences of the primers used for detection of activity-dependently expressed genes and control genes were:

Arc: mArc347for: 5'-GGAGGGAGGTCTTCTACCGTC-3'
mArc460rev: 5'- CCCCCACACCTACAGAGACA-3'

c-Fos: mc-Fos330for: 5'-AATGGTGAAGACCGTGTCAGGA-3'
mc-Fos433rev: 5'- CCCTTCGGATTCTCCGTTTCT-3'

Homer1a: mHomer1a1757for: 5'- ATGCCAGCAGAAGGAAGGCTT-3'
mHomer1a1867rev: 5'- AGTCCAGTAATGCCACGGTACG-3'

β-actin: mbactin1201for: 5'- GCTTCTAGGCGGACTGTTACTG-3'

	mbactin1301rev: 5'- GCCATGCCAATGTTGTCTCT-3'
α-tubulin:	Tuba550for: 5'- GAGTTCTCCATTTACCCAGCCC-3'
	Tuba652rev: 5'- AGGCACAATCAGAGTGCTCCAG-3'
18SrRNA:	18SrRNA876for: 5'-ACCGCGGTTCTATTTTGTTGGT-3'
	18SrRNA979rev: 5'-CGCCGGTCCAAGAATTTCA-3'

All primer pairs were used with the following cycling parameters: 50°C 2', 95°C 2', 40 cycles of (95°C 15'', 55°C 30'', 72°C 30'').

GFP-tagged expression plasmids. Expression plasmids for profilin II-GFP and GFP-tagged versions of actin have been described before (Ackermann and Matus, 2003; Kaech et al., 1997). The construction of profilin IIF59A is described elsewhere (Ackermann, 2003). Profilin IIG120F-GFP was constructed using pEGFPN2-profilin II and the Quik change mutagenesis system (Stratagene). Mutant primers were 5'-GGGAAAAGAAGGGGTGCATTTCGGCGGATTGAATAAGAAGGC-3' and the corresponding reverse complementary primer.

Cell culture and transfection. Hippocampal pyramidal neurons were cultured according to Goslin and Banker, with minor modifications. In brief, hippocampi from E18/E19 rat or E17 mouse embryos were dissected in HBSS, briefly trypsinized and triturated to dissociate cells. The cells were gently pelleted, resuspended in HBSS and counted. For transfection using the Amaxa Nucleofector, ca. 1.5 million neurons were used for one electroporation together with 3 μg of DNA, following the manufacturer's instructions, and plated on 18 mm coverslips in a 10 cm bacterial culture dish. For cultures of untransfected neurons (e.g. neurons from profilin II-GFP transgenic mice), between 3×10^5 and 5×10^5 neurons were plated per dish. Neurons were grown on top of a glia feeder layer on glass cover slips coated with poly-L-lysine and maintained in serum-free medium consisting of MEM (Invitrogen) and an N2 supplement(Goslin and Banker, 1991) .

Pharmacological reagents. N-methyl-D-aspartate (NMDA) was from Sigma or Tocris; D(-)-2-amino-5-phosphopentanoic acid (APV) was from Sigma or RBI; 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 1,2,3,4-Tetrahydro-6-nitro-2,3-dioxo-benzo(f)quinoxaline-7-sulfonamide (NBQX) were from RBI; W-7 and and phorbol 12-myristate 13-acetate (PMA) were from Alexis; nifedipine was from Sigma.

V. GENERAL DISCUSSION

V.1. Discussion

This work shows phenomenological data on activity-dependent changes in the actin cytoskeleton and the possible implications for neuronal plasticity. In the discussion, I want to start by looking at the microscopy data on activity-dependent localization changes, discuss their relevance and meaning in a cell biology context, and then move on to implications for cellular plasticity and finally neurobiology.

V.1.1. Nuclear transport of profilin and actin

In this thesis, I describe the nuclear accumulation of the small actin-binding protein profilin in response to neuronal activity and the subsequent activation of NMDA receptors. Judged simply by its size (ca. 15 kDa) and its compact protein structure (Nodelman et al., 1999), profilin should be able to enter the nucleus by normal diffusion through the nuclear pore. The nuclear pore is a proteinacious structure in the nuclear envelope encircling a central channel of about 50 nanometers (nm) (Pante, 2004). Based on this channel size, it is assumed that the molecular cut-off for molecules being able to diffuse freely through the pore is at around 40 kDa (Becskei and Mattaj, 2005). Thus it is unclear whether a fusion protein of profilin II and GFP (calculated molecular weight: 43 kDa) as used in this work would still be able to enter the nucleus passively. However, Stuven and colleagues provide convincing evidence for passive diffusion of profilin-GFP fusions through the nuclear pore in their work on the identification of exportin 6 (Stuven et al., 2003). They show that isolated nuclei prepared in a way that neither nuclear transport factors nor energy resources are present can be supplied with profilin-GFP in the medium so that the fusion protein equilibrates between the medium and the nucleus. Upon addition of exportin 6 protein and ATP, the fluorescent protein shifts to the medium with the nucleus showing no

fluorescent signal, effectively demonstrating passive nuclear import by diffusion and energy-dependent, exportin 6-mediated nuclear export of profilin-GFP. Nuclear export of profilin depends on its interaction with actin; this is demonstrated by my observation that two different profilin point mutations accumulate in the nucleus in the absence of neuronal activity (III.1, Fig.3). Furthermore, Stuven and colleagues demonstrated that exportin 6 binds to actin, which in turn binds profilin, leading to nuclear export of the profilin-actin (profilactin) complex. Interestingly, profilin binding also greatly facilitates nuclear export of actin, making complex formation of these two proteins a prerequisite for efficient nuclear export of both. Therefore I asked the question whether actin would accumulate in the nucleus of neurons under conditions of profilin nuclear accumulation, possibly due to either sequestering of profilin by nuclear binding partners or a general inhibitory effect on nuclear export. In this respect, it has recently been shown that a *Drosophila* profilin homologue is necessary for nuclear export (Minakhina et al., 2005). A widespread effect on nuclear transport following LTP stimuli has been demonstrated by Thompson and colleagues, who showed that importins translocate to the nucleus in response to strong NMDA receptor activation, effectively inhibiting further classical nuclear localization signal-dependent nuclear import (Thompson et al., 2004). Therefore also a general effect on nuclear export cannot be excluded a priori, however my data show no nuclear accumulation of actin-GFP or GFP-actin following NMDA receptor activation (III.1, Fig. 4). The mechanism of nuclear import of actin is unknown, although none of the actin isoforms contains a classical nuclear localization signal. Passive entry through the nuclear pore is possible, but other nuclear import pathways cannot be ruled out.

The data presented in the results section argue for profilin nuclear accumulation being a consequence of less availability of actin for shuttling through the nucleus. A decrease in perinuclear actin leads to less actin entering the nucleus and supporting profilin export, thus causing profilin to accumulate in the nucleus over time. The reason for the decrease in perinuclear actin is the accumulation of

actin at the cell cortex in response to NMDA receptor activation, a very rapid process preceding profilin nuclear accumulation (III.1, Fig.5). A model illustrating these observations and their potential interplay is given in Figure D1:

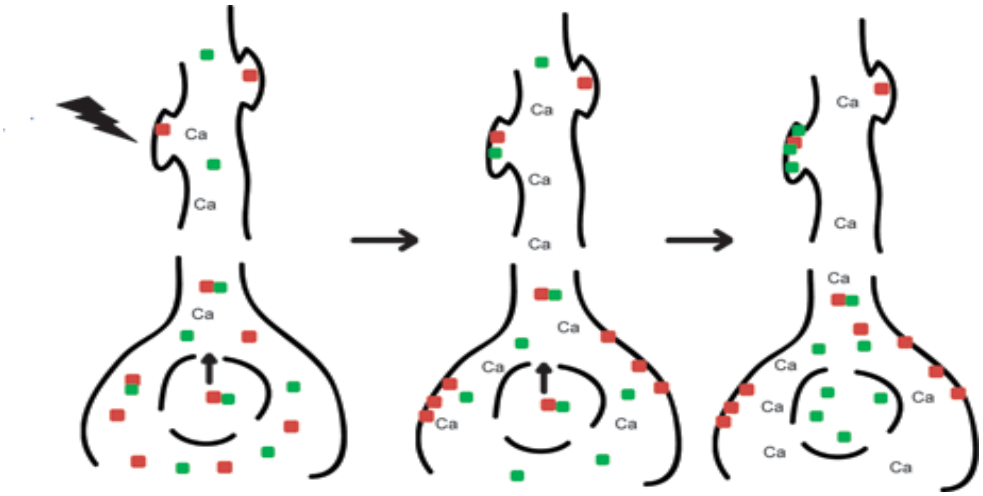


Figure D1: Model for cytoskeletal changes in response to NMDA receptor activation (hypothesis): *Left*, strong synaptic activity (lightning bolt) leads to opening of NMDA receptors and influx of calcium (Ca), which rapidly spreads throughout the cell (*left, middle*). *Middle*, in the soma actin (red) polymerizes at the cell cortex and therefore less actin is available to transport profilin (green) out of the nucleus. *Right*, consequently profilin accumulates in the nucleus, in addition to its targeting from the dendrite to activated synaptic sites.

In my experiments, GFP-tagged γ–cytoplasmic actin showed activity-dependent redistribution to the cell cortex, although it cannot be ruled out that β–actin shows the same effect when tagged N-terminally with GFP (III.1., Fig. 4). However, there is reason to think that γ–actin may be the dominant isoform undergoing this redistribution *in vivo*, as γ–actin is expressed evenly throughout the cell while β–actin localizes to peripheral sites in neurites (Bassell et al., 1998; Micheva et al., 1998).

GFP-tagged γ–actin shows a very rapid redistribution to the cell cortex in response to NMDA receptor activation and Ca^{2+} influx. What may be the mechanisms? To discuss this, I want to take a look at what we know about actin cytoskeleton regulation in response to calcium signals.

V.1.2. Calcium and the actin cytoskeleton

Despite a number of actin binding proteins known to be regulated by Ca^{2+}, a detailed understanding of the relationship of actin to calcium is still lacking. Actin binding proteins regulated by calcium (directly or indirectly) fall into a variety of categories, ranging from bundling proteins (α-actinin, spectrin, fimbrin) to severing proteins (gelsolin, ADF/cofilin), motor proteins (myosin), or proteins with mixed functions (calponin, gelsolin, caldesmon, ADF) (Dent and Gertler, 2003; el-Mezgueldi, 1996; Mangeat and Burridge, 1984; Sarmiere and Bamburg, 2004; Silacci et al., 2004). This list is by no means complete but illustrates how difficult it is to predict the effect of a change in calcium concentration on actin filament assembly, as this will depend on the expression of calcium dependent actin regulatory proteins in a certain celltype or cellular subdomain, among other factors.

One of the best studied systems for actin cytoskeleton changes in response to calcium is the neuronal growth cone, which uses actin filament regulation to steer axons in the direction of growth (Henley and Poo, 2004). A rise in internal calcium in the growth cone inhibits retrograde actin flow (Welnhofer et al., 1999). Moreover, local calcium increase by calcium uncaging induces filopodia formation from axons near growth cones, indicating that calcium is capable of inducing actin polymerization (Lau et al., 1999). Conversely, an increase in calcium levels can also induce growth cone collapse by disruption of F-actin bundles (Zhou and Cohan, 2001). These results demonstrate that even within the same cellular domain, the actin cytoskeleton can react differently to a change in calcium levels, possibly due to different degrees of calcium increase (Henley and Poo, 2004). For actin accumulation at the cortex of the neuronal cell soma, the phenomenon presented in this work was previously observed by Furuyashiki and colleagues using slightly different cell culture systems and stimulation paradigms (Furuyashiki et al., 2002). A fast and reversible polymerization of actin at the cell cortex is in line with other reports showing that actin can undergo drastic

rearrangements on a very fast timescale (Dramsi and Cossart, 1998; Vicker, 2002; Wang, 1991).

Conversely, the actin cytoskeleton has been shown to influence intracellular calcium levels by regulating Ca^{2+} channels as well as Ca^{2+} release from internal stores (Lader et al., 1999; Leach et al., 2005; Rosado and Sage, 2000; Wang et al., 2002). Strikingly, the actin cytoskeleton has also been shown to influence Ca^{2+} dependent transcription. The transcriptional activity of NFAT (nuclear factor of activated T cells) is modulated by actin dynamics influencing the duration of intracellular Ca^{2+} increase in response to various stimuli (Rivas et al., 2004). This is one example of the translation of actin dynamics into gene expression patterns which I want to discuss further with respect to a putative role for nuclear profilin.

V.1.3. The actin cytoskeleton and gene expression

The cytoskeleton mediates changes in cell morphology in response to extra- and intracellular signals, often maintaining altered cell shapes for long periods of time. Actin assembly is regulated by controlling the balance between polymerized and non-polymerized actin, implying the need for new actin monomers in cells undergoing actin assembly (cf. chapter II.2). Therefore it has been proposed that general changes in the organization of the cytoskeleton can control cytoskeletal gene expression (Ben-Ze'ev, 1991).

The actin gene itself, although often referred to as a “housekeeping gene” in gene expression studies, has been shown to be transcriptionally regulated in response to growth factor stimulation and adhesion to the substratum, both having an immediate effect on actin organization (Dike and Farmer, 1988; Farmer et al., 1983; Greenberg and Ziff, 1984).

Experiments using actin-modulating drugs demonstrated that altered levels of monomeric actin are the most significant correlate for changes in expression of the actin and vinculin genes (Bershadsky et al., 1995). The primary transcription factor influenced by actin monomers was shown to be the serum response factor

(SRF) (Sotiropoulos et al., 1999). In fibroblasts, cytoplasmic actin monomers can bind to the SRF coactivator MAL, which is released and enters the nucleus upon integration of the actin monomer into filaments (Miralles et al., 2003). Of note, MAL can also enter the nucleus of cortical neurons in a Rho-dependent manner leading to modulation of SRF activity (Tabuchi et al., 2005). SRF in turn has been found to be necessary for expression of several neuronal immediate early genes, including c-Fos, Egr1/zif268, Arc and β-actin (Ramanan et al., 2005).
These data suggest a model in which transcriptional modulators are sequestered in the cytoplasm and enter the nucleus upon actin polymerization.
Adding to this, it is interesting to note that the actin-regulating protein migfilin also accumulates in the nucleus upon cytoplasmic calcium influx (Wu, 2005). Strikingly, migfilin contains a proline rich sequence analogous to profilin-binding proteins. In the nucleus, migfilin interacts with the transcription factor CSX/NKX2-5 and increases its transcriptional activity in cardiomyocytes (Akazawa et al., 2004).

Profilin redistribution follows a similar pathway: Calcium influx leads to actin polymerization, which causes profilin to accumulate in the nucleus and enable it to interact with nuclear binding partners involved in gene expression. Thus it is tempting to speculate that profilin carries out a similar function in neurons as the other transcriptional modulators described above in various cell systems. However, knowledge about nuclear profilin binding proteins is not sufficient to suggest potential target genes. In the following section I will summarize the knowledge on nuclear profilin with respect to its possible involvement in gene expression.

V.1.4. Gene expression in response to NMDA receptor dependent nuclear accumulation of profilin

As the presence of actin in the nucleus has emerged to be not mainly an artifact but coupled to specific functions, nuclear actin-binding proteins (ABPs) have

been suggested to mainly serve the function of regulating actin (Gettemans et al., 2005; Pederson and Aebi, 2005). On the other hand, nuclear ABPs may also be independent of actin in their nuclear function and primarily act via specific nuclear interaction partners of their own. These options are not mutually exclusive, since actin is thought to perform different nuclear functions involving distinct actin pools (cf. chapter II.2.4). These pools could involve distinct actin structures and therefore different binding partners, highlighted by the fact that different monospecific actin antibodies recognize distinct nuclear actin pools (Schoenenberger et al., 2005).
In the case of profilin, regulation of actin-dependent transcription was the first nuclear function demonstrated, namely transcription of the respiratory syncytial virus genome (Burke et al., 2000). Since profilin is necessary for nuclear export of actin, a role in modulating actin functions by regulating nuclear actin levels seems obvious. However, the export model of Stuven and colleagues implies that profilin-actin complexes may be present inside the nucleus as long as the binding to exportin 6 is blocked by other interactions (Stuven et al., 2003).
On the other hand, the interactions of profilin with survival of motor neuron protein (SMN) and the Myb-type transcriptional repressor p42POP, together with the direct influence of profilin protein on p42POP dependent repression in reporter gene assays, imply a role for actin-independent profilin interactions in nuclear profilin functions (Giesemann et al., 1999; Lederer et al., 2005).

“Myb-type” transcription factors are defined solely by sequence homology to the myb proto-oncoprotein and have mainly been described in differentiating and proliferating cells (Oh and Reddy, 1999). They regulate distinct sets of target genes, highlighted by the fact that c-myb and its highly related viral homolog v-myb display strikingly different transcriptional activities (Liu et al., 2005). Combined with the finding that myb DNA binding domains recognize sites defined by a highly abundant sequence (PyAAC$^{T}/_{G}$G, Py=pyrimidine base) (Ganter et al., 1999), this demonstrates that it is not feasible to predict target

genes of a novel myb-type transcription factor exclusively by an *in silico* approach.

The high expression level in brain and the direct modulation of activity by profilin make p42POP a good candidate for mediating profilin nuclear activity. However, the growing number of profilin ligands implies that other proteins might be functionally interacting with nuclear profilin. To this end, a collaboration with Michael Rebhan (FMI Bioinformatics) was set up to determine novel transcriptionally active profilin ligands. A set of genes implicated in transcription was screened by the following criteria:

(1) presence of a poly-L-proline stretch of at least 10 prolines interrupted by only one other amino acid.

(2) more stringent criterion: presence of a poly-L-proline stretch of at least 11 prolines interrupted by only one other amino acid.

(3) poly-L-proline site interrupted by no other amino acid than either glycine or leucine

(4) expression in brain

(5) absence of any defined secondary structure in regions around the poly-L-proline site

The reason for imposing these criteria on the genes was that a number of known profilin ligands fulfill them, according to analysis of their protein sequence and predicted structure (Michael Rebhan, personal communication).

Additionally, genes and proteins fulfilling these criteria were screened for expression data in array experiments (GEO (Gene Expression Omnibus) database), known protein/protein interactions and cellular processes involved as being of interest with respect to a function in neurons.

Table D1 lists the genes which fulfilled all the criteria mentioned and their known features/functions in relation to brain or neurons.

Uniprot accession #	Gene name	Fuzzpro 10 P, 1 mismatch	Fuzzpro 11 p, 1 mismatch	SymAtlas brain expression	Poly-L-Proline interrupted by only G or L
Q61329/Q15911	ATBF1, alpha-fetoprotein enhancer binding protein	+	+	+	+
P17483	Homeobox protein Hox-B4	+	+	n.d.	+
Q9Y467	Zinc finger protein SALL2 Spalt-like TF	+	+	+	+
Q61345	Foxd1 (Forkhead box protein D1)	+	+	+++	+

Uniprot accession #	SABLE No flanking 2ndary structures (N-term/C-term)	Protein/protein interaction data	GEO	Homologues, cellular function, expression
Q61329/Q15911 (continued)	10/100	Myb proteins	Sp1 transcriptional activation in myoblasts	Myoblast differentiation, STAT3 pathway, liver regeneration, embryonic brain, cell differentiation, neuronal maturation
P17483 (continued)	70/70	Other homeobox proteins	Regulation by antipsychotic drugs	Cell renewal and differentiation
Q9Y467 (continued)	10/90	-	Co-regulated with calsenilin1	Highest levels in adult brain, probably TF
Q61345 (continued)	100/130		Regulated in Circadian rhythm	Predominantly expressed in brain and temporal half of the retina; early development

Table D1: potential profilin ligands implicated in transcription. Abbreviations used: TF = transcription factor, STAT = signal transducer and activator of transcription. Adapted from Michael Rebhan (unpublished; personal communication)

V.1.5. Profilin as a synaptic tag

Taken together, published data on the p42POP-profilin interaction and the putative interactions above suggest a role for profilin in modulating transcription.

A function for profilin in gene expression would mean that this protein embodies properties of a molecule setting a synaptic tag (Frey and Morris, 1998a) and influencing the production of macromolecules enabling long-term plasticity as outlined in the introductory chapter II.2.5 (Fig. I1). However, profilin nuclear accumulation seems to be functionally linked to actin polymerization at the soma, not at distant spines where depletion of monomeric actin could hardly have an impact on nuclear export of profilin. Nevertheless, somatic actin polymerization could occur at functional synapses; axo-somatic synapses do occur on pyramidal neurons, but they tend to show anatomical aspects of inhibitory rather than excitatory synapses (Gray, 1959). It is tempting to speculate that nuclear profilin could influence cytoskeletal gene expression in a fashion similar to actin-filament assembly in other cell systems (Ben-Ze'ev, 1991).

On the other hand, even if strong calcium influx mediated by NMDA receptors alters the cytoskeleton at somatic synapses, gene products expressed in response to activity can still target to distal sites. In fact, if profilin represents a synaptic tag, it would recruit macromolecules to synapses where it is enriched in an activity-dependent manner, which has so far only been described for spine synapses (Ackermann and Matus, 2003). One property of a synaptic tag is that it can promote long-term changes in transmission strength in the synapses expressing it. Consequently, synapses have been shown to be potentiated by stimuli normally insufficient for expression of LTP if paired with a strong stimulus converging on the same set of cells (Frey and Morris, 1997; Frey and Morris, 1998b). The idea is that the “weak” stimulus sets a tag at its synapses which is only converted to a long-lasting change in transmission strength because the strong stimulus leads to activity-dependent gene expression. Thus the cellular mechanisms leading to synaptic tagging on the one hand and gene expression on the other may occur together in many cases of *in vivo* activity, but are not causally linked. In this respect, it is conceivable that in a culture system certain stimuli trigger gene expression but do not lead to synapse tagging, e.g. via the activation of extrasynaptic receptors.

Profilin is a good candidate as a putative synaptic tag, since its activity-dependent concentration is restricted to spine heads. On the other hand, synaptic targeting has not been shown to be reversible so far which would not be in agreement with the current models of a temporally restricted tag (Frey and Morris, 1998a; Martin and Kosik, 2002). However, long-term reversibility following *in vivo* activity has not been investigated, leaving open the possibility that bath application procedures in a cell culture system may activate certain signaling pathways to a degree that does not allow recovery below a threshold level. Profilin, in contrast to other candidates for synaptic tags, has a potential impact on nuclear events and shows activity-dependent nuclear accumulation. This accumulation is reversible and can be re-induced (III.1, Fig.1), in agreement with the idea that different synapses within a cell could be potentiated at different times.
In conclusion, profilin embodies several properties of a synaptic tag, yet work in organized tissue will be needed in the future to determine whether it fulfills all the criteria imposed on a synaptic tag from electrophysiological experiments.

Of note, a nuclear function for profilin II may not be restricted to activity-dependent gene expression in pyramidal neurons: Although profilin II is primarily expressed in neurons of the central nervous system, lower expression levels can be detected in thymus, spleen, kidney and gut (Witke et al., 2001). The isoform profilin IIb, created through alternative splicing, makes up only ca. 5% of total profilin II in brain tissue but can constitute more than 50% in kidney and ES cells (Di Nardo et al., 2000). Profilin IIb has been shown to possess severely reduced binding to actin and to phosphoinositides, which should lead to steady-state localization to the nucleus as shown for actin-binding mutants F59A and G120 F of profilin II (Di Nardo et al., 2000)(chapter III.1, Fig.4).

V.2. Outlook on future experiments

The data presented in this thesis gives us some answers to the questions we asked before starting the project, but more significantly enables us to ask more precise questions for future work. As already mentioned in chapter III.2, activity-dependent nuclear accumulation of profilin is a phenomenon which can be further investigated by the help of tools created in this work and provided by collaborators. In this section I try to outline approaches which I believe to be able to contribute to an understanding of the data presented in this thesis.

V.2.1. The impact of actin polymerization on nuclear accumulation of profilin

As described in chapter III.1, nuclear accumulation of profilin can be explained by activity-dependent polymerization of actin at the cell cortex. However, a direct link between these two observations would strengthen the argument made in chapter III.1 and the hypothesis put forward in V.1 that profilin is a mediator of changes in gene expression in response to alteration of actin filament assembly. Actin filament assembly can be influenced by cell-permeable drugs which can either decrease or increase the amount of actin filaments by different mechanisms. One interesting class of drugs with respect to the intended follow-up experiments in this project are latrunculins, metabolites from the sea sponge *Latrunculia magnifica* (Spector et al., 1983), which bind actin monomers and prevent their incorporation into filaments (Coue et al., 1987). On the other hand, latrunculin does not interfere with actin-binding of profilin since the two binding sites do not overlap (Yarmola et al., 2000). However, it cannot be excluded that latrunculin would interfere with binding of profilin.actin to exportin 6. Therefore the use of another class of actin-depolymerizing drugs, cytochalasins, should be useful to confirm results obtained with latrunculins. Cytochalasins cap the barbed end of actin filaments, leading to net depolymerization from the pointed end and interference with actin-dependent processes (Cooper, 1987).

On the other hand, actin filament-stabilizing drugs such as phalloidins or jasplakinolide can be used to shift the equilibrium between filaments and monomers towards filaments (Estes et al., 1981; Visegrady et al., 2005). In conclusion, actin-depolymerizing drugs can be used to look for prevention of activity-induced nuclear accumulation of profilin, while actin filament-stabilizing drugs may induce nuclear accumulation in the absence of activity. The use of different classes of drugs to account for possible nonspecific effects of particular drugs is preferred.

V.2.2. Long term changes in synaptic transmission strength and nuclear accumulation of profilin

As already mentioned in introductory chapters, profilin is necessary for activity-dependent stabilization of postsynaptic dendritic spines, a putative anatomical correlate of changes in transmission strength. The concomitant accumulation in the nucleus suggests an involvement in different aspects of long-term plasticity as put forward by the synaptic tagging hypothesis (cf. chapter II.2.5, Figure I1 and chapter V.1.5). Therefore it is of interest to investigate the relationship of nuclear profilin and changes in transmission strength, long-term potentiation (LTP) and long-term depression (LTD). The main model system for the cell biological work in this thesis have been hippocampal pyramidal neurons, and LTP and LTD in the hippocampus have been particularly well studied (Lynch, 2004). LTP/LTD can be evoked in CA1 neurons by stimulation of the axon bundle (Schaffer collaterals) from CA3 neurons, an experiment which can be performed on acutely cut slices *in vitro*. Transgenic mice expressing profilin II-GFP as well as wild-type mice coupled with antibody staining can be used (cf. chapter III.2.1). Since profilin nuclear accumulation is rapidly reversible (chapter III.1., Figure 1) it is important to check profilin distribution at different points in time, also during the induction period. It is possible that a certain threshold of somatic calcium concentration necessary for nuclear accumulation of profilin is only reached temporarily.

On the other hand, it will be interesting to determine whether the expression of long term potentiation or depression is altered in profilin knockout mice. For instance, SRF knockout mice, which have impaired expression of several immediate early genes, show a reduction in both the early and late phase of LTP (Ramanan et al., 2005). In this respect, the absence of a putative synaptic tag and transcriptional modulator should have a profound effect on at least the late phase of LTP. However, care has to be taken with respect to upregulation of potentially redundant molecules such as profilin I.

V.2.3. Experience-dependent plasticity and nuclear profilin

Specific sensory experience leads to activity in defined neuronal pathways, which can be highlighted by staining for activity-dependently expressed genes such as c-Fos (cf. chapter III.2.3). In this context, it will be necessary to relate the accumulation of profilin in neuronal nuclei to neurons activated upon sensory experience in order to obtain an indication for the relevance of this phenomenon *in vivo*. Production of an isoform-specific antibody for tissue section stainings (chapter III.2.1) provides the technical prerequisite for performing these experiments. Behavioral paradigms which can be used to evoke activity in a known subset of neurons include whisker stimulation after trimming of certain whiskers, fear conditioning and dark/light rearing (Barth et al., 2000; Campeau et al., 1991; Mower and Kaplan, 1999; Staiger et al., 2002). Again, as mentioned in the section above, timing of analysis could be crucial in order to be able to visualize differences in nuclear profilin levels.

V.2.4. Nuclear profilin and gene expression

Chapter III.2.3 gave an introduction to my approach of looking at candidate gene expression in neurons. The rationale for choosing these genes has been outlined there, explaining the involvement of gene products in synaptic plasticity and their upregulation in response to both electrical and sensory stimulation. However, it is

likely that other genes not described so far are expressed in an activity-dependent manner and can be influenced by putative transcriptional modulators such as profilin. Therefore a screening approach would add to an understanding of a possible role of nuclear profilin in gene expression. Microarray experiments provide a wealth of data; however, their results have to be evaluated by follow-up experiments with quantitative approaches such as real-time PCR (Rajeevan et al., 2001). Moreover, any readout on gene expression comparing tissue from wildtype and knockout animals (e.g., neuronal cultures from wildtype versus profilin II -/- mice) does not directly link the absence of the protein in question (i.e., profilin) to a nuclear function; a significant difference in expression of a specific gene may as well be due to influence on a cytoplasmic signaling pathway. For instance, neurons from profilin II knockout animals have been reported to show increased neurite branching during early development and increased endocytosis (Da Silva et al., 2003; Gareus et al., 2005). Therefore it is necessary to back up data obtained from studies of cells deficient in profilin II with data from cells showing increased nuclear profilin II. In this respect, the actin binding mutants F59A and G120 F (cf. III.1, Figure 3) may prove to be valuable tools. Candidate genes emerging from a screening approach could be tested for increased expression by antibody stainings on cells transfected with nuclear profilin mutants.

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Appendix A: Abbreviations used

ABP	actin-binding protein
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid
APV	D(-)-2-amino-5-phosphopentanoic acid
Arc	activity-regulated cytoskeletal-associated protein
Ca^{2+}	calcium
CaMKII/IV	calcium/calmodulin-dependent protein kinase II / IV
CMV	cytomegalovirus
CREB	cAMP responsive element binding protein
GABA	gamma-aminobutyric acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
GST	glutathione-S-transferase
HBSS	Hank's balanced salt solution
IPTG	isopropyl β-thiogalactoside
kDa	kilodalton
LTD	long term depression
LTP	long term potentiation
MAP kinase	mitogen-activated protein kinase
MBP	maltose binding protein
MEM	minimal essential medium
n.a.	not applicable
n.d.	not determined
nm	nanometer
NMDA	N-methyl D-aspartate
μm	micrometer
p42POP	partner of profilin, molecular weight 42 kDa
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PIP_2	phosphatidylinositol-4,5-bisphosphate

PLF	pore-linked filament
ROCK	Rho-dependent protein kinase
RSV	respiratory syncytial virus
RT-PCR	reverse transcription polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SEM	standard error of the mean
siRNA	small interference ribonucleic acid
SMN	survival of motor neuron protein
SRF	serum response factor
STAT	signal transducer and activator of transcription
shRNA	small hairpin ribonucleic acid
TF	transcription factor

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APPENDIX C: Curriculum vitae

Curriculum vitae

Andreas Birbach
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11th july 1975	Born in Vienna, Austria
1981 – 1993	Primary school and high school in Perchtoldsdorf, lower Austria; final exam (Matura) with distinction
october 1994 – july 2000	Studies of biochemistry at the university of Vienna, Austria
february 1997	Completion of 1st part of undergraduate studies (Studienabschnitt). Scholarship awarded by the university of Vienna for outstanding success during 1st part of studies.
february 1998 – july 1998	Studying abroad as part of the SOCRATES/ERASMUS program of the european union. Scholarship awarded for staying at the university of Coimbra, Portugal.
may 1999 – may 2000	Master thesis (Diplomarbeit) conducted at the Institute for Vascular Biology and Thrombosis Research, Vienna International Research Cooperations Center. Topic: Visualizing signal transduction in the NF-kappaB pathway. Supervision: Prof. Johannes A. Schmid
july 2000	Completion of studies of biochemistry with distinction.
september 2000 – present	PhD thesis at the Friedrich Miescher Institute (FMI) of the Novartis research foundation, Basel, Switzerland, after being accepted to the international PhD program of the FMI. Topic: The actin-binding protein profilin II in neuronal plasticity. Supervision: Prof. Andrew Matus