Regulation of dendritic spine morphogenesis and synapse formation by copines

Inauguraldissertation

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

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Basel, 2006

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List of abbrevations

Ach	Acetylcholine
ADF	actin depolymerizing factor
AKAP79	A kinase (PRKA) anchor protein 79
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
ARP 2/3	actin related protein 2/3
CREB-1	cAMP responsive element binding protein 1
CA 1	Cornu Ammonis field 1
CaMKII	calcium/calmodulin-dependent kinase II
cAMP	cyclic adenosine monophosophate
CBP	Calcium binding protein
CDC42	cell division cycle 42 (GTP-binding protein)
CNS	central nervous system
COS	Monkey Kidney Fibroblast Cells
CREB-1	cAMP responsive element binding protein 1
DIV	days in vitro
E18	embryonic day 19
EPSP	excitatory postsynaptic potential
F/G-actin	filamentous/globular actin
GFP	green florescent protein
GNF	Genomic Institute of the Novartis Research Foundation
GKAP	discs, large (Drosophila) homolog-associated protein 1
GluR	glutamate receptor
GTPase	enzymes that bind and hydrolyze GTP
K50	concentrations of ions giving half-maximal ion binding
kDa	Kilo Dalton
LIMK	LIM-domain-containing protein kinase
LTD/LTD	long-term depression / long-term potentiation
MAPK	mitogen-activated protein kinase
mGluR	metabotropic glutamate recepor
Munc13	mouse unc-13 homolog
nAChRs	nicotinic acetylcholine receptor
NEDD8	neural precursor cell expressed, developmentally downregulated gene 8
NMDA	N-methyl-D-aspartic acid
NMJ	neuromuscular junction
NO	nitric oxide
NRA-1-	copine homolog in *C. elegans*
Pak1	p21-activated kinase
PDZ	PSD-95/Discs large/zO-1
PKA	Protein kinase A
PKC	Protein kinase C
PSD	postsynaptic density
Rac1	ras-related C3 botulinum toxin substrate 1
Rho A	ras homolog gene family, member A
RIM	regulating synaptic membrane exocytosis
RNAi	RNA inhibition
shRNA	short hairpin RNA
SV's	Synaptic Vesicles
SynGAP	synaptic Ras GTPase activating protein
UBC12	ubiquitin-conjugating enzyme E2M

Zusammenfassung

Primäre hippocampale Kulturen aus der Ratte sind ein weit verbreitetes System um molekulare Mechanismen während der Synapsenbildung in Neuronen zu untersuchen. Eine detaillierte Analyse des Erscheinens der synaptischen Eiweisse Bassoon, SynGAP, PSD-95 und GluR2 haben es uns ermöglicht, den zeitlichen Ablauf der Synapsenbildung in verschiedene Module zu unterteilen. Als erstes erkennen wir gleichmässig im Axon verteilt Aggregate des presynaptischen Eiweisses Bassoon. Es handelt sich hierbei um die bereits bekannten „80 nm Vesikel“, mobile Komplexe bestehend aus allen notwendigen Komponenten einer Presynapse. Erst anschliessend beginnen die Neuronen postsynaptische Strukturen auszubilden welche die synaptischen Strukturproteine PSD-95 und SynGAP jedoch keine AMPA Rezeptoren beinhalten. Räumlich betrachtet erscheinen diese „stummen Synapsen“ zuerst in der Nähe des Zellkörpers und sind erst später in weiter entfernten Regionen des Dendriten nachzuweisen. Etwa zur gleichen Zeit wie die Synapsen in distalen Regionen erscheinen kommt es auch zu einer Zunahme in der Zahl und im Durchmesser der Synapsen. Praktisch alle Postsynapsen zeigen ab diesem Zeitpunkt eine Kolokalisierung mit presynaptischen Strukturen. Zu guter letzt, jedoch zeitlich getrennt, steigt schliesslich die Zahl der Synapsen an in denen der AMPA Rezeptor vorhanden ist – ein Indiz, dass die Synapse nun aktiv ist.

Durch ein Such-Test Verfahren mit dem Ziel Gene zu finden die während der Synapsenbildung in primären Kulturen hochreguliert sind, stiessen wir auf Mitglieder der Copine Familie. Um die Rolle der einzelnen Familienmitglieder auf die Synapsenbildung zu untersuchen wurde die endogene mRNA durch RNAi reduziert. Der Verlust von Copine 3 führt zu einer Reduktion der dendritischen Auswüchse und anschliessend zum Zusammenfallen des gesamten dendritischen Baumes. Im Gegensatz dazu führt der Verlust von Copine 6 zu einer Zunahme von Aktin positiven dendritischen Auswüchsen und erhöht Zahl, Dichte, Grösse und Aktivität der Synapsen. Copine 6 erfüllt somit eine Rolle als Synapsen-Unterdrücker. Diese Effekte konnte in sich entwickelnden und auch in reifen Synapsen gezeigt werden. Copine 6 ist ausschliesslich im Hirn exprimiert und findet sich dort vor allem im Hippocampus, Amygdala und im Riechkolben. Wenn man eine einzelne Nervenzelle betrachtet, so findet man Copine 6 nur im Dendriten, und dort wiederum in Spines angereichert. Copine 3 hingegen hat keine dermassen spezialisierte Expression und wird auch ausserhalb des Hirns produziert.

Zu guter letzt zeigen wir das Copine 3 und Copine 6 aneinander und an die Rho GTPasen Rac 1 and Pak 1 binden. Des weiteren wird der Effekt von Copine 6 durch Pak1 umgekehrt. Aufgrund dieser Daten kann man davon ausgehen, dass Mitglieder der Copine Familie zur synaptischen Plastizität beitragen. Es ist anzunehmen, dass dies durch die Regulation der kleinen Rho GTPasen Rac 1 und Pak 1 geschieht, die wiederum die Aktin Polymerisation regulieren.

Summary

Primary rat hippocampal culture is a well established system to study molecular mechanisms occurring during synapse formation and maturation. By closer analysis of the temporal appearance of presynaptic Bassoon and the postsynaptic proteins SynGAP, PSD-95 and GluR2 we were able to dissect synapse formation into distinct modules. Cultured neurons first show axonal clusters of the presynaptic protein Bassoon in the previously described 80 nm dense core vesicles, mobile aggregates of presynaptic proteins. In a second phase, neurons start to form PSD-95 and SynGAP positive synaptic structures that are absent for AMPA receptors. These “silent synapses” appear first in the somato-dendritic region and extend during time into more distal dendritic regions. In the same extend as the synapses appear at more distal regions, also the number, density, size and the colocalization of pre- and postsynaptic markers increases. Finally, in a third step, the number of synapses with incorporated AMPA receptors starts to rise, suggesting active synapses.

In a screen, aimed to detect genes upregulated during initial synapse formation in primary hippocampal culture we detected various copine family members. We then investigated the role of copine family members Knockdown of endogenous copines by RNAi during the period of synaptogenesis unveiled opposing roles on synapse formation. Loss of copine 3 reduced dendritic protrusions and caused a collapse of the dendritic tree during synapse formation. In contrast, knockdown of endogenous copine 6 triggered ectopic polymerisation of the actin cytoskeleton on dendritic filopodia and increased synapse size, number and activity. Thus, copine 6 appears to act as a synapse-suppressor. Interestingly, copines also affect mature spines in adult cultures. Copine 6 is expressed exclusively in the brain and within the brain mainly in the amygdala, hippocampus and the olfactory bulb. On the level of a single neuron, copine 6 is localized exclusively in the somato-dendritic compartment and therein it is enriched in spines.

Furthermore, by co-immunoprecipitation, we show that copine 3 and copine 6 interact with each other and with the actin-modulating small GTPases Rac 1 and Pak 1. Moreover, a knockdown of Pak 1 revert the effect of copine 6 on spine formation. These data suggests that copines contribute in morphological synaptic plasticity by regulating the actin cytoskeleton trough direct interaction with small Rho GTPases.

CHAPTER 1

General Introduction:
Regulation of spine morphology during synaptic plasticity

Galic Milos

General introduction

The human brain has about 100 billion (10^{11}) neurons and 100 trillion (10^{14}) synapses. Somewhere in this number terms like awareness and conciousness are decoded. Where and how lies beyond the scope of this thesis. Yet, another aspect of brain function can more easily be dissected into its molecular components – learning and memory. Since Donald Hebb postulated 1949 that coactivity of pre- and postsynaptic elements results in increased efficacy of their synaptic contacts, knowledge has accumulated that a closer understanding of dendritic spines formation, modulation and elimination and the thereof resulting changes of wiring into networks is key for a proper understanding of learning and memory [1, 2]. Altough dendritic spines were described already a hundred years ago by Ramon y Cajal, the molecular mechanism regulating spine structure are starting to unfold but now [3-5].

On the following pages I attempted to summarize the current view of the role of changes in the spine morphology on synaptic plasticity and the thereby caused broader effects namely network remodeling and in consequence learning and memory. The following survey will start with a short section on the hippocampus, since all our studies were performed in this region of the brain. Next, we will adress the phenomenon of synaptic plasticity at its different level with emphasis on the molecular mechanisms affected by calcium. Upon this, we will discuss how morphological changes of dendritic spines are induced, how they attribute to synaptic plasticity and what effects misregulation of spine stucture can cause. The chapter will then be finished with the introduction of copines, a novel protein family involved in calcium dependent signal transduction.

The hippocampus, a model system

The hippocampus is generally accepted to be important for different forms of learning and memory [6, 7]. Anatomically, it is part of the temporal lobe of the cerebral cortex. It receives input from the entorhinal cortex, the contralateral hippocampus, the hypothalamus, and the basal forebrain. Output fibers project to the entorhinal cortex and the contralateral hippocampus. The hippocampus has only one cell layer, the stratum pyramidale which contains mainly pyramidal neurons. Input into the hippocampus is transmitted via the granule cells of the gyrus dentatus to pyramidal neurons in CA3.

Axons of CA3 neurons, the Schaffer collaterals, project to area CA1 where they form synapses with the apical and basal dendrites of pyramidal neurons in the stratum radiatum and stratum oriens, respectively. This relatively simple trisynaptic pathway of excitation and its importance for learning and memory processes make the hippocampus well suited to study synaptic transmission and plasticity. Therefore we analyse the role of genes in spine development and formation in primary rat hippocampal cultures. This culture, derived form prenatal rat embryo offer unique advantages for the study of neuronal development and synaptogenesis [8, 9]. When maintained under specific culture conditions, primary hippocampal neurons extend axons and dendrites by a stereotyped sequence of developmental events [8, 10]. During the second week in culture, they form physiologically active synaptic contacts which have all the feature of synapses including the characteristic presynaptic accumulation of SV's and the clustering of postsynaptic receptors [11, 12]. Synaptogenesis and spine formation in these cultures is highly synchronous and synaptic contacts are easily accessible and detectable by light microscopy.

From synapses to synaptic plasticity

Synapses are anatomically and functionally specialized structures, where action potentials are transmitted from the axon of one neuron to the dendrite or cell body of another neuron [13, 14]. They consist of a presynaptic, axonal and a postsynaptic, dendritic specialization which are spatially separated by the synaptic cleft. The postsynaptic site can be located directly on dendrites or on tiny protrusions emerging from the dendrites called spines [13]. On the presynaptic site action potentials cause fusion of small membranous vesicles with the presynaptic membrane and release of neurotransmitter from these vesicles. The neurotransmitter molecules diffuse across the synaptic cleft to the postsynaptic membrane where they bind to receptor molecules. The type of receptor activated at the synapse dictates the postsynaptic response.

Many forms of synaptic plasticity have been observed in the cerebral cortex but they all have in common, that alterations of the input cause changes in the transmission properties of synapses. The strength of a synapse is defined by the change in transmembrane potential resulting from activation of the postsynaptic neurotransmitter

receptors and activity-dependent changes in synaptic strength are called synaptic plasticity. Synaptic plasticity can be divided into three broad categories:

(1) Long-term plasticity, involving changes that last for hours or longer, is thought to underpin learning and memory [15-17].

(2) Short-term plasticity, occurs over milliseconds to minutes and allows synapses to perform critical computational functions in neural circuits [18].

(3) Homeostatic plasticity of both synapses and neurons allows neural circuits to maintain appropriate levels of excitability and connectivity despite changes caused by protein turnover and experience-dependent plasticity [19-21].

Long term plasticity

Long-term changes are widely believed to be a key feature in the cellular basis of learning and memory formation [22]. Of the several models used to identify the changes which accompany plasticity in synaptic connections, long-term potentiation (LTP) has received most attention, and although it is not yet clear whether the changes that underlie maintenance of LTP also underlie memory consolidation, significant advances have been made in understanding cell signalling events that contribute to this form of synaptic plasticity. Signalling mechanisms made in LTP were also shown in other forms of synaptic plasticity and impairment of LTP due to misregulation of a protein also affects other forms of synaptic plasticity [23, 24]. However, impairment of LTP does not necessary perturb other forms of synaptic plasticity [25].

Long-term potentiation (LTP) and long-term depression (LTD) occur as a result of correlated or uncorrelated activity of two coupled neurons. LTP, is dependent on the activation of postsynaptic NMDA receptors, a subtype of glutamate receptors that can be regarded as a coincidence detector [26-28]. Upon activation of NMDA receptors, two distinct forms of synaptic plasticity can be described: an early, protein synthesis-independent phase (E-LTP) that lasts between one and five hours, and a late, protein synthesis-dependent phase (L-LTP) that lasts from days to months [29].

E-LTP can be induced experimentally by applying a few trains of tetanic stimulation. Repeated presentations cause the postsynaptic cell to be progressively depolarized until

the magnesium block is removed and successive stimuli promote calcium entry through the NMDAR channel into the postsynaptic cell. Within the spine, calmodulin then binds calcium and the calcium-calmodulin complex directly activates CaMKII which then phosphorylates voltage-gated potassium channels increasing their excitability. CamKII also phosphorylates intracellular AMPA receptors, SynGAP and the MAPK cascade, thereby facilitating the insertion of AMPA receptor into the postsynaptic membrane [30-33]. In parallel, PKA becomes activated by cAMP as a result of the calcium dependent activation of adenylyl cyclase-1 [34]. PKA then phosphorylates voltage-dependent potassium channels and calcium channels enhancing their excitability to future stimuli [34]. Furthermore, PKA increase the number of AMPA receptor at synapses via activity-dependent changes in AMPA receptor trafficking [31, 35-38]. In addition, another component of LTP direct phosphorylates of AMPA receptor and causes modification of the biophysical properties [39, 40]. Taken together, E-LTP leads to increased synaptic strength due to calcium-dependent relocalization and activation of postsynaptic receptors.

The late phase of LTP is dependent upon gene expression and protein synthesis, regulated largely by CREB-1 [41-43]. The synthesis of gene products is driven by kinase dependent activation of transcription factors that mediate gene expression. cAMP response element binding protein-1 (CREB-1) is both necessary and sufficient for late LTP. In its phosphorylated, thus active form CREB induces the transcription of immediate-early genes, including c-fos and c-jun and the products of CREB-1-mediated transcription and protein synthesis give rise to new synaptic proteins [41, 44]. In consequence, synapses at which LTP has occurred undergo structural remodelling. Morphological changes include growth of new dendritic spines, enlargement of pre-existing spines and their associated postsynaptic densities (PSDs), and the splitting of single PSDs and spines into two functional synapses [45, 46].

Long-term depression (LTD), in neurophysiology, is the weakening of a neuronal synapse that lasts from hours to days. The induction of LTD curiously is also mediated through a calcium dependent mechanism. Homosynaptic LTD of basal synaptic responses at Schaffer collateral synapses in the CA1 region of hippocampal slices is induced by low-frequency stimulation activation of NMDA receptor [47, 48], a rise in postsynaptic calcium ion concentration [48], and activation of a serine-threonine protein

phosphatase cascade [48, 49]. LTD can be observed in the neocortex of several species [50, 51]. LTD is thought to result from changes in postsynaptic receptor density, since AMPA-Rs are rapidly internalized in response to LTD-inducing stimuli via a dynamin- and clathrin-dependent mechanism [52-57]

In summary, a growing body of evidence is accumulating concerning the molecular mechanisms underlying long term synapse remodelling. Yet, caution is warranted accepting findings obtained from LTP experiments as a general mechanism. NMDA receptor-dependent LTD and LTP is but one possible mechanism. It is likely that other, independent plasticity mechanisms play a role as well [58]. Recently, mechanistically distinct forms of synaptic plasticity that dependent on mGlu receptor [59, 60] and mossy fiber LTP that is independent of NMDA receptors have been described [61]. Furthermore, it has been suggested that different LTP induction protocols may activate distinct signalling cascades that generate synaptic plasticity with different molecular mechanisms [62-64], and the molecular mechanisms of LTP have been shown to change during development [65-68]. In conclusion, it is likely that various forms of plasticity in the CNS share some underlying mechanisms, but to what extend remains elusive.

Short term plasticity

Changes occurring directly after the applied stimulus and persisting for a relatively short period of time are called short-term changes. Short-term changes depend on presynaptic mechanisms and support a variety of computations [69]. Depending on whether the modulation causes an increase or decrease of the postsynaptic signal, these changes are termed facilitation, augmentation or depression [70]. Facilitation reflects an increase in the probability of neurotransmitter release that lasts for up to hundreds of milliseconds. Synaptic facilitation is observed when the presynaptic neuron is subjected to a short train (5-10 pulses) of stimuli in rapid succession and results in an increased postsynaptic potential. This effect is due to increased transmitter release probability [70] caused by an increase in the presynaptic calcium concentration [71].. Synaptic augmentation is also inducible by conditioning trains of stimuli. Its induction is due to an accumulation of sodium which slows down the extrusion of calcium from the presynapse, resulting in an

elevated calcium concentration, which explains the longer persistence of augmentation compared to facilitation [70]. Posttetanic potentiation can be induced by longer trains of stimuli (in the range of several thousand pulses). In contrast to facilitation which decays within several hundred milliseconds and augmentation which decays after seconds– posttetanic potentiation can last for minutes to hours. Similarly to facilitation and augmentation, the effect is presynaptic in origin and dependent on calcium entry to the presynaptic terminal [72]. Synaptic depression – the contrary to facilitation - also seems to be presynaptic in origin. Facilitation and depression seem to coexist at synapses, with their relative weight depending largely on the initial probability of neurotransmitter release high probability favours depression, low probability favours facilitation. The fact that a large amount of transmitter release is necessary for its induction led to the assumption that synaptic depression may be caused by a depletion of releasable synaptic vesicles in the presynaptic terminal [70].

In summary, short-term synaptic plasticity can drastically influence to what extend an action potential activates its postsynaptic targets [73, 74]. An important consequence of these forms of synaptic dynamics is that responsiveness to different forms of firing pattern is altered [75]. The implementation of changing transmission properties on networks will be discussed later in this section.

Homeostatic plasticity

Without stabilizing mechanisms operating at the level of neural circuits, activity-dependent forms of plasticity such as long-term potentiation (LTP) and long-term depression (LTD) could drive neural activity towards runaway excitation or quiescence [76]. Similarly, without these mechanisms operating at the level of single cells, the complex interplay of inward and outward conductance that subserve each neuron's unique pattern of electrical activity would be difficult to maintain in the face of morphological change and protein turnover [77]. Homeostatic forms of synaptic plasticity are ubiquitous in the developing nervous system [20]. Intensive study of these important phenomena has revealed a palette of mechanisms that contribute to the maintenance of overall excitability. One mechanism is the adjustment of synaptic excitability so that firing rates remain relatively constant [78]. This is achieved by changes in postsynaptic receptor localization and numbers [79-81], presynaptic transmitter release [82, 83] or

reuptake [83, 84], or the number of functional synapses [78, 81]. In consequence the postsynaptic response changes upon release of a single neurotransmitter vesicle. Evidence accumulates that these mechanisms are important *in vivo* [85].

Signal direction

Like the previous results indicate, multiple factors influence the transmission properties of the postsynaptic structure. However, the flow of information across a synapse can also be bidirectional. Synaptic plasticity can depend on feedback from the postsynaptic neuron through the release of retrograde messengers [86-88]. Several retrograde messengers have been identified that once released from dendrites act on presynaptic terminals to regulate the release of neurotransmitter [88-90]. Furthermore, postsynaptic increase of calcium triggered by NMDA receptor activation has an impact on presynaptic neurotransmitter release [91-93]. One candidate for a NMDA dependent messenger is arachidonic acid, which augments synaptic transmission when coupled with presynaptic stimulation. In addition, platelet-activating factor (1 O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) selectively enhances excitatory postsynaptic currents in hippocampal neurons by a presynaptic mechanism upon NMDA dependent calcium influx [91]. Another example is the activity dependent activation of NO synthase, leading to the enhanced production of the putative retrograde messenger, NO [92, 94, 95]. NO leads to a chain of molecular events that facilitate the presynaptic response to subsequent stimuli [92, 94]. And finally, the endocannabinoid system mediates retrograde signalling at GABAergic and glutamatergic synapses [89]. Endocannabinoids are released from the postsynaptic cell following the cleavage of lipid precursors. Endocannabinoid release can be triggered by increased concentrations of calcium in postsynaptic cells and by activation of second messengers systems [96, 97] and leads to the inhibition of presynaptic GABA release [96].

From spines to networks and back again

In summary we can conclude that plasticity is the result of synaptic changes at the biochemical (e.g. changes in ion channel currents) and morphological level (e.g. changes in shape, size numbers or location of synapses on the dendrite). These

changes are achieved by modifications of protein level (relocalization, degradation and de novo synthesis) and protein activity (phosphorylation and dephosphorylation) at the pre- and postsynaptic side. Each synaptic transmission contains information about the previous history of spiking. Synaptic plasticity assures that current activity reflects both the current state of a stimulus and the previous history of activity within the neural circuit. On the synaptic level it means that an identical, basic signal transmitted from one neuron to another can vary enormously in the output, depending on the recent history of activity at either or both sides of the synapse, and such variations can last from milliseconds to months [18], In consequence, synapses from the same neuron can express in parallel different forms of plasticity [98, 99].

How do changes of single synapses affect the state of neuronal activity? Initially, synaptic integration was assumed to result from simple algebraic summation, with dendrites considered only to spatially isolate synaptic inputs and as conduits by which synaptic potentials are delivered to the site of integration [100, 101]. Changes in synaptic receptivity and transmission thus facilitate or inhibit the action potential. However, recent evidence indicates that dendrites are not passive structures, but significantly modify the dynamics of synaptic integration in dendrites. In vitro and in vivo preparations have demonstrated that action potentials actively propagate from the soma into dendrites, where the depolarization they produce can have important influences on synaptic plasticity [102], synaptic integration [103], and dendritic release of neurotransmitter [104]. Furthermore, calcium dependent regenerative events in dendrites can occur in isolation from the soma [105, 106]. In consequence this means that dendrites might modulate synaptic properties globally. Moreover, different neuronal types express specific sets of voltage-gated channels that are highly regulated, undergo developmental changes [107, 108] and can be modulated by intracellular signalling pathways [109].

On the network level, changes in the responsiveness of synapses and their modulation by dendrites decode the filtering characteristics of a neuron. Low depolarization capacity upon neurotransmitter release, such as parallel fibre synapses, functions as a high-pass filter, whereas synapses with a high initial capacity of depolarization, such as climbing fibre synapses, act as low-pass filter. Changes of the synaptic transmission [73] can convert a synapse from a low-pass filter to a band-pass filter, or from a band-pass filter to a high-pass filter [75]. In consequence the coding behaviour of the neuronal network changes.

In summary, changes of synaptic transmission alter the output (firing pattern) and the input (postsynaptic responsiveness to different forms of firing pattern) of neurons. Given that there are many more synapses than neurons in a typical circuit, the state of a neural network might better be described by specifying the state of its synapses. Neural responses typically arise from the summation and interaction of several synaptic inputs. To predict how a circuit will respond to a stimulus and to interpret that response, we therefore need to know the dynamic state of its synapses.

From synaptic plasticity to spine morphology

The dendritic spine is the postsynaptic compartment of most excitatory synapses and some inhibitory synapses [110]. A dendritic spine consists of a bulbous head with the postsynaptic density, an electron-dense structure of densely packed ion channels and cell surface receptors and the spine neck, a narrow structure that links the spine head to the dendritic shaft [110-112]. Spines are dynamic structures that can change shape during lifetime [110, 112]. In consequence, dendritic spines vary in sizes and shapes, even on the same dendrite [110, 112]. In most regions of the developing brain, the formation of dendritic spines coincides with the main period of synaptogenesis in the first few weeks after birth [113]. As synapses mature, the number of filopodia declines and the number of stable spine-like structures increases, suggesting that filopodia are the precursors of dendritic spines [114]. Dendritic spines and synapses in general remain plastic in the adult brain. Spine formation, pruning, and remodeling in mature neurons can be induced by many factors, such as certain patterns of synaptic activity, learning and memory formation, hormonal fluctuations and changes in temperature [13, 115, 116]. Furthermore, synaptic plasticity occurs at single spine level and is regulated by local protein trafficking, synthesis or degradation [117-122].

Actin and spine morphology

It is generally believed that actin rearrangements drive the formation and loss of dendritic filopodia and spines as well as their morphological plasticity [123]. The constant turnover of actin filaments in dendritic spines most likely involves the treadmilling of existing filaments, with polymerization occurring at the fast growing ‘‘barbed’’ ends, which are

predominantly oriented towards the surface of the spine, and depolymerization occurring at the ‘‘pointed’’ ends [124]. The changing spine head contains a variety of proteins in the postsynaptic density regulating the actin filament. We can distinguish two protein families which are controlling actin dynamics independently to achieve this function: actin depolymerizing factors (ADF) and capping proteins. These proteins are regulated by small GTPases of the Rho family. RhoA, Rac1, and Cdc42 are ubiquitously expressed but present at high levels in neurons [125, 126]. Constitutively active Rac1 causes a reduction in the size of the dendritic spines but increases their density, in parallel with increasing the number of synapses [125]. Consistent with these in vivo data, overexpression of constitutively active Rac1 in cultured hippocampal and cortical slices induces the formation of irregularly shaped protrusions resembling membrane ruffles and lamellipodia-like ‘veils’, which may consist of densely packed very small and thin protrusions [125, 127]. Furthermore, constitutively active Rac1 causes the formation of long and fine processes on the cell body and proximal dendrites of pyramidal neurons [125]. In cultures of dissociated hippocampal neurons, constitutively active Rac1 also promotes the formation of lamellipodia-like protrusions, but disrupts synapse formation in contrast with its in vivo effects [128, 129]. On the other hand, overexpression of a mutant form of Rac1 that blocks exchange factors, and therefore acts as a dominant-negative, drastically decreases the number of both spines and synapses in cultured hippocampal slices and dissociated hippocampal neurons [125, 128]. Taken together, these data suggest that Rac1 promotes the development of new spines and that an optimal level of Rac1 activity is required for proper spine morphogenesis and the maintenance of normal spine morphology.

Misregulation of spine morphology

Deformed dendritic spines and deficient spine density are a hallmark of many neurological conditions, notably in virtually every disease in which cognitive performance is impaired. Alzheimer’s disease is perhaps the best characterized neurological disease with significant learning and memory dysfunction. Substantial decreases in dendritic spine density in pyramidal cells of the neocortex and hippocampus can be observed in human tissue from Alzheimer’s patients [130]. Dendritic spine loss is reported in other non-Alzheimer’s type dementias, and may represent a pathological acceleration of the normal decrease in dendritic spine density observed in senescence [131]. Furthermore,

pyramidal cells in several different forms of mental retardation have a lower than normal density of spines, including Down's syndrome and fragile X syndrome [132, 133]. Decreases in spine density and structural synaptic abnormalities are also common in human tissue from psychotic schizophrenic patients [134], and in hippocampi from patients suffering from uncontrolled epileptic seizures [135].

From spine morphology to calcium

The spine represents the smallest computational unit of the brain and calcium compartmentalization in spines is likely to be functionally important, because calcium mediates input-specific forms of synaptic plasticity [136, 137]. Increases in calcium concentration can have opposite effects on spine morphology depending on their magnitude and duration. Moderate and transient elevations in intra-spine calcium level induce spine elongation. In contrast, large and sustained increases in calcium levels due to high concentrations of glutamate cause spine shortening and in some cases collapse [138].

Calcium decay kinetics in spines is controlled on one site by duration and amount of calcium influx and on the other side by diffusion of calcium across the spine neck and active removal of calcium from the spine cytoplasm [139]. In consequence, the morphology of the spine neck and the expression and regulation of calcium pumps and buffers control the duration of calcium transients in spines. Generally, about 80% of the calcium ions that enter the cell are rapidly buffered by CBPs. CBPs, distributed throughout the cytoplasm, bind and buffer calcium. Single, unpaired action potentials or EPSPs result in sharp increases in calcium which both peak at 1 mM. The action potential-induced increase in calcium decays within 20 ms. Calcium entry, particularly through NMDA-Rs, which are localized on the synaptic face of the spine, will create a concentration gradient across the spine, with high concentrations, as high as hundreds of µM, near the mouth of the channel. If these proteins are concentrated very near the NMDA-Rs, the probability of their activation can be orders of magnitude greater than if the same number calcium ions are uniformly distributed through the spine. Thus the localization of proteins near the source of calcium might strongly influence the function of this protein complex. In this context, the calcium dependent delocalization of proteins might be crucial to the function of proteins.

The biochemical pathways required for translating the calcium signal into a change of the underlying cytoskeleton are not known. In dendritic spines, calcium functions both as a charge carrier and as a signaling molecule that influences the activities of many proteins, including several actin regulatory proteins [140]. Therefore, changes in calcium concentration affect the organization of the actin cytoskeleton with consequences on spine shape and synaptic strength [123, 141, 142]. In this manner, activation of neurotransmitter receptors can induce the formation and remodeling of dendritic spines and influence their stability

From calcium to copines

Copines are a scarcely described family of cytosolic proteins that show calcium-dependent phospholipid-binding properties [143]. The copine family is conserved in organisms reaching from Paramecium to human and the functions attributed to members of the copine family range from cell death repression and increased disease resistance in Arabidobsis over gonadal cell division in C. elegans to neural tube closure in mouse [144-151]. In the following we will summarize the observed effects and will discuss possible implementations in spine formation. Copines are cytosolic proteins of 50-60 kDa size. Although no direct structural information is available, the sequence homologies between copine family members allow prediction of their domain organization. Copine 1-9 all share a similar organization, with a linear sequence of two C2 domains followed by one A domain (Figure 1A). C2 domains are calcium-dependent, phospholipid-binding domains that regulate calcium or lipid binding properties on the proteins in which they reside and via the A domain copines are capable of interacting with a wide variety of "target" proteins, that are themselves components of intracellular signalling pathways [152]. Recent publications suggest that copines are present at low calcium levels as monomers [143]. Upon increase of calcium concentration, copines undergo conformational changes and multimerize into higher order homo- and heteromere [143, 150] (Figure 2). Thus, copines can receive calcium-changes originating at the cell surface and convert them into changes in the localization and activity of interacting proteins [152].

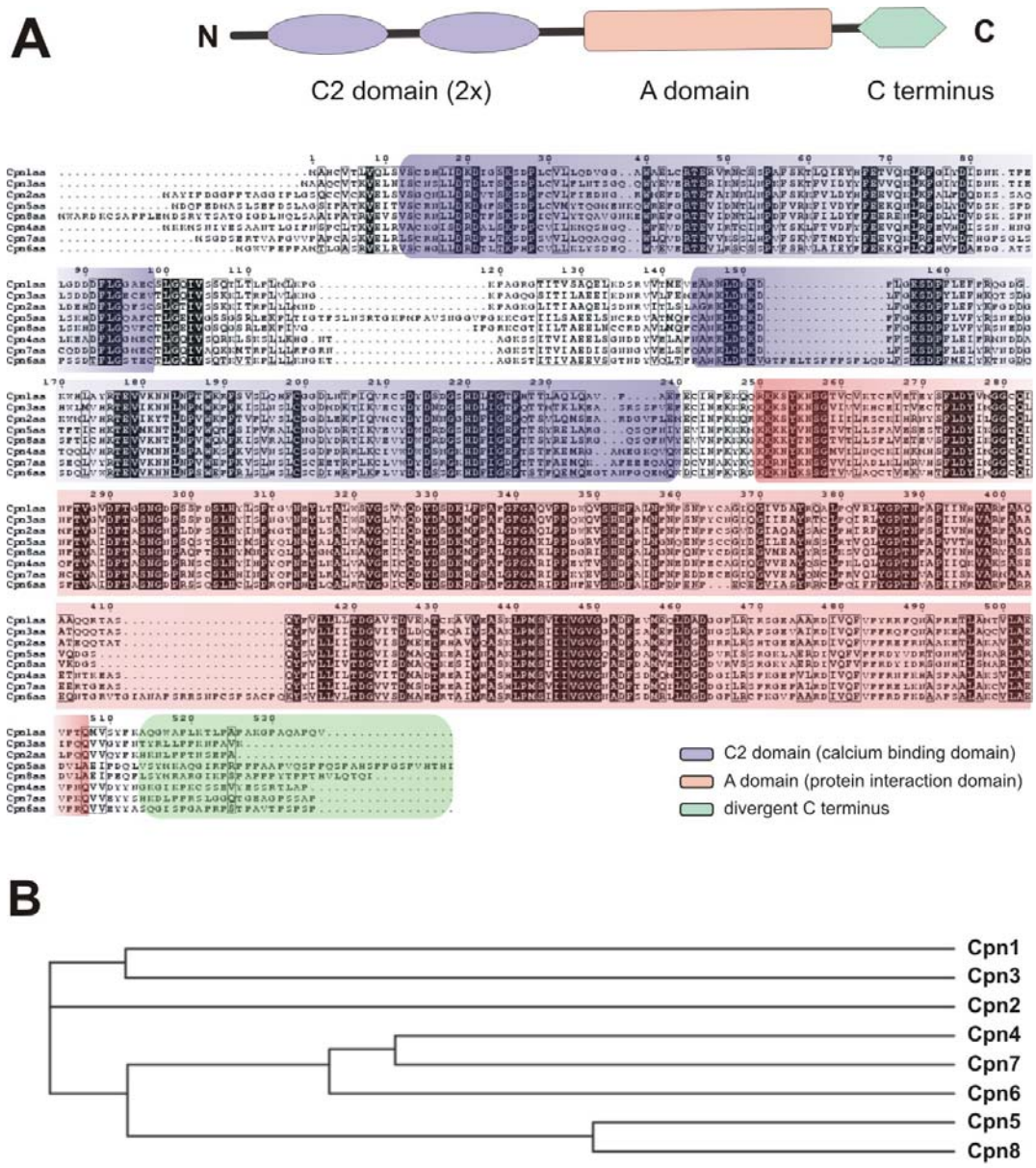


Figure 1 The copine protein family

(A) Structure of copine proteins and alignment of the predicted amino acid sequences. The copine protein consists of three functionally distinct structures: The two C2 domains at the amino-terminal part are responsible for calcium-dependent plasma membrane interaction. On the C-terminal part, copines have one A domain involved on protein-protein interaction, presumably via a coiled-coil structure that is followed by a highly divergent C terminal ending, presumably involved in copine-specific protein interactions. Below, sequences were aligned using ClustalW from the EMBL-EBI homepage Asterisk mark the consensus homologous in all nine family members, conservative substitutions are encircled. The C2 and A domain are highlighted in purple and red, respectively. As indicated in the alignment, all copines share these structures but are highly divergent at the very C terminal part that is highlighted in green. (B) Cladogram showing the relative copine homologies between the individual family members. According to the amino acid sequence homologies, the copine family can be divided into 3 major families. Copine 9 is missing, since no amino acid sequence is available. Note that CNS specific members (copine 4, 6 and 7) share highest homology.

In *Dictyostelium* changes in calcium caused a very transient membrane localization of a GFP-copine fusion protein [150]. The transient localization of copine often occurred multiple times within the same cell, suggesting that the translocation from cytosol to membranes and back to the cytosol is a respond to fast intracellular calcium spikes or waves [150]. .This suggests that copine rather “bind and react” to changing calcium concentrations rather than just “bind and buffer” it. Given that independent copine members bind to specific proteins [152], then an increase of calcium orchestrates the relocalization of cytosolic proteins to plasma membranes. As a consequence copine-interacting proteins accumulate calcium-dependently at plasma membranes in spines. As an extension of this idea, calcium can also cause the assembly of copine heteromere [153]. Each copine binds to independent interacting proteins and a calcium dependent accumulation might promote biochemical reactions by spatial enrichment of interacting partners (discussed later). In the following we will discuss functions attributed to copines that are calcium-dependent and might affect spine formation upon multimerization.

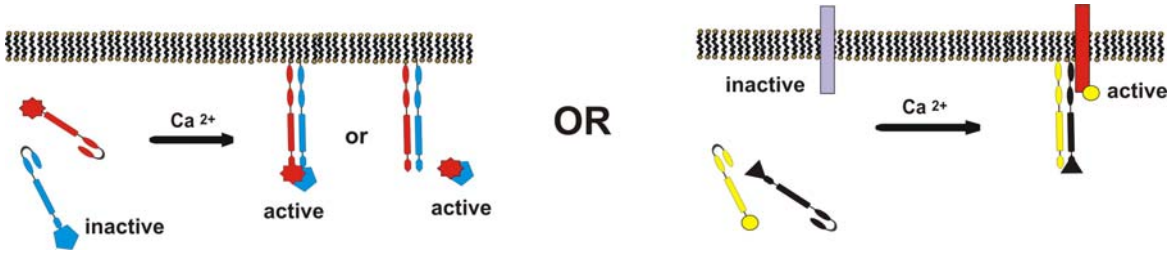


Figure 2 Model of copine function

Calcium dependent relocalization of copines and interacting proteins. For reasons of simplification copine are illustrated to form hetero-dimer instead of hetero-multimer. At low calcium concentrations, individual copines are present as monomers in the cytosol. As a result of increasing calcium concentration, copines undergo a conformational change at the N-terminal part as indicated by the opening of the loop. In consequence, copine multimerize and bind to the plasma membrane. Due to the multimerization of copines *inter se* proteins that bind to individual copines accumulate. As a result, these proteins can interact within the complex (left picture). Alternatively, target proteins can be localized at the plasma membrane (right picture), since the relocalization of the complex to the plasma membrane accumulates copine-interacting proteins in the vicinity of the plasma membrane (right picture).

However, calcium is not essential for copines to bind to lipid membranes composed of phosphatidic acid [153] or plasma membranes isolated from Arabidopsis cells [148]. Furthermore, copine protein was constitutively localized to the plasma membrane in transfected leaf protoplasts [148] and copine 6 in the brain was not completely removed from plasma membranes upon calcium depletion [154]. Thus, copine proteins might also be constantly localized at plasma membranes and serve as scaffolds mediating the assembly of receptors and synaptic proteins. In *C elegans*, copine was shown to be required for maintenance of normal levels of nAChRs at synaptic sites [155]. They show the association of the copine with the levamisole receptor, thus they argue that the copine homolog NRA-1 may recruit proteins that interact with the levamisole receptor, possibly in an activity-dependent manner [155]. Deletion of copine caused resistance to cholinergic agonists and reduced synaptic levamisole receptor levels; thus, copine may play a relatively specific role in targeting or stabilizing the levamisole receptor at the plasma membrane [155]. Besides supporting the targeting of other proteins at plasma membrane, copines might also support the targeting of vesicles at plasma membrane. Interestingly, members of the Munc and Rim family as well as piccolo and synaptotagmin contain C2 binding domains alike copines [156-158]. Munc13-1 [159, 160], RIM [161] Synapsins [156, 162] and Piccolo [163] are presynaptic proteins organizing the exocytic machineries at the transmitter release site. Synapsins are peripheral SV membrane proteins that are firmly established as regulators of neurotransmitter release [156-158]. Intriguingly, in Arabidopsis, copine gene function is required for exocytosis [148]. It is assumed, that copine could function either by acting catalytically (increasing the fusion of vesicles with the membrane) or structurally (by associating with the plasma membrane to maintain membrane function at low temperature). Copine function in exocytosis might, in theory, also extend to the presynaptic side.

In Arabidopsis, the *copine* family regulates cell death by repressing a number of *R* genes [146]. One possibility is that copines directly influence transcriptional or translational regulation at the level of nucleic acids. The other possibility is that copines bind to regulators of these events and therefore indirectly regulate protein expression. However, copines appear to regulate gene expression, thereby increasing protein levels. Interestingly, copines seem also to contribute to protein degradation. A possible direct link between copines and ubiquitination pathway is represented by the interaction

between the NEDD8-conjugating enzyme UBC12 and the copine I A domain [152]. NEDD8 is an ubiquitin-like protein that is covalently attached to proteins targeted for degradation through the co-ordinated action of the conjugating enzyme UBC12 and other enzymes. Recent data suggests, that copines may regulate NF *κ*B signalling calcium dependently by promoting I*κ*B degradation via an activatory effect on UBC12 [164]. Possibly, endogenous copine binds UBC12 and promotes its association with other components of the signalling pathway on the membrane surface, or regulates its activity directly in a calcium-dependent fashion. Recent publications suggest that copines might exceed purely scaffolding properties (calcium dependent and independent, respectively) to an active participation in the modulation/activation of effector proteins in spine formation. Copine 3 shows intrinsic kinase activity [165]. In vitro kinase assays were performed with immunoprecipitated endogenous copine 3, chromatography-purified endogenous copine III, and recombinant copine 3. The exogenous substrate myelin basic protein was phosphorylated in all in vitro kinase assays containing copine 3 immunoprecipitate or purified copine 3 [165]. Interestingly, a search for kinase protein motifs did not identify the classical kinase catalytic domain. Copine 3 may therefore represent the first member of a novel unconventional kinase family. Phosphorylation can act as a posttranslational modification to rapidly alter protein function, and phosphorylation-mediated activation can produce some of the changes attributed to copines.

In summary, the function of the copines is mainly decoded in the expression and - in consequence – in the composition and responsiveness of the individual copine multimers. It is not clear whether individual copines are responsible for specific functions, but copines bind to individual interacting partners [152], show a tissue specific expression (discussed before) and becomes transcriptionally upregulated upon synaptic activity [166]. Taking this into account, changes in relative amounts of copines might cause alterations in the composition and function of complexes. With other words, the presence or stochiometric changes of individual copines within a complex might cause changes in the protein composition and alter the receptivity to diverse upstream pathways and in consequence the outputs originating from the complex.

Topic of this thesis

Aim of this thesis was to find and describe the role of novel genes involved in synapse formation in the CNS. Starting material was a list of genes derived from a microarray study to analyze changes in gene expression profile during synapse formation at the neuromuscular junction. To achieve this goal, we first designed a novel system to detect genes involved in synaptogenesis of the CNS and investigated the expression profiles of individual genes derived from the previously described list during initial synapse formation. With this approach we detected copine family members to be transcriptionally upregulated during synapse formation. In a second part we then focused on the role of the copine family members on synapse formation. By transfection of overexpression and knockdown constructs of the copine family members into primary hippocampal culture we further dissected the role of copines in synapse formation. We found that copine 3 and copine 6 are involved in various aspects of synapse formation. Since we find copine dependent changes in spine morphology, we next focussed on the involvement of copine on actin rearrangements. We find that copine 3 and 6 are able to bind to small GTPases and thereby modulate the underlying actin cytoskeleton in developing and mature spines.

CHAPTER 2

Regulation of dendritic spine morphogenesis and synapse formation by copines

Galic M, Kriz A, Vigot R, Bentzinger CF, Zhang YP, Bezakova G, Stebler M, Oertner T, Bettler B, Ruegg MA

[2006]

In Preparation

Summary

We investigated the role of copine family members during synapse formation in primary hippocampal cultures. Genes from the copine family are upregulated during this critical period. Knockdown of endogenous copines by RNAi during the period of synaptogenesis unveiled opposing roles on synapse formation. Loss of copine 3 reduced dendritic protrusions and caused a collapse of the dendritic tree during synapse formation. In contrast, knockdown of endogenous copine 6 triggered ectopic polymerisation of the actin cytoskeleton on dendritic filopodia and increased synapse size, number and activity. Copine 6 is enriched in spines and binds in a calcium dependent manner to plasma membranes. Finally, by Co-Immuoprecipitaion, we show that copine 3 and copine 6 interact with each other and with Rac 1. These data suggests that copines contribute in morphological synaptic plasticity by regulating the actin cytoskeleton trough direct interaction with small Rho GTPases.

Introduction

Spines, which protrude from the dendritic branches are the principal site of excitatory synapses and may function as the basic unit of synaptic integration [1, 2]. Formation of spines is established by sequential cellular events [3, 4] and is accompanied by morphological changes of dendritic filopodia into mature spines [5, 6]. Even after the establishment of these contacts, spines are still motile and change their shape and size [7-9]. *De novo* synapse formation and activity-dependent changes of synaptic structures can also be observed in adult animals. Structural changes of spines in adult animals are thought to allow functional changes in synaptic strength [10] and provide neural circuits with the ability to rewire [11-14]. Thus, structural changes of spines are thought to contribute to learning and memory [14]. Several molecules have been identified as potential regulators of spine development [15-17]. To induce formation, elaboration or elimination of dendritic spines these factors exert their effects by signalling to the actin cytoskeleton [18-20] and the function of these proteins is often regulated by activity-induced changes in intracellular calcium concentration [15].

Recent studies have suggested a possible function for copines as calcium sensors. Copines are cytosolic proteins characterized by two C2 domains at the amino-terminus and an A domain at the C-terminus. C2 domains are calcium-dependent, phospholipid-binding domains that regulate calcium or lipid binding properties on the proteins in which they reside and via the A domain copines are capable of interacting with a wide variety of "target" proteins, that are themselves components of intracellular signalling pathways [21]. It is assumed that copines bring calcium dependently their interacting proteins in the immediate vicinity of the membranes. Thus, proteins that were spatially separated accumulate due to multimerization of copines inter se and biochemical events within the multimolecular complex can affect localization, amount and activity of target proteins. It is noteworthy to mention at this place that copine 3 shows intrinsic kinase activity [22]. In vivo and in vitro studies have shown that copines are involved in a wide range of biological activities including exocytosis, gene transcription, protein degradation, cytoskeletal organization and targeting or stabilizing of receptors at the plasma membrane [23, 24].

So far 9 members have been described based on their structure. While most of the copines are expressed ubiquitously, copine 4, 6 and 7 have been shown to be expressed exclusively in the brain. Copine 4 recently was reported to interact via the A-domain with Cdc42 binding protein MRCKβ and β-actin [21]. Cdc42 belongs to the family of small GTPases and dendritic morphogenesis [25]. Copine 6 expression in hippocampal neurons was upregulated upon increased synaptic stimulation by kainate injection and LTP [26]. Interaction partners and physiological function of copine 7 are unknown [27].

Although many studies illustrate localization and the biochemical function of copines, it remains unclear whether they also affect dynamic changes in spines. To address this question we examine the role of all copine members during synapse formation in cultured hippocampal neurons. We find that several copines are upregulated during synapse formation. We report that loss of copine 6 increases the number, size and activity of dendritic spines. Knockdown of copine 3, however, causes a loss of synapses and a retraction of the dendritic tree. Moreover, we provide evidence that these effects on spine morphology are caused by copine-dependent regulation of the actin cytoskeleton through the small Rho GTPase Rac 1.

Results

Neurons develop functional synaptic contacts during the second week in culture

Recent publications describe mobile vesicles in axons and dendrites filled with synaptic proteins [28, 29]. These mobile units, composed of preformed scaffold protein complexes, serve as predetermined synaptic hotspots for establishment of new functional excitatory synapses [28]. An increase in postsynapstic structures is observed at DIV 12–14 [4] and the number of active zones able to fuse synaptic vesicles increases between DIV 11 and 14 [4, 30]. To visualize the formation and maturation of synapses in our system, we first performed co-staining of various pre- and postsynaptic markers and evaluated their content and location at different stages of neuronal development. At day in vitro 7 (DIV 7), staining of PSD-95 and SynGAP, prominent proteins of the postsynaptic compartment, was limited to diffuse staining in the soma and proximal dendritic shafts and did not extend into distal regions of the dendritic shaft (Figure 1A, B, left panel). Between DIV7 and DIV15, there was an increase in the diameter of PSD-95 and SynGAP positive puncta (Figure 1A, B, middle panel), followed by a significant increase in the total number of clusters that spread over the entire dendritic tree (Figure 1A, B, right panel). Furthermore, colocalization studies for PSD-95 and SynGAP show a high percentage of overlap for SynGAP and PSD-95 suggesting that all postsynaptic structures were stained (data not shown). For both proteins the number of postsynaptic structures remained constant between DIV 7 and DIV 11 and increased nearly three fold between DIV11 and DIV 15. The diameter of postsynaptic structures stained for PSD-95 and SynGAP in both cases increased between DIV 7 and DIV 15 gradually as indicated in the graphs to the left. The diameter corresponds to serial electron microscopy data of 3D reconstruction of rat hippocampal dendritic segments from stratum radiatum of area CA1 [31]. The increase in spine diameter is of particular interest, since an increase of +25 in diameter reflects almost a doubling in volume.

We next examined whether postsynaptic clusters of SynGAP colocalize with the presynaptic scaffold protein Bassoon (Figure 1B). We find numerous Bassoon positive puncta at DIV7, representing Piccolo/Bassoon transport vesicles (PTVs) (Figure 1B). Previous findings indicate that 2–3 PTVs need to be incorporated at a nascent synapse to supply enough active zone proteins and membrane to constitute an active zone [28, 32].

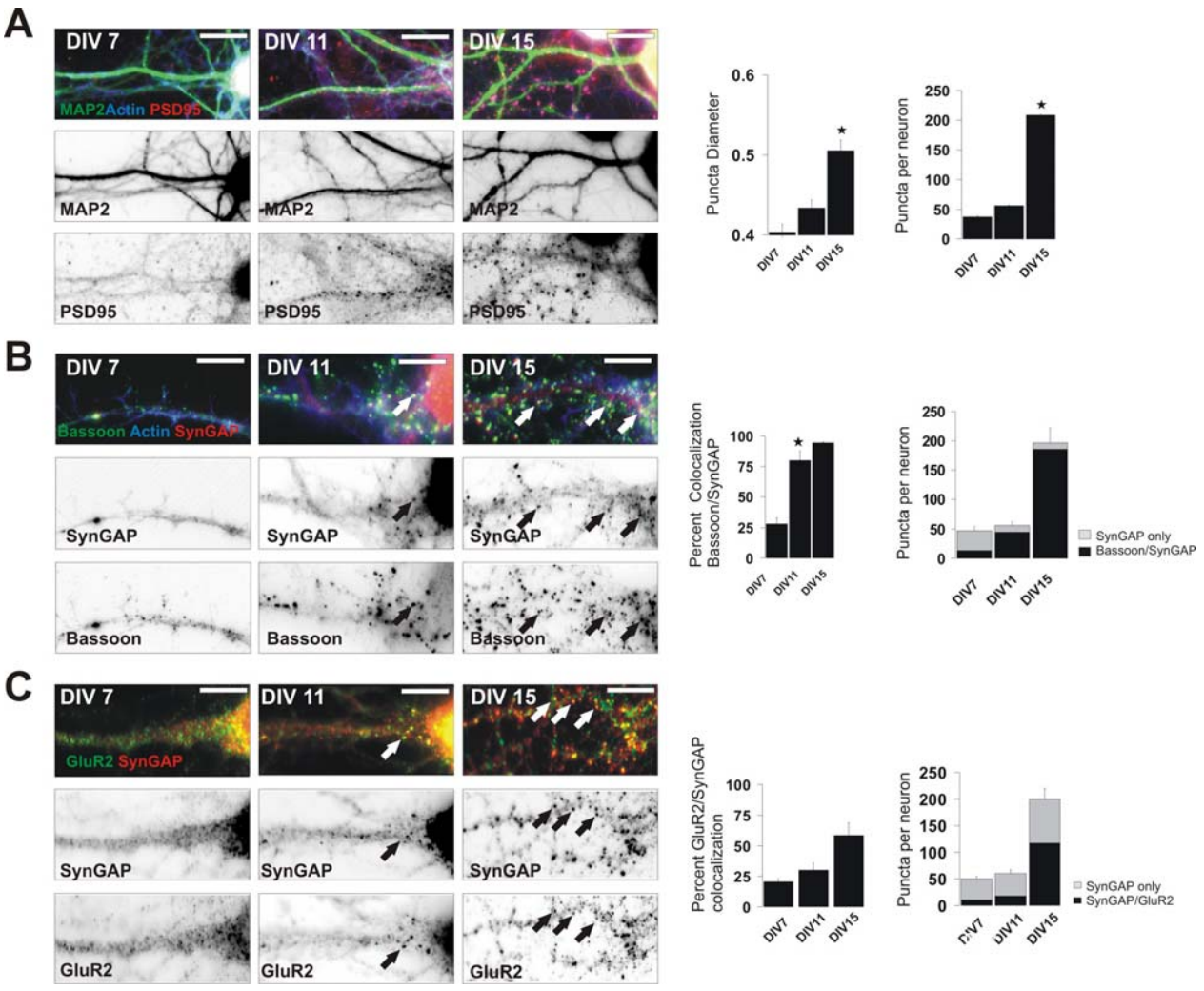


Figure 1 Localization of individual synaptic protein in primary hippocampal culture during synapse formation as revealed by immunostaining

(A and B) Primary hippocampal cultures were stained at DIV7 (left panels), DIV11 (middle panels) and DIV15 (right panels) against the postsynaptic scaffolding molecules PSD-95 (A; red) or SynGAP (B; red). (A) Quantification of changes in puncta diameter (left) and in puncta number (right) at the three time points are shown next to it. Both, PSD-95 and SynGAP staining first appear at somato-dendritic regions and, accompanied by an increase in number and size, exceed to more distal regions. Note that puncta size increase continuously whereas puncta number increases mainly between DIV11 and DIV15. (B) Quantification of co-clustering of pre/postsynaptic marker. Colocalization of pre/postsynaptic staining increases between DIV7 and DIV11. Arrows indicate colocalization. Analysis of the colocalization of pre- and postsynaptic structures (B, left) and a graph summarizing the absolute SynGAP puncta number with the percentage of Bassoon/SynGAP costaining (B, right) is shown next to the pictures. (C) Quantification of synapses with incorporated GluR2 receptors. Colocalization of SynGAP (red) GluR2 (green), as indicated by the arrows, increases continuously between DIV7 and DIV15. Quantification of relative GluR2/SynGAP colocalization (left) and the absolute number of contacts positive for SynGAP and GluR2 (right) are shown next to the pictures. Data represent the analysis of neurons from at least two experiments, n = 15–20 neurons per group, ≥800 clusters per group. Error bars represent mean ± SEM. The asterisk denotes significance values of $p < 0.01$ compared to the previous timepoint. Scale bar = 10 µm.

In accordance with this finding, we see between DIV7 and DIV11 an increase in the colocalization of pre- and postsynaptic marker from 25% to 75% colocalization that is accompanied by an increase in diameter of Bassoon positive puncta (Figure 1B). This suggests that the percentage of colocalized pre and postsynaptic structures increases between DIV7 and DIV11, whereas the number of colocalized pre and postsynaptic structures mainly increases between DIV 11 and DIV 15.

To address the question if these colocalized structures are able of electrical transmission we stained for AMPA-R incorporation into excitatory synapses (Figure 1C). We observe that 23 % of GluR2 colocalize with SynGAP at DIV7 and 59% of SynGAP positive structures appear also GluR2 positive at DIV 15. These data correspond with previous findings that, the hippocampal glutamatergic network becomes gradually functional during the first postnatal week owing to the transformation of pure NMDA receptor-based synaptic contacts into conducting AMPA/NMDA-receptor-type synapses [33, 34].

Copine family members are upregulated during synapse formation *in vitro* and *in vivo*

Synapse formation in primary hippocampal culture is accompanied by gene transcription [35-37]. In previous work, we analyzed changes in gene expression during synapse formation at the neuromuscular junction. Interestingly, we find upregulation of copine family members (data not shown). We then asked if some of these genes might also be upregulated during synapse formation in primary hippocampal culture. We isolated mRNA from primary hippocampal culture, transcribed it reversely and performed quantitative PCR (Figure 2A). The expression levels of the individual copine genes were compared at four time points, namely at DIV8, DIV10, DIV12 and at DIV14. Expression profiles were normalized to a housekeeping gene. The first detection was set to the value 1, thus absolute concentrations can not be compared between the individual copines.

Expression changes among the individual copine family members during development can be grouped into three types. Type I include copine 2 and copine 5 and exhibit no significant up-regulation, whereas Type II and Type III clusters show increased expression, reaching detection levels either at DIV 10 or DIV 12. Type II copines (copine 1, 3, 4, 6 and 8) reach detection level at DIV 10, whereas the Type III copine (copine)

exhibit transcriptional upregulation above threshold at DIV12. Except copine 5, no other copine was detected at DIV 8. In Figure 2B we check the system by analyzing the expression profile of phosphoglycerolkinase 1 (PGK-1), another housekeeping gene and the postsynaptic protein SynGAP. To confirm the real-time data, we next measured protein levels during synapse formation. Protein was extracted from primary hippocampal culture at DIV 7, 11 and 14. The western data corresponds with the upregulation observed at the translational level (Figure 2C). Next, we examined protein from cortex of rats at postnatal day 0, 3, 6, 9, 12 and 28. We find copine 3 and copine 6 to be upregulated in parallel with SynGAP during the second postnatal week (Figure 2D). The remarkably similar upregulation of copines and synaptic genes like SynGAP during synapse formation *in vitro* and *in vivo* suggests an involvement in synapse formation. To further probe a possible role of copines in synapse formation we decided to knock down all copine family members that were significantly upregulated during synapse formation using shRNA in primary hippocampal cultures.

Knockdown of copine family members causes changes in spine morphology

We identified 21 bp shRNA sequences that specifically reduced overexpressed copine-GFP fusion proteins in COS cells (Supplementary 1). To assess the involvement of copines in synapse formation, primary hippocampal neurons (DIV7) were transfected with shRNA against copines or against CD4 in combination with an enhanced green fluorescent protein (GFP) expression vector. Neurons were fixed and imaged 4, respectively 7 days after transfection (DIV11 and DIV14) and analysed. Knockdown of copine 1, 3, 4, 7 and 8 but not of copine 6 or CD4 led to swelling of neuronal soma, vesiculation of neurites and accumulation of green fluorescent cell debris in the culture when examined at DIV14, suggesting that cells with a knockdown for 7 days undergo apoptosis (data not shown). Except for copine 8, neurons survived upon reduction of the transfection period to 4 days (DIV7-11). At the superficial level the knockdown of copine 1 and 7 appeared to have no affect on neuronal morphology at DIV11 (Figure 2C). Interestingly, a knockdown of copine 3 showed aspiny dendrites whereas a knockdown against copine 6 show the opposing effect, namely an ectopic outgrowth of dendritic filopodia (Figure 2C). Copine 4, however, did not affect protrusions but altered dendritic arborisation. Neurons lacking copine 4 show a collapse of the dendritic tree accompanied by an ectopic lamelipodial outgrowth along the entire dendrite (Figure 2C).

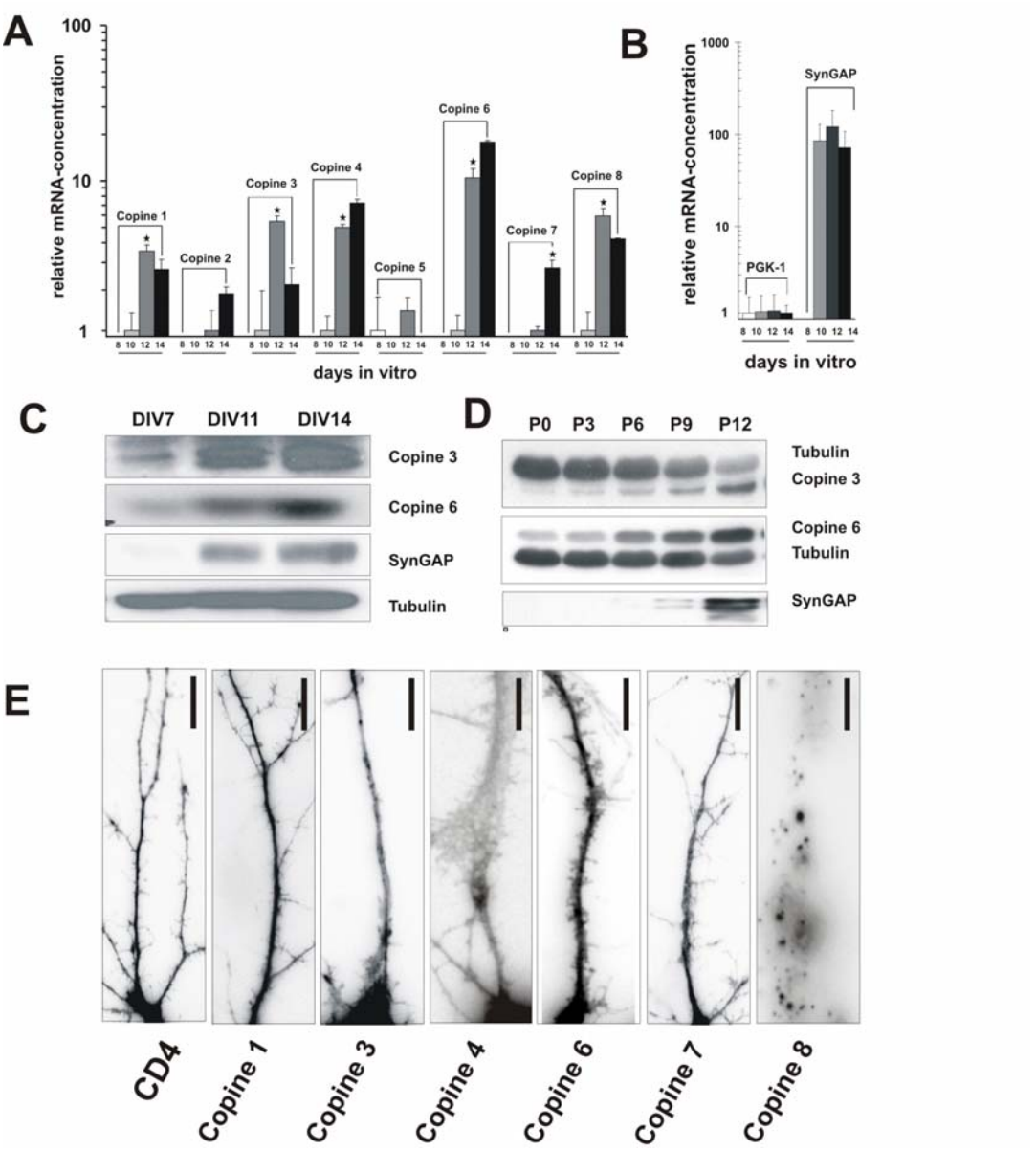


Figure 2 Copines are expressed during synapse formation and affect neuronal morphology

(A) Expression level of copines during synapse formation in primary hippocampal cultures as determined by quantitative real-time PCR. Copines are blotted in a semi-logarithmic scale showing the relative mRNA concentration in relation to the housekeeper. (B) PGK-1, another housekeeping gene, does not change during synapse formation, whereas SynGAP expression is upregulated. Note that expression of SynGAP and most copines is upregulated during the period of synapse assembly. Data represent the analysis of three independent experiments, each point was analysed by qRT-PCR in triplicates. The asterisk denotes significance values of $p < 0.01$, Error bar represent mean ± SEM. (C) Western blot of endogenous protein concentration of copines in primary hippocampal cultures at DIV7, DIV11 and DIV14. Protein level of copine 3 and copine 6 become upregulated during synapse formation. Note the changes in protein level of SynGAP, copine 3 and copine 6 correspond with the changes in expression described above by real time. (D) Western blot of developing rat cortex homogenates from postnatal day 0, 3, 6, 9 and 12. Copine 3 and 6 are upregulated during the second postnatal week. Note, that synapse formation occurs in various regions of the neocortex during the second postnatal week *in vivo* [38]. (E) Representative examples of neurons transfected with a knockdown against the individual copines at DIV7 and analyzed at DIV12. Spines on control neurons (left picture) transfected with a knockdown against CD4 reveal filopodia and first mushroom-like spines on arborized dendrites. The knockdown of copine 1 and 7 do not alter neuronal morphology at the superficial level. Knockdown of copine 3 leads to smooth, aspiny dendrites, whereas the knockdown of copine 6 causes ectopic spine formation and a strong ruffling on soma and neurites. The knockdown of copine 4 causes a dendritic collapse and filopodial outgrowth along the dendrites. Only cell debris can be observed of copine 8. Scale bar represents 10 µm.

Since we were interested to dissect the functional implementation of copines in spine morphology, we decided to look more closely on copine 3 and copine 6. Analysis of the number and size of protospines at the distal part of the dendritic tree showed for copine 3 a reduction of 36 % in protrusion density and 21 % in protrusion length (Supplementary 1). The decrease in dendritic complexity caused by copine 3 shRNA appears to be specific and is not due to the activation of the shRNA machinery per se, since expression of CD4 shRNA did not result in the described effect (Figure 2C). Protrusion number in neurons transfected with a knockdown against copine 6 remained constant but caused increased outgrowth of the actin cytoskeleton (Supplementary 5). When the protrusion length was measured, a subtle but significant reduction in length by 9 % could be observed.

Copine 3 affects protospine development

To obtain insights into the function of copine 3, we next overexpressed a series of fusion proteins, all of which contain the full length copine 3 gene coupled to enhanced green fluorescent protein (GFP) directly or separated by an internal ribosomal entry site (IRES) in primary hippocampal culture during synapse formation. The transfected cells died within 2 days, independent of the fusion protein and of the duration of the transfection (data not shown). We then focussed on the copine 3 knockdown. As described previously, reducing copine 3 levels in neurons results in a loss of spines followed by a collapse of the dendritic tree. Analysis of neurons 3, 5 and 7 days after transfection unveiled a continuous progression where the loss of spines precedes the collapse of the dendritic tree and apoptosis (Figure 3A, B).

To exclude the possibility that any effects seen with copine 3 shRNA were due to apoptosis, we analyzed the presence of active caspase 3 and picnotic cell bodies at DIV11, when the loss of protrusions occurs. We could not see an increase of picnotic cell bodies (data not shown). When we look for the pro-apoptotic marker caspase 3 in cells lacking copine 3 we see an upregulation by +110% compared to untreated adjacent cells (Figure 3C-E). However, since the pro-apoptotic pathway is an “all-or-nothing”-decision and cells lacking copine 3 survive after DIV11 for at least another 3-4 days, we assume that a loss of copine 3 protein first triggers the loss of protrusions and of the dendritic tree and that – in consequence – the so caused increased stress finally accumulates a critical amount of active caspase 3 that causes a fast apoptosis of the cell [39].

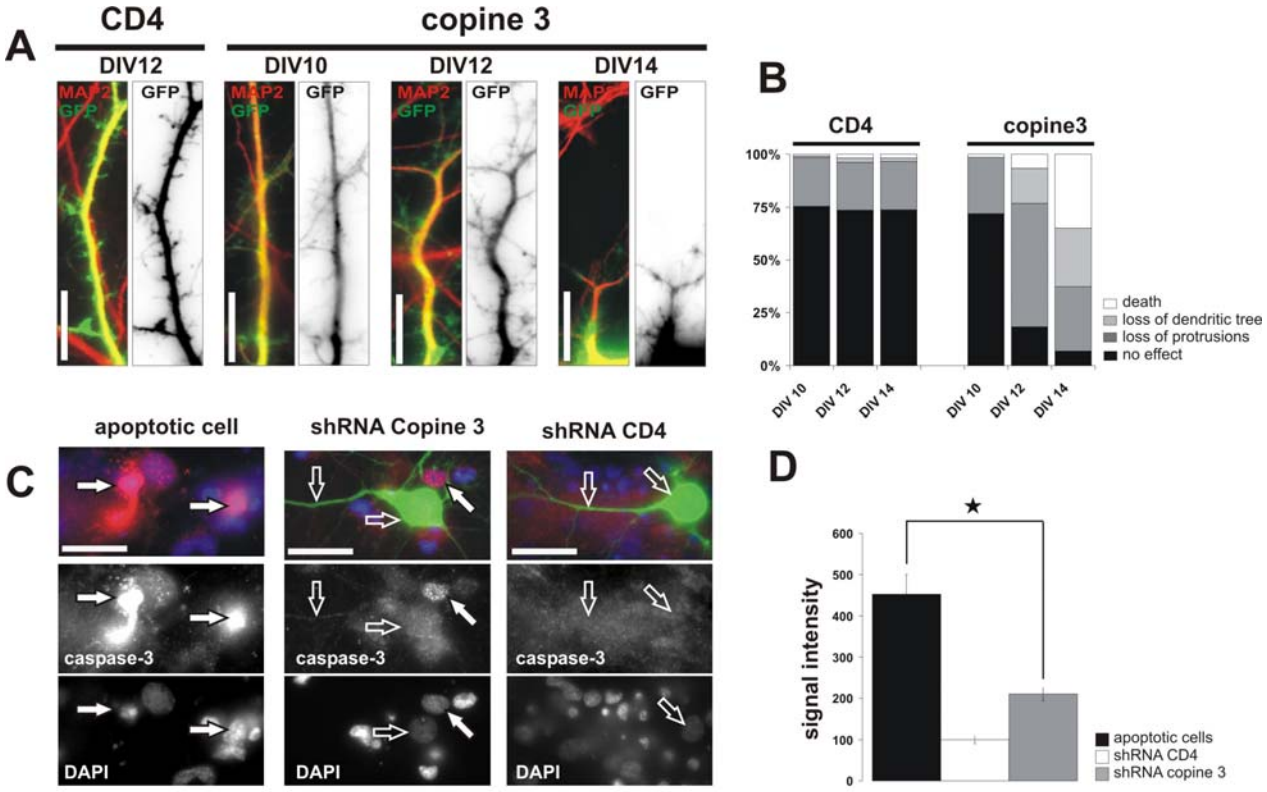


Figure 3 Knockdown of copine 3 reduce dendritic protrusions and dendrite complexity

(A and B) Morphological changes of neurons transfected at DIV7 and examined at DIV10, DIV12 and DIV14 upon knockdown of copine 3 and CD4, respectively. (A) Representative pictures of neurons illustrate the progressive loss of filopodial structures followed by the retraction of the dendritic tree upon knockdown with copine 3. (B) Quantification show no changes in the knockdown of CD4 (B, left) whereas the knockdown of copine 3 unveils an increasing percentage of abnormal morphology with time (B, right). Data represent the analysis of three independent experiments, n = 25-30 neurons per group, Scale bar = 10 µm.(C and D) Comparison of apoptotic cells, control cells and copine 3 knockdown by active caspase (C, red) and DAPI (C, blue) staining. The knockdown of copine 3 at DIV11 (C, middle panel, transparent arrows) shows an increase of activated caspase activity compared to normal cell (C, right panel, transparent arrows) but significantly lower levels than an apoptotic cell defined by the picnotic cell body (C, left panel, full arrows). (D) Quantification of the intensity of active caspase 3 staining. Note the significant difference in caspase3 activity of copine 3 knockdown compared to apoptotic cells (D, asterisk). Data represent the analysis of two independent experiments, intensity of n= 15-20 neurons per group was measured. The asterisk denotes significance values of $p < 0.01$, Error bar represent mean ± SEM. Scale bar = 20 µm.

Copine 6 regulates spine maturation

Alike with copine 3, the overexpression of full length copine 6 in cultured hippocampal neurons led to apoptosis of neurons within 2 days (data not shown). Since copine 6 is localized in soma and dendrites, we focussed on the effects of a knockdown on the spine morphology. To quantify the effect of copine 6 knockdown on spine morphology, we first used the postsynaptic marker PSD-95 to outline the number, shape and dimensions of postsynaptic sites (Figure 4A). PSD-95-positive puncta at the proximal 100 µm of the dendritic tree were analyzed at DIV12. Knockdown of copine 6 causes a significant increase of spine number by + 66% and a slight over all increase of spine head diameter by + 9% compared with neurons transfected with RNAi against CD4 (Figure 4A). PSD-95 staining does not necessarily report spine head diameter in the absolutely quantitative sense, however: it shows relative changes in the PSD-95 accumulation on the postsynapse and therewith a measurable parameter of the change. Analysis of high resolution confocal pictures of synapses in the distal 100 µm show no abnormal shaped synapses. Spine neck and spine head appear normally formed, although bigger. (Supplementary Figure 5). When compared to CD4, copine 6 knockdown shows an increase of artificial actin-positive structures from the dendritic shaft (Supplementary Figure 5).

To see whether the knockdown of copine 6 also affects the presynapse, we next performed a costaining of the presynaptic marker Bassoon together with SynGAP (Figure 4B). The analysis unveiled a significant increase of +32% in the costaining of the two markers, indicating an increase of total synapse number (Figure 4B).

Enlargement of dendritic spines has been shown to correlate with an increase in surface glutamate receptors [40] and thus might represent a change of the synaptic properties. To investigate the effect shRNA copine 6 might have on excitatory synapses, we transfected pyramidal neurons and performed mEPSC analysis by whole-cell patch-clamp recording. Neurons were transfected at DIV7 and analyzed at DIV12, during the period when synapse formation occurs (Figure 4C). We see no change in mEPSC amplitude compared with neurons where CD4 was transfected. Since changes in amplitude represent changes in the postsynaptic size, we assume that copine 6 knockdown does not alter the amount of incorporated AMPA at the postsynapse.

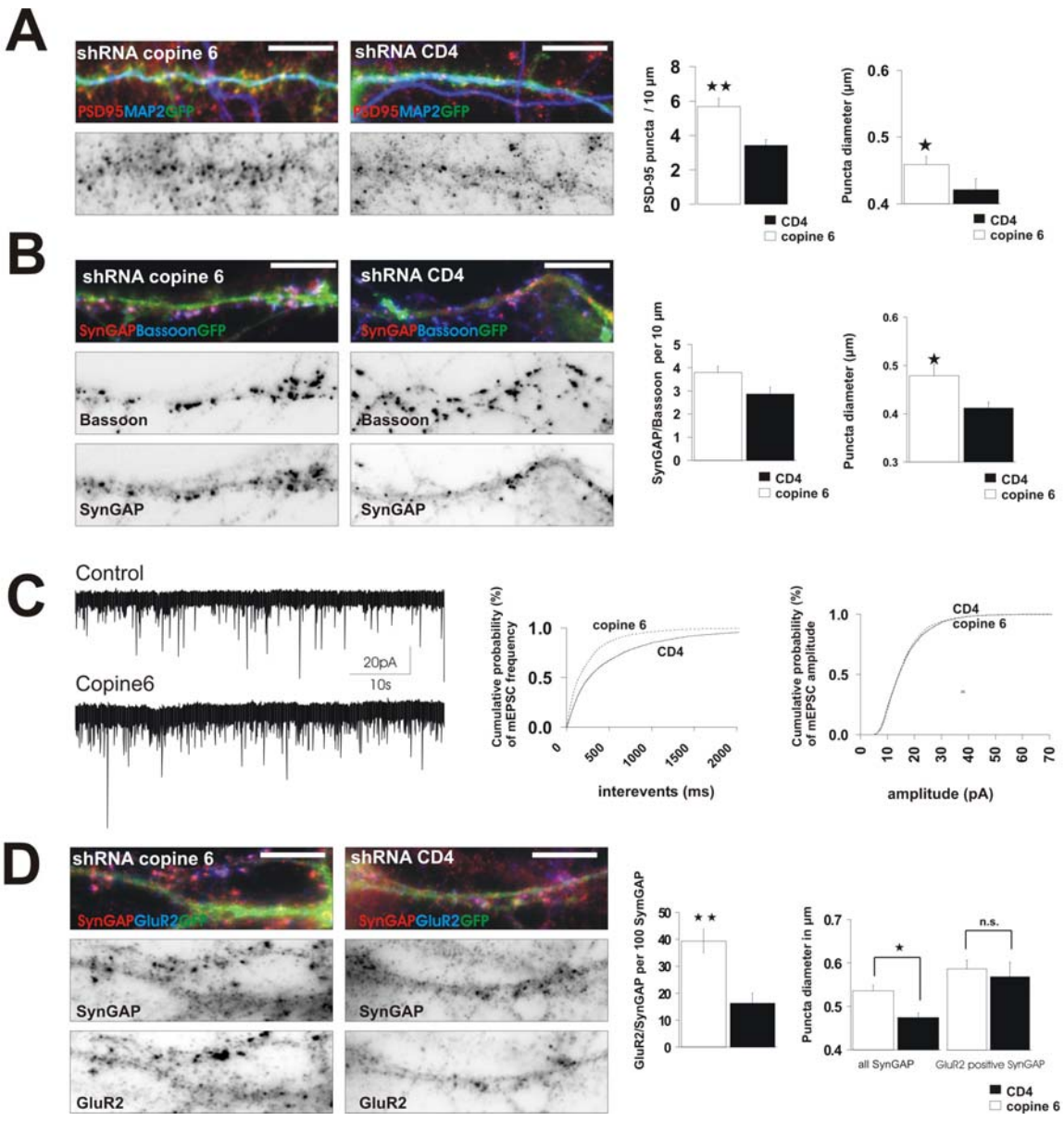


Figure 4 Knockdown of copine 6 promotes spine morphogenesis and activity

(A and B) Morphology of GFP-labelled hippocampal neurons after knockdown of copine 6 (left panel) or CD4 (right panel). The hippocampal neurons were transfected at DIV7 and stained at DIV12 against the postsynaptic scaffolding molecules PSD-95 (A; red) or SynGAP (B; red). The quantification shows a significant increase in puncta density (A, left graph) and increased puncta size (A and B, right graph) for both postsynaptic markers in the copine 6 knockdown compared to CD4, as indicated in the diagrams next to the pictures. (B) Quantification of SynGAP/Bassoon costaining shows a slight increase in the colocalization of pre- and postsynaptic structures upon loss of copine 6 (B, left graph). (C) The knockdown of copine 6 resulted in an increased mEPSC frequencies but constant amplitudes when compared with neurons transfected with CD4 (C, left). Cells were transfected a DIV7 and analyzed at DIV12. 800 mEPSC's per neuron and 6 neurons per condition were analyzed. Left illustrates representative traces. To the right the cumulative probability of the mEPSCs is plotted. No changes for the mEPSC amplitude can be observed; $p < 0.01$ for the frequency in the Kolmogorow-Smirnow test. (D) Knockdown of copine 6 shows an increase of GluR2 incorporation into synapses (D, left graph). Comparison of the diameter of GluR2-containing synapses shows no significant increase (D, right graph). Note, the increase of average spine size (D, right graph) observed in A and B is caused by a higher percentage of the bigger spines containing GluR2-receptors but not due to an overall increase of spine size. Data represent the analysis of neurons from at least two experiments, n = 20-25 neurons per group, ≥1000 clusters per group. ★★$p < 0.01$, ★$p < 0.05$. Data represent mean ± SEM. Scale bar = 10 µm.

To test this idea, we next focussed on the synaptic localisation of endogenous AMPA receptors. Transfected hippocampal neurons, stained with an antibody specific to identify the extracellular part of the GluR2 subunit of the AMPA receptors, exhibited a noticeable increase by 241% in GluR2/SynGAP costaining relative to neighbouring untransfected cells (Figure 4D). Closer analysis unveiled an increase in AMPA positive postsynaptic terminals with a constant AMPA intensity, suggesting no change in synaptic size but an increase in synapses with incorporated AMPA receptors. In accordance with this idea, cells with a knockdown of copine 6 exhibited a significant enhancement in the frequency of mEPSC's when compared with control cells (Figure 4C).

Taken together, a knockdown of copine 6 during synapse formation causes an increase of synapse number and an increase in the percentage of active synapses suggesting a synapse-inhibiting role of copine 6.

Functional interaction of copine 3 and copine 6

Since copines are able to form higher order multimers, the possibility emerges that interaction between copine 3 and copine 6 might affect regulate synapse formation [21]. To test this, we next checked the expression pattern in coronal sections of adult rat brains. Copine 6 immunoreactivity was detected mainly in the hippocampus and dentate gyrus and at lower levels in the cortex. Copine 3, by contrast, is expressed widely throughout most of the brain (Figure 5A). Highest expression occurs in the cortex, dentate gyrus and hippocampus. Previous studies suggested that copine 6 protein is localized mostly around the cell body and in dendrites [41]. To clarify this point, the subcellular distribution of copine 6 was further examined in primary hippocampal cultures. Neurons were transfected with cytosolic GFP and stained against endogenous protein. Copine 6 (Figure 5B) localizes to somatodendritic compartments of neurons and is absent in axons. No significant localization was observed with copine 3 (data not shown).

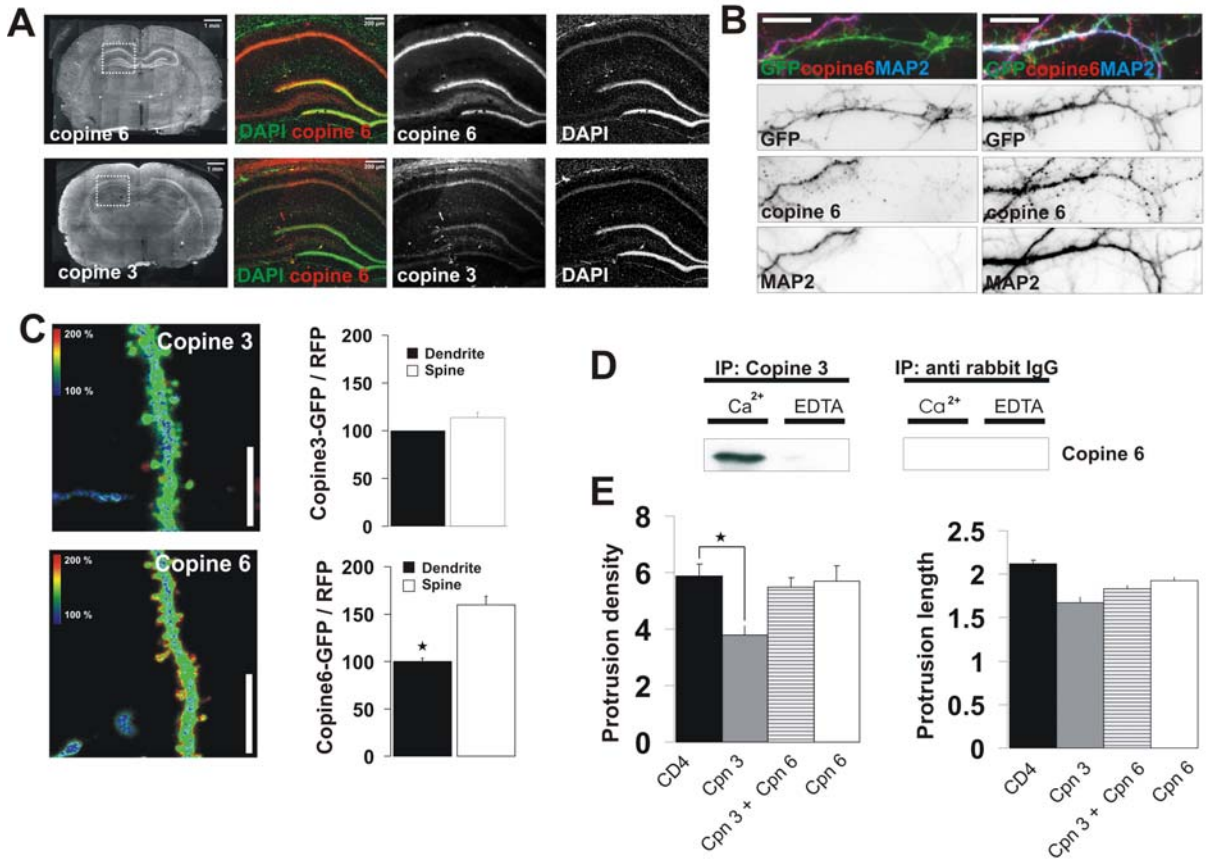


Figure 5 Localization, expression and interaction of copine 3 and copine 6

(A) Localization of endogenous copine 3 and copine 6 protein in coronal sections of adult rat brain. Copine 6 is mainly found in the hippocampus and dentate gyrus, but also enriched in cortical layers. Copine 3 is enriched in the same regions but appears to be present at lower levels. Scale bar left picture = 1 mm, right picture = 200µm. (B) Localization of copine 6 protein in pyramidal neurons at DIV18. Copine 6 (red) colocalizes with MAP2 (blue) in somato-dendritic compartments of the neuron but is absent from axons (right) Scale bar = 10 µm (C) Ratio images (green/red) of CA1 pyramidal cells expressing GFP-tagged 3 (left) and copine 6 (right), respectively, upon normalization to cytosolic RFP. Blue depicts low copine density, and red depicts high density (2-fold higher concentration). Quantification of ratio images shows an enrichment for GFP-tagged copine 6 in spines by 60 % and by 10 % for copine 3 when compared to dendrites., n = 40 spines, five cells; Scale bar, 10 µm. asterisk denotes significance values of $p < 0.01$ (D) Co-Immunoprecipitation of endogenous protein from lysates of 4 week old rat cortex. Pulldown with copine 3 as baid shows a calcium-dependent interaction of copine 3 with copine 6. No copine 6 is detected when anti rabbit IgG is used as baid. (E) Effect of double knockdowns of copine 6 and copine 3. Cells were transfected at DIV7 and analyzed at DIV13. Knockdown of copine 3 causes a reduction of protrusion length and protrusion density, whereas copine 6 has no effect on spine number compared to control cells. The effect of a knockdown of copine 3 is reverted when copine 6 is downregulated at the same time. Data represent the analysis of three independent experiments, n = 25-30 neurons per group, asterisk denotes significance values of $p < 0.01$.

We next asked whether copines are present in spines. Hence, we co-transfected organotypic rat hippocampal slice cultures with a cytosolic RFP and a copine-GFP fusion protein. The concentration of copine-GFP was measured and normalized to cytosolic RFP. High-resolution optical stack images of dendritic regions revealed that the copine 3-GFP and copine 6-GFP signal was fairly homogeneous along the dendrite and enriched in dendritic spines (Figure 5D). Copine 6 is significantly enriched in spines when normalized to dendrites (Figure 5D), whereas copine 3 is slightly but not significantly enriched, when normalized to dendrites (Figure 5C). To check these findings, we purified synaptosomal fractions from rat whole brain lysate. We find copine 3 and copine 6 localized in synaptosomes. Indeed, we find copine 3 and copine 6 present in synaptosomes (Supplementary Figure 4).

Taken together, copine 3 and copine 6 are present in the same neuron at the same time, although copine 6 expression appears more restricted than copine 3. Since there is evidence that copines might hetero multimerize [21], we next performed co-Immunoprecipitations of whole brain lysates in the presence and absence of calcium. We find that copine 6 binds in a calcium-dependent manner to copine 3. (Figure 5D). To analyze the impact of this biochemical interaction on spine formation, we performed knockdown experiments. Again, primary hippocampal cultures were transfected at DIV7 and analyzed at DIV14. We find that a double knockdown of copine 3 and copine 6 during synapse formation causes the same effect as a knockdown of copine 6 alone. (Figure 5E). These data suggest that copine 6 functions epistatically downstream of copine 3 since the apoptotic effect of copine 3 is rescued and converted to the presynaptic effect observed with copine 6 only. It is of particular interest in this context that copine 6 binds calcium-dependently to plasma membranes of synaptosomal fractions (Supplementary Figure 4). In summary, these data indicate a biochemical and a functional interaction of copines in a neuron within which copine 3 and copine 6 take in opposing roles on synapse formation.

Copines are calcium-dependent regulators of the actin cytoskeleton in spines

GTPases of the Rho family play an important role in dendritic spine morphogenesis and remodeling since they regulate the underlying actin cytoskeleton [42]. The effects of Rac 1 are in our context of particular interest, since the effects resemble the ones observed

for a knockdown of copine 3 and copine 6. The constitutively active form of Rac 1 causes a reduction in the size of the dendritic spines but increases their density, in parallel with increasing the number of synapses [43]. The overexpression of a dominant negative form of Rac 1 that blocks exchange factors, drastically decreases the number of both spines and synapses in cultured hippocampal slices and dissociated hippocampal neurons [20] [44]. There is evidence that Rac 1 regulates spine morphogenesis in a signaling module composed of GIT1/PIX/Rac/PAK [45]. To test an implementation of copines on this signalling module we first performed CO-Immunoprecipitation of total brain lysate using Pak 1, Rac 1 and rabbit total IgG as baid (Figure 6A). Western blot analysis of the pulldowns showed binding of copine 6 and copine 3 to Pak 1 in a calcium dependent manner. Calcium dependent binding to Rac 1, however, was detected for copine 3 but not for copine 6 (Figure 6A).

If binding of copines to Rac 1 really influences the functional properties, then the knockdown effect of copine 6 affecting the Rho complex should be reverted by loss of Pak 1. Rac 1 acts in a complex wherein it activates Pak 1, a serine–threonine kinase which in consequence reduce actin filament turnover and cell motility [45, 46]. Thus, we next analyzed a knockdown of copine 6 in combination with a knockdown against Pak 1. Indeed, a loss of copine 6 and Pak 1 within the same neuron leads to aspiny dendrites, suggesting that the biochemical interaction between copine 6 Rac 1 might affect the composition and/or activity of individual proteins of the multiprotein complex (Figure 6B). Interestingly, the double knockdown of copine 6 and Pak 1 did not alter ectopic outgrowth originating from the soma or PSD-95 accumulation, suggesting that Pak 1 is only partially responsible for the effects of copine 6 knockdown (Figure 6B, C).

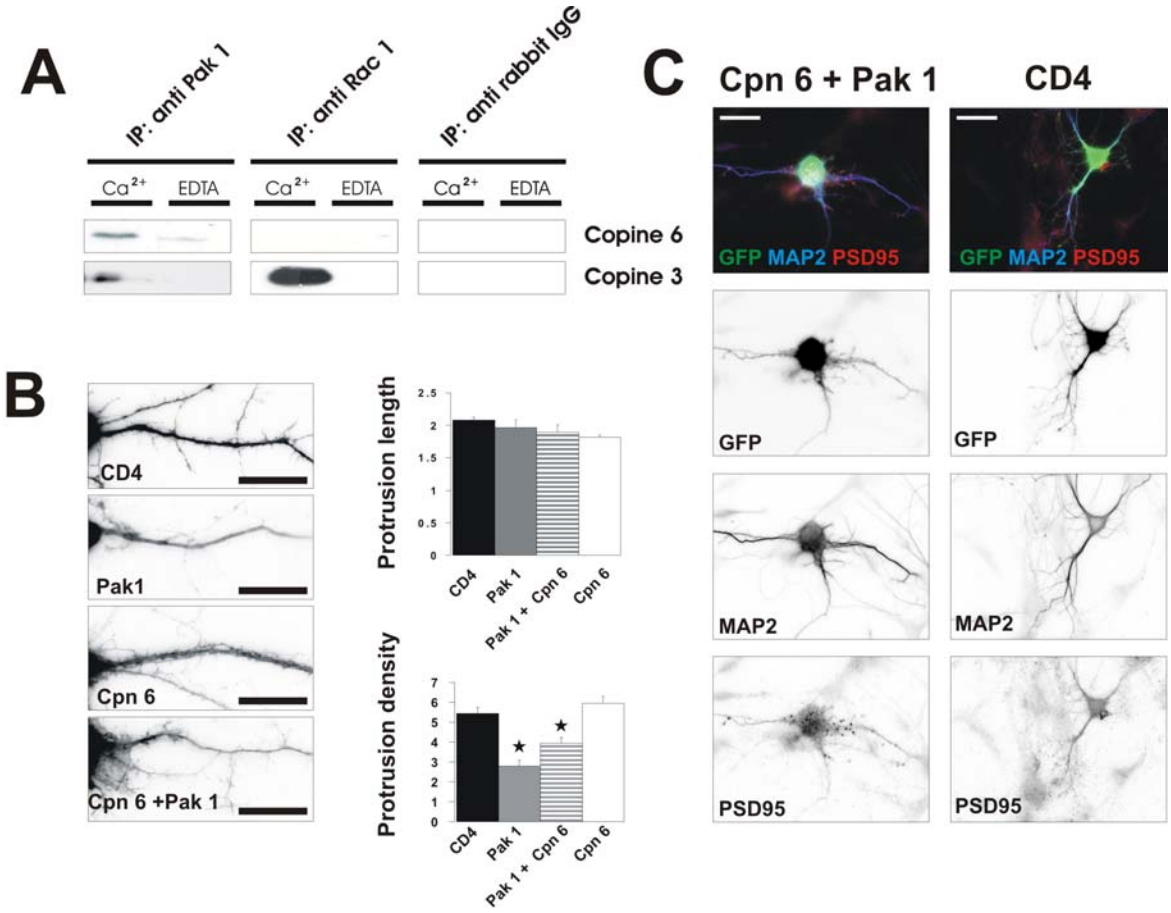


Figure 6 Interaction of copines with small GTPases

(A) Co-Immunoprecipitation of endogenous protein from lysate of 4 week old rat cortex. Pulldown with Pak 1, Rac1 and rabbit IgG as baid in the presence of calcium or EDTA, respectively. Copine 3 and copine 6 both interact with Pak 1 in a calcium dependent manner. No signal is detectable for copine 6 upon pulldown with Rac 1. (B) Representative pictures of neurons upon knockdown with CD4, Pak 1, copine 6 and double knockdowns of copine 6 and Pak 1, respectively. Cells were co-transfected with GFP and the knockdown at DIV7 and analyzed at DIV11. On the superficial level, the knockdown of Pak 1 causes complete loss of protrusions (B, left). Analysis of protrusion length and density (B, Right) shows tha knockdown of copine 6 leads to ectopic outgrowth on soma and dendrites but does not alter protrusion density and length, whereas knockdown of Pak 1 reduces protrusion density. Note that the double knockdown of Pak 1 and copine 6 reverts the spine promoting effect of the copine 6 knockdown on dendrites. Data represent the analysis of two independent experiments, n = 25-30 neurons per group, asterisk denotes significance values of $p < 0.01$. (C) Transfected neurons (green) were stained for PSD-95 (red) and MAP2 (blue) Note that the double knockdown of copine 6 and Pak 1 (left) does not impair copine 6 dependent PSD-95 accumulation. Scale bar = 20 µm.
(A)
(B)

Discussion

In the first part we analyzed the onset of synapse formation in primary hippocampal culture. Our analysis shows that synapse formation in primary hippocampal culture is achieved by a gradual accumulation of pre- and postsynaptic components during the second week *in vitro* and is accompanied by transcriptional upregulation of genes. What does this data tell us regarding the nature of these synapses? Although we can conclude that synapse formation coincides with gene expression, we can not determine whether gene transcription is influenced by synapse formation.

Gene transcription does not necessarily depend on synaptic events. There is evidence that reduction of neuronal activity in hippocampal neurons by TTX results in a substantial but reversible reduction of gene expression and delayed synapse formation [35-37]. However, it is not clear whether this gene transcription is caused by synapse dependent transcriptional activation. Immature neurons are able to undergo glutamate release and this does not necessarily occur at synaptic sites [47, 48]. In accordance, NMDA receptor-mediated transmission occurs before receptor subunits become localized in apposition to presynaptic terminals [49]. Furthermore, electrophysiological recordings have demonstrated the presence of functional glutamate receptors on neurons shortly after terminal cell division [47, 48] and a role for glutamatergic signalling in regulating development of both dendritic [50] and axonal [51] processes. And finally, even after excitatory synapses are formed and become “unsilenced” by postsynaptical incorporation of AMPA receptors [10, 52, 53] synaptic transmission can still be functionally silent in developing synapses, possibly due to a reduced flux of transmitter from immature terminals [54].

On the other side, synaptic plasticity does not necessarily have to be regulated by excitatory glutamatergic contacts. GABAergic synapses are formed before glutamatergic synapses [55] and activation of GABAergic synapses in young neurons produces depolarization instead of the characteristic hyperpolarization, because of a relatively high concentration of intracellular chloride ions. Thus, in immature neurons GABA alters the affinity of NMDA receptors for magnesium, leading to more calcium influx [56]. More important, synaptic networks of GABA generate a primitive pattern of activity, which helps to modulate neuronal growth and synapse formation [57, 58].

In summary, synapse formation and gene expression at excitatory synapse are closely synchronized processes in developing hippocampal neurons in culture [35, 37], yet these events do not necessarily have to be linked to electrical activity within the same synapse.

Nevertheless, hippocampal expression profile *in vitro* highly resembles the gene expression *in vivo* although the program of gene expression is accelerated *in vitro* as compared to the situation *in vivo* [36]. Comparison of the expression profile of synaptic markers in the developing hippocampus *in vivo* and *in vitro* has demonstrated that the programs of gene expression are highly correlated [36, 37]. Consistent with this notion, we show here that members of the copine family are upregulated at the mRNA and protein level during synapse formation in primary rat hippocampal culture and in whole brain lysate. The coincidence of transcriptional regulation and synapse formation in vitro and in vivo raises the question to which extend genes upregulated during this time are involved in synapse formation.

Using transfection of knockdown plasmids perturbing the expression of individual copines in hippocampal neurons during synapse formation we have shown that they play important roles in maintenance and reorganization of spine structures. Copine 3 appears to be implicated in the growth of spines and limiting for the arborization of the dendritic tree. Although we can conclude that the effects caused by the loss of copine 3 are not due to the apoptotic effect, we could not determine by what mechanism it is achieved. Surprisingly, a knockdown of copine 3 in glia cells causes an ectopic outgrowth of filopodia-like actin positive structures (Supplementary Figure 3). These data suggest that copine 3 plays a role in the local regulation of the actin cytoskeleton since remodeling of the actin cytoskeleton affects spine shape and number [42]. Further evidence concerning a function of copines on the actin cytoskeleton was gained from the knockdown of copine 6. Copine 6 appears to be a negative regulator of spine development, since the knockdown shows an increase in spine number and spine head diameter. This could be due to an increase in the generation of new spines or trough an increased stability of existing spine structure. In both cases spine number and size would increase.

Several lines of evidence suggest that copines act as regulators of spine formation by direct regulation of the small Rho GTPases. First, we have shown that copine 6 binds to

copine 3, Rac 1 and plasma membranes at the postsynaptic site in a calcium dependent manner. This leads us to the model, discussed in Figure 7. According to this model, activity dependent changes in postsynaptic calcium levels contribute to multimerization of copines and relocalization of copines and its binding partners to plasma membranes within spines. Thus, a temporal and spatial coordination of the activities of Rho GTPases might be achieved by interaction with copines. Function of Rac 1 in this model depends on its state of activity which in turns is regulated by the multimolecular complex. If this model is correct, the effect should be reverted by knockdown of the main downstream target of the Rac 1 signaling pathway in spine formation. Indeed, we were able to revert the increase of protrusions on dendrites caused by the knockdown of copine 6 by parallel downregulation of Pak 1, a downstream target of Rac 1. This suggests that the actin remodeling properties of copine 6, indeed, might be achieved trough the small GTPase Rac 1.

Copines might affect Rac 1 function in multiple ways. First, copines might aggregate Rac 1 with other proteins important for actin remodeling and thereby promote activity. It is worth mentioning at this place that β-actin interacts with the A domain of copine 4 and copines are hetero-multimerizing calcium dependently [21]. Second, copines can increase local protein concentrations by relocalization of the multimolecular complex to the plasma membrane. Furthermore, relocalization could bring components of a signaling pathway in immediate vicinity of structures located at the plasma membrane and thereby promote activity. Third, copines have been described to affect transcriptional activation or protein degradation [24, 59]. Thus, copines might calcium dependently alter global protein concentrations. Forth, copines can act as kinases. This opens the possibility that copines regulates directly the activity of interacting proteins by phosphorylation [22] (Supplementary Figure 3). In summary, copines bind to Rac 1 and regulate the response of this protein trough a so far not described process. Further experiments will solve the question by what mechanism this is achieved.

However, there exists an alternative explanation. Copine might regulate synaptic activity and thereby cause spine remodelling (Figure 7D). Interestingly, we observe an increased percentage of synapses with incorporated AMPA receptors in copine 6 knockdown. Measurement of miniature EPSC representing spontaneous receptor release show an increase in the release probability resulting in a higher frequency but no changes in the amplitude. These data reflects an increase in the number of functional synaptic contacts

on a single neuron without affecting the synaptic size. In addition, PSD-95 accumulation caused by knockdown of copine 6 was not affected by the double knockdown of copine 6 and Pak 1, suggesting that small Rho GTPases might represent only one target of copines during synapse formation among others.

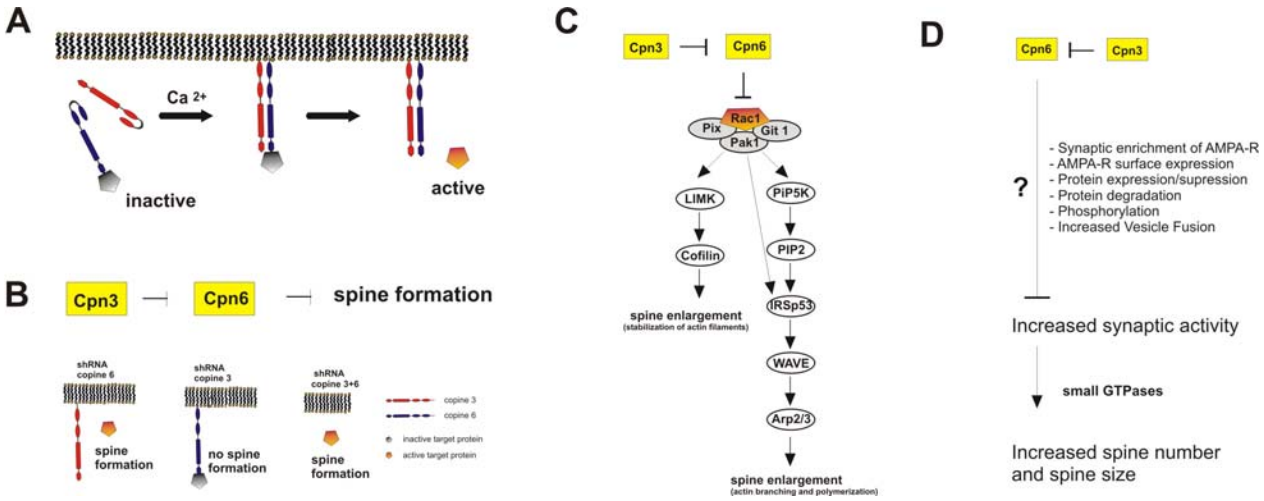


Figure 7 Model of copine function

(A) Putative model of copine-dependent protein activation. At low calcium concentrations, copines are localized as monomeres in the cytosol bound to individual target proteins. Upon increase of cytosolic calcium, copines undergo conformational change at the N teminus, as indicated by opening of the loop. In consequence, copines multimerize and relocalize to the plasma membrane. In parallel copine-interacting proteins accumulate at the plasma membrane. Accumulation or activation of these proteins, respectively, affects proteins in the vicinity, causing broader changes. (B) Epistatic model of copine 3 as negative regulator of copine 6 which blocks spine formation. As illustrated below, changes caused by knockdown of copine 3, copine 6 and copine 3 plus copine 6 support this model. Knockdown of copine 3 reduces spine number and spine size whereas knockdown of copine 6 causes the opposite effect. Reversion of the copine 3 effect by double knockdown suggests that copine 3 acts epistatically upstream of copine 6. This effects might be achieved by binding of copine 6 to a target protein. Knockdown of copine 6 would release and/or activate the target protein. (C) Rac 1/Pak1/Pix/Git 1 signalling module as putative target. Within the complex, Rac 1 activates Pak 1, a serine– threonine kinase that activates LIM kinases 1 and 2. The LIM kinases, which are also serine– threonine kinases, in turn phosphorylate and inhibit the actin depolymerizing proteins ADF and cofilin to reduce actin filament turnover and cell motility. Another pathway by which the signalling module likely promotes actin nucleation and branching in the dendritic spine head is by Rac 1 dependent Arp2/3. Activated Arp2/3 complex nucleates actin polymerization and branching, which may be the mechanism leading to spine head enlargement. Rac 1 can also promote actin polymerization by binding to the adaptor insulin receptor substrate p53 (IRSp53), which is localized in spines and known to regulate the actin cytoskeleton in nonneuronal cells. (D) Alternative model, where copines affect synaptic activity. As consequence of the increased synaptic activity, small GTPases modulate spine number and shape.

There are various possibilities how copines might cause synaptic accumulation of AMPA receptors. Recent evidence describe copines to enhance exocytosis [60]. Thus, copines might promote the fusion of AMPA containing vesicles with the plasma membrane. Alternatively, copines might also be involved in receptor trafficking or stabilization at synaptic sites. In accordance with this idea, copine was shown in *C elegans* to play a relatively specific role in targeting or stabilizing the levamisole receptor at the plasma membrane [61]. Unfortunately, it is not possible to conclude whether loss of copine 6 first promotes incorporation of AMPA receptors into synapses before increasing spine size or vice versa. It will be interesting to determine whether copine 6 directly regulates the incorporation of AMPA receptors at the postsynaptic density or whether it acts on the structure of the dendritic spine itself.

Copines might also be involved in the elaboration of dendritic trees. We show here that knockdown of copine 3 as well as of copine 4 in primary hippocampal culture causes a collapse of the dendritic tree. Interestingly there is evidence that copine 4 binds to the Cdc42 binding protein MRCKβ and to β-actin [21]. Cdc42 is thought to be involved in dendrite initiation since a dominant-negative form of Cdc42 causes significant reduction in the number of primary dendrites in cortical neurons [25, 62, 63]. Furthermore, dendrite branching is also controlled by Cdc42. The dominant-negative forms of Cdc42 reduce the dendrite branching of *Xenopus* retinal ganglion neurons [64]. Thus, copines might also play a role as a negative regulator of dendritic arborisation.

Alike changes of calcium concentration, changes of copine concentration can also influence the effect of copines. It is not clear whether individual copines are responsible for specific functions, but copines bind to individual interacting partners [21]. In consequence, changes in relative amounts of copines will cause changes in the protein composition of multimolecular complexes and alter the receptivity to diverse upstream pathways and in consequence the outputs originating from the complex. In this context changes in the stochiometry of individual copines might affect synapse activity. Supporting this assumption, copine 6 expression is upregulated in adult rats upon increased synaptic activity triggered by kainate injection and LTP [26]. Since copine 6 levels negatively regulate synapse number and maturation, this might represent an

autoregulation loop of the spine to regulate synaptic transmission. The fact that copines are expressed in adults and also affect mature spine morphology (Supplementary Figure 6) implies that copine protein in neurons is also responsible for modulations of synapse structure at later stages.

As a consequence of these findings, misregulation of copines would affect spine number and transmission. Dendritic spines are irregularly shaped and have abnormal densities in a number of neurodevelopmental disorders, such as Down's syndrome, Fragile X syndrome, William's syndrome, Rett syndrome and autism [65-68]. It would be interesting to dissect whether changes in copine expression can contribute or are involved in neurodevelopmental disorders.

Experimental procedures

DNA constructs and antibodies

shRNAs were designed according to Elbashir et al. [69, 70] and cloned under a U6 promotor into a SK(-)-vector. All siRNAs target the open reading frame. Sequences for the sense strand of the central 21-nt double-stranded region are listed in Supplementary Figure 1. Overexpression constructs were cloned into pEGFP(N3), pEGFP(C1), pIRES2-GFP (BD Bioscience, Clontech), pcDNA3-1(+) and pcDNA3.1(-) (Invitrogene), pMH4 (gift fromThomas Oertner, FMI). Following antibodies were used. PSD-95 (ABR, MA1-045), SynGAP (ABR, PA1-046), copine 6 (BD Bioscience, CG8695), Tubulin (BD Bioscience, 556321), MAP2 (Chemicon, AB5622), GFP (Chemicon, AB16901), c-fos (Calbiochem, PC05), Bassoon (Stressgen, VAM-PS003), copine 3 (raised in rabbit), GluR2 (BD Bioscience, 556341), Rac 1 (Santa Cruz, sc-217), Pak 1 (Cell Signaling, 2602), active caspase-3 (Chemicon, AB3623).

Hippocampal cultures

For colocalization studies and expression profile experiments low density cultures (~150 cells mm^{-2}) were used. Primary astrocyte feeder layer were obtained from newborn P1-wistar rats. Cortical hemispheres were treated with 0.25% trypsin in Hank's solution for 10 min at 37°C. A single-cell solution was prepared by dissociation with a narrow polished Pasteur pipette and plated in a density of 5.0×10^4 cells/cm^2 in 12-well-plates in HC-MEM (1xMEM with Glutamax, 0.3% glucose (w/v), 10% horse serum and 1% P/S.) On the day of preparing neuronal cultures the medium was aspirated and replaced by B27-MEM (1xMEM with Glutamax, 0.3% glucose (w/v), 1% B27 Formulation and 1% P/S). Hippocampal primary low density cultures were established from 18-day-old fetal Wistar rat hippocampi. Tissue was trypsinized as described above and cells were seeded on coverslips coated with 1 mg/ml poly-L-lysine hydrobromide (Sigma) in HC-MEM containing petridishes at a density of 5.0×10^3 cells/cm^2. After 4 hr, the coverslips were put on top of the Astrocyte feeder cells, separated by 1-2 mm high paraffin dots. After 2 days in culture 5µM AraC was added to the cells to prevent further growth of glia cells.

For spine morphology studies, hippocampal primary neuronal cultures prepared from embryonic day (E) 18–19 rat embryos were plated at high density (~750 cells mm^{-2}) and directly plated into B27-MEM.

Quantitative real-time PCR

Total mRNA was transcribed by using Superscript II (Life Technologies) enzyme, following the manufacturer's instructions using oligo dT. The real-time PCRs were carried out on a ABI 7700 and 7700 Sequence Detection system (Applied Biosystems). Primer sequences were designed using Primer Express software (PE; Applied Biosystems). We selected primers close to the 3′ end of the target genes with primers localized on different exons. Amplicons were 150 bp (+/- 10%) in size. The reactions were performed using the SYBR Green PCR Core Reagents (Applied Biosystems). To verify that the SYBR Green dye detected only one PCR product, all the reactions were subjected to gel electrophoresis or to the heat-dissociation protocol following the final cycle of PCR. All the samples were normalized against glyceraldheyde 3-phosphate dehydrogenase (GAPDH), and phosphoglycerolkinase (PGK-1) -reference genes previously described not to be differentially regulated during synapse formation. Each RT-PCR quantitation experiment was performed in triplicates for 2-3 independently generated cDNA templates. The calculations were performed with Microsoft Excel.

Copine 3 AB

We generated polyclonal antibodies by constructing a peptide of the 16 C-terminal amino acids of Copine 3 and KLH and raised them in rabbits. These antibodies recognized a single band from rat whole brain lysate corresponding to the appropriate size of copine 3 (Supplementary Figure 3).

Western blot analysis.

Hippocampal cultures were harvested in sample buffer. Equal amounts of protein were separated by 10% PAGE, transferred to nitrocellulose membrane, and immunostained using antibodies previously described.

Transfection of hippocampal culture

In 24 well plates we used per well 1 µl Lipofectamine2000 in 50 µl OptiMEM and incubated for 5 min at room RT. This was mixed with 1 µg total DNA in 50 µl OptiMEM and incubated for 20-30 min at RT. The mix was then added on the cultures, and allowed to sit for 4–6 h until medium was changed. This resulted in a transfection efficiency of 1-2%.

Immunocytochemistry

Neuronal cells were fixed with 4% paraformaldehyde in PBS w 120 mM sucrose for 20 min at RT and washed 3 times in PBS. Cultures were then permeabilized with 0.25% Triton X-100 in PBS for 10 min at RT, washed 3 times with PBS. To block unspecific binding we washed with 10 % BSA in PBS for 1h at 37°C and, subsequently, we incubated with primary antibody overnight at 4°C in PBS w 3% BSA. Finally, cells were treated with appropriate secondary antibody for 1 hr at RT and the immunolabeled cells were mounted with Cervol.

Imaging and analysis

Pictures were made on Leica DM5000 and analysed using the “analySIS” software.

Co-IP assay

COS7 cells were transfected with copine 3 and copine 6. Cells were harvested 48 h after transfection and lysed in NP-40 Lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris, PMSF, pH7.4) containing 10 µM Ca2+ or 1mM EDTA. Insoluble materials were removed by centrifugation at 10,000g for 10 min at 4°C. Anti-copine 3 antibody (10 µg) or Preimmunserum was then applied to the supernatant and rocked for 2 h at 4°C. Then, Protein A-sepharose was added to the lysate for another 2 h. Bound materials were washed four times with the same buffer, followed by centrifugation at 10,000g for 15 sek at 4°C. Western blotting analysis using an anti-copine 6 antibody was performed using

the final pellet vs. the supernatant. Brain lysates were prepared from adult rat brain cortex in a similar manner. Material was then incubated with anti-copine 3 antibody or preimmunserum coupled to protein G-sepharose for 2 h as described above.

Preparation of membrane fraction

All procedures were done with pre-cooled reagents at 4°C. Brain regions of interest were dissected into ice-cold homogenization buffer (0.32 M sucrose, 10 mM HEPES pH 7.4, 2 mM EDTA, protease inhibitors, phosphatase inhibitors) and homogenized using 10-15 strokes of a motor-driven glass-teflon homogenizer. Nuclear fraction was removed with centrifugation at 1000g for 15 min (P1). The supernatant (S1) was centrifuged at ~200,000g to yield crude cytosol (S2) and crude membrane pellet (P2). Upon resuspension of the pellet in HEPES-Lysis buffer (50 mM HEPES pH 7.4, 2 mM EDTA, protease/phosphatase inhibitors). protein concentration was measured by BCA or Coomassie.

Synaptosome preparation

According to Cohen *et al.* [71] we used the P2 fraction described previously and put it onto 4 ml of 1.2 M sucrose. Upon centrifugation at 230,000g for 15 we collected gradient interphase and dilute to ~7-8 ml with ice-cold HEPES-buffered sucrose (0.32 M sucrose, 4 mM HEPES pH 7.4). The resuspension we layer onto 4 ml of 0.8 M sucrose and centrifuged at 230,000g for 15 min. The pellet contains pure synaptosomes.

Organotypic slice cultures

In all experiments, organotypic slice cultures were obtained from wistar rat at postnatal day 5. Slices were transfected between day *in vitro* 5-7 using a biolistics gene gun. After transfections, the cultures appeared healthy, and the expression of copine-EGFP and RFP was analyzed under epifluorescence illumination between DIV21-28.

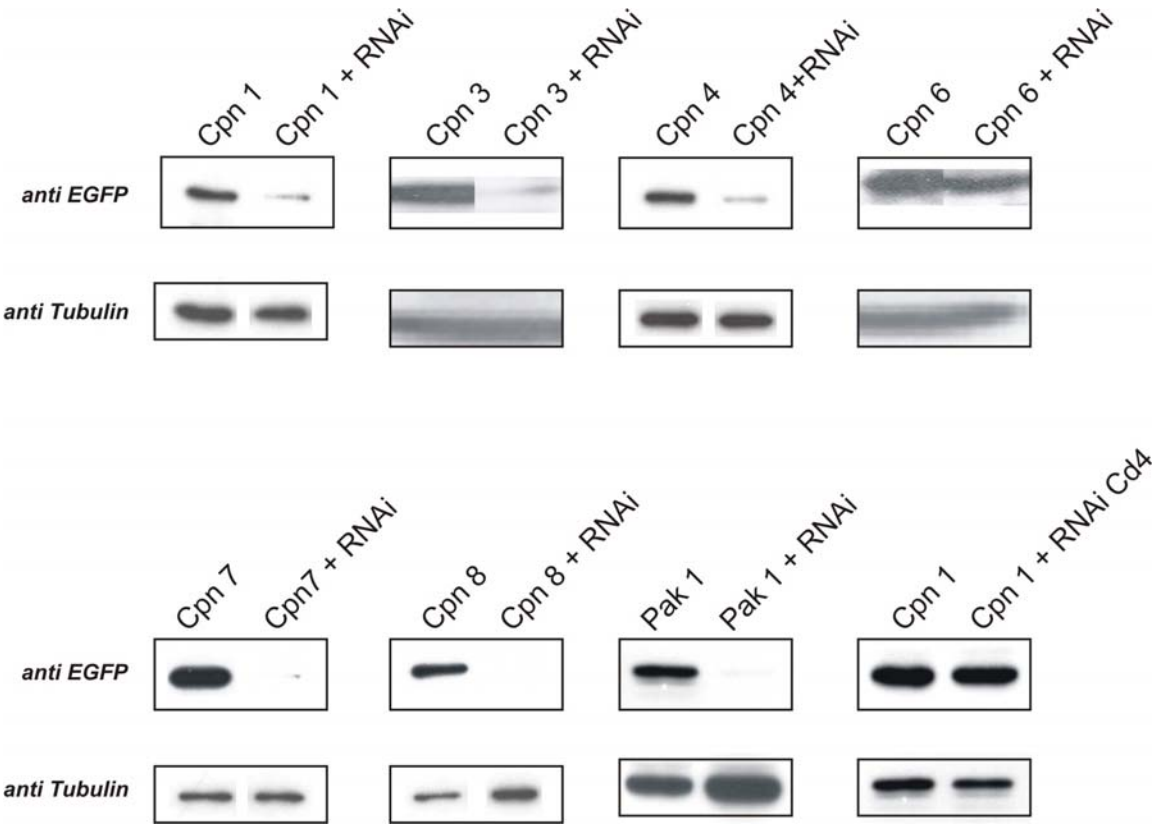
Two-photon laser imaging

We used a custom-built 2-photon laser scanning microscope (2PLSM) with an Olympus objective (60×, 0.9 NA), Zeiss scan lens, and a Ti:sapphire laser tuned to λ = 910 nm for excitation. Fluorescence was detected using photomultiplier tubes. Image acquisition was controlled by custom software (MatLab7).

Electrophysiology

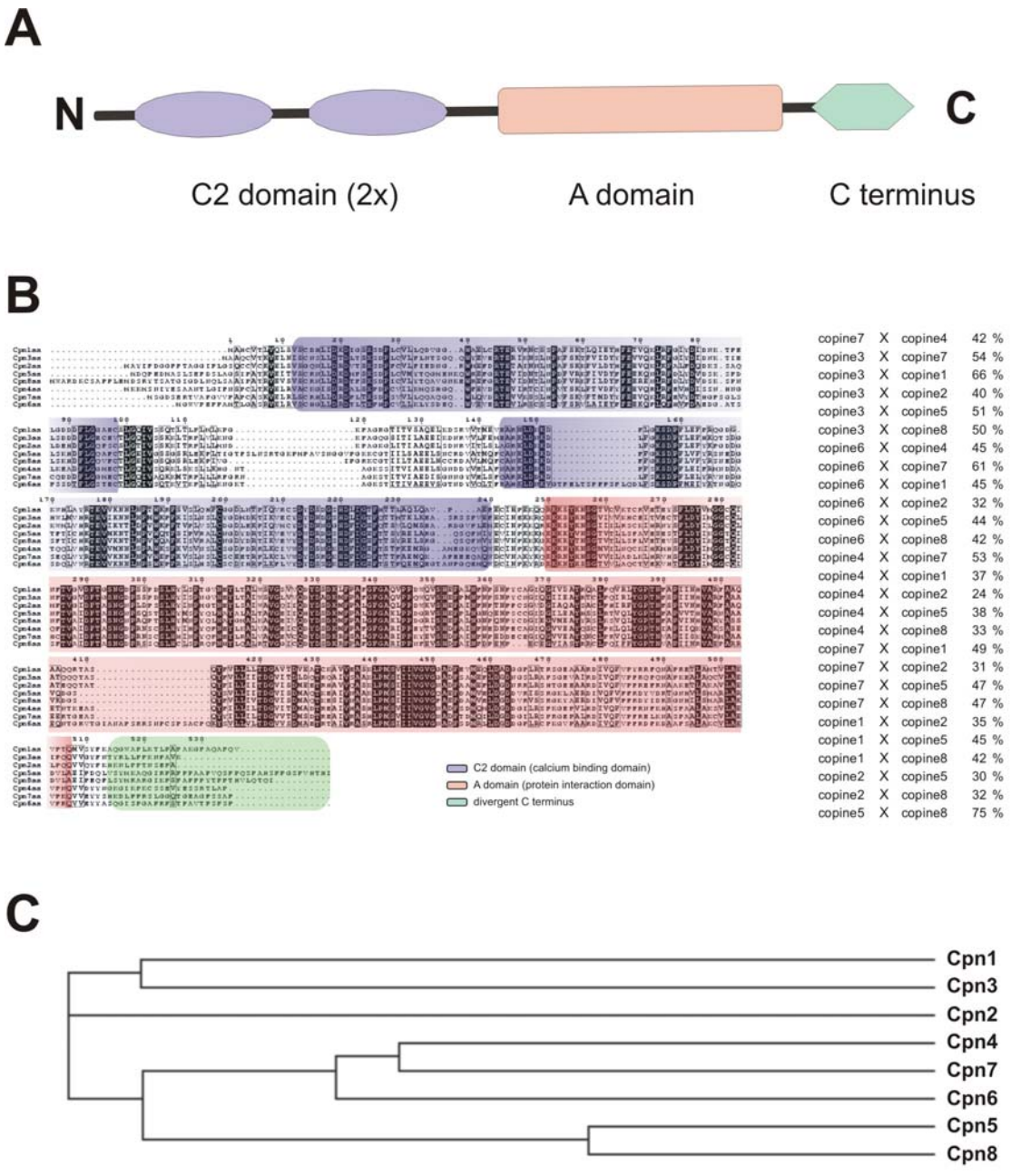
High density cultures (DIV12) were perfused with an ACSF solution containing (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgCl_2$, 2.5 $CaCl_2$, 1 NaH_2PO4, 26.2 $NaHCO_3$, 11 glucose, equilibrated with 95%O2/5%CO_2 at room temperature (25°C) and delivered at 1.5ml.min^{-1}. Whole-cell patch-clamp recordings were performed from the somata of visually identified neurons. The recording electrode (3-5MΩ) was filled with a solution containing (in mM): 135 $CsMeSO_4$, 8 NaCl, 10 HEPES, 0.5 EGTA, 5 QX-314, 4 Mg-ATP, 0.3 Na-GTP (pH 7.25, 285 mOsm). Miniature EPSC (mEPSC) were recorded at -70mV in the presence of 0.5μM TTX (Latoxan, Valence, France) and 100□M picrotoxine (Fluka/Sigma, Buchs, Switzerland). Detection and analysis of mEPSC were done using the MiniAnalysis software (Synaptosoft, Decatur, GA, USA). 600 consecutive events from each cell were used for the cumulative histograms/Kolmogorov-Smirnov tests. Data were obtained with an Axopatch 200B (Axon Instruments, Union City, CA, USA), filtered at 2kHz and digitized at 10kHz, acquired and analyzed with pClamp9 (Axon Instruments, Union City, CA, USA). Values are expressed as mean±sem.

Supplementary material



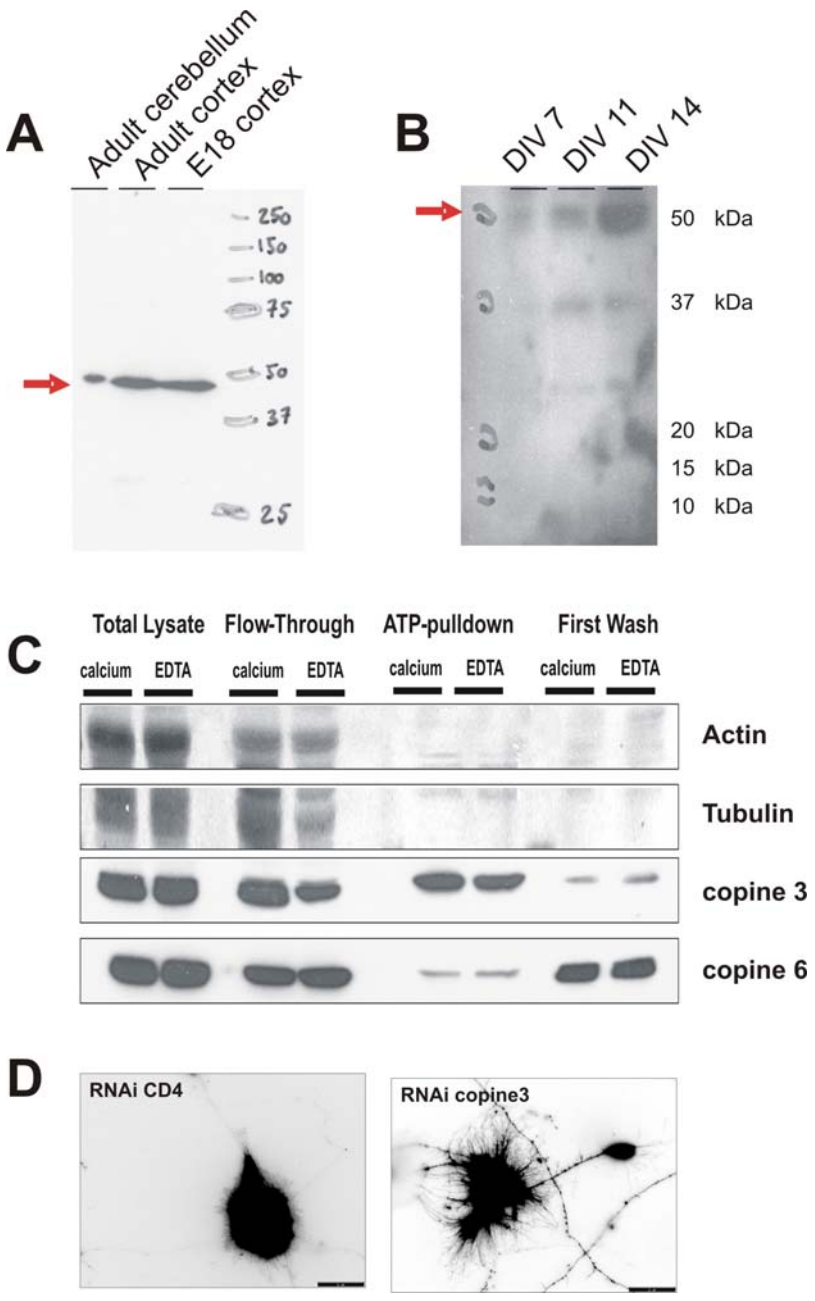
Supplementary 1 Knockdown of copines in COS cells

(A) COS cells grown in a 35 mm dish were co-transfected with a copine-GFP overexpression construct plus a shRNA against copine 1, 3, 4, 6, 7, 8 and Pak 1 in a ratio of 1:3. 48 hours following transfection, the cells were harvested and analyzed by western blot against GFP. Tubulin serves as loading control. In all cases, knockdown of the particular copines and Pak 1 resulted in a significant reduction of the GFP fusion protein. No reduction was observed upon knockdown of copine 1 with CD4. Sequences for the sense strand of the central 21-nt double-stranded region are listed in the Supplementary 7.



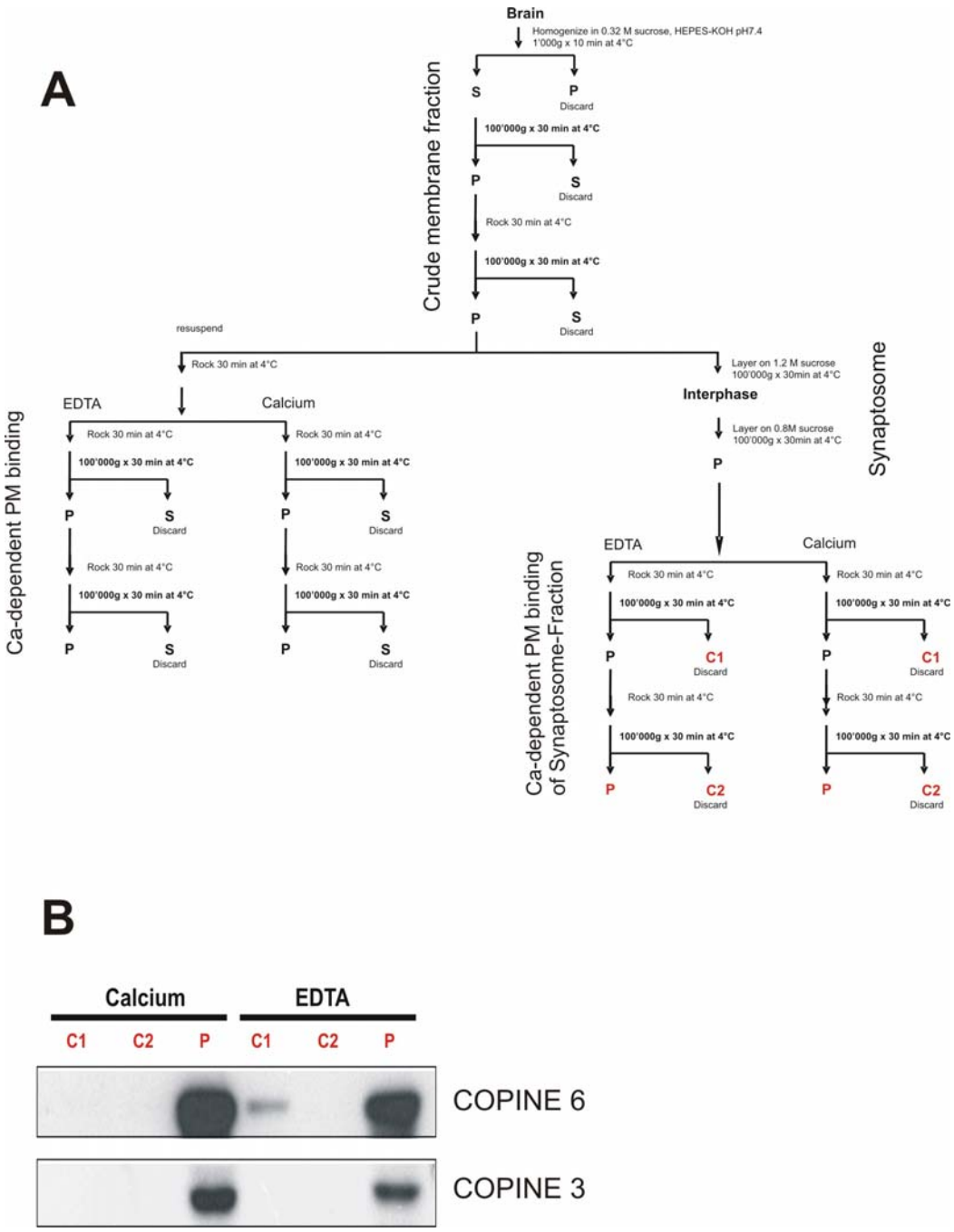
Supplementary 2 The copine family

(A) Structure of copines. Copines are cytosolic proteins of 50-60 kDa size that share at the N-terminus two C2-binding domains (purple). On the C-terminus, copines share an A-domain (red), responsible for protein-protein interaction followed by a highly divergent terminus (green). (B) Alignment of amino acid sequences of the individual family members. Domains are highlighted in the colours as described above. Sequences were aligned using ClustalW from the EMBL-EBI homepage. The sequences are retrieved from the NCBI homepage and doublechecked with nucleotide blast on the rat genome published on the UCSC Genome Bioinformatics Site. Copine 9 is missing due to the lack of a clear prediction. Note that the highest divergence between the various members of the copine family can be found at the C-terminus, responsible for copine-specific protein-protein interaction. At the right the over all amino acid homologies are listed. (C) Dendrogram of the rat copine family. Note that brain specific copines (cpn 4, 6 and 7) represent an independent branch within the family.



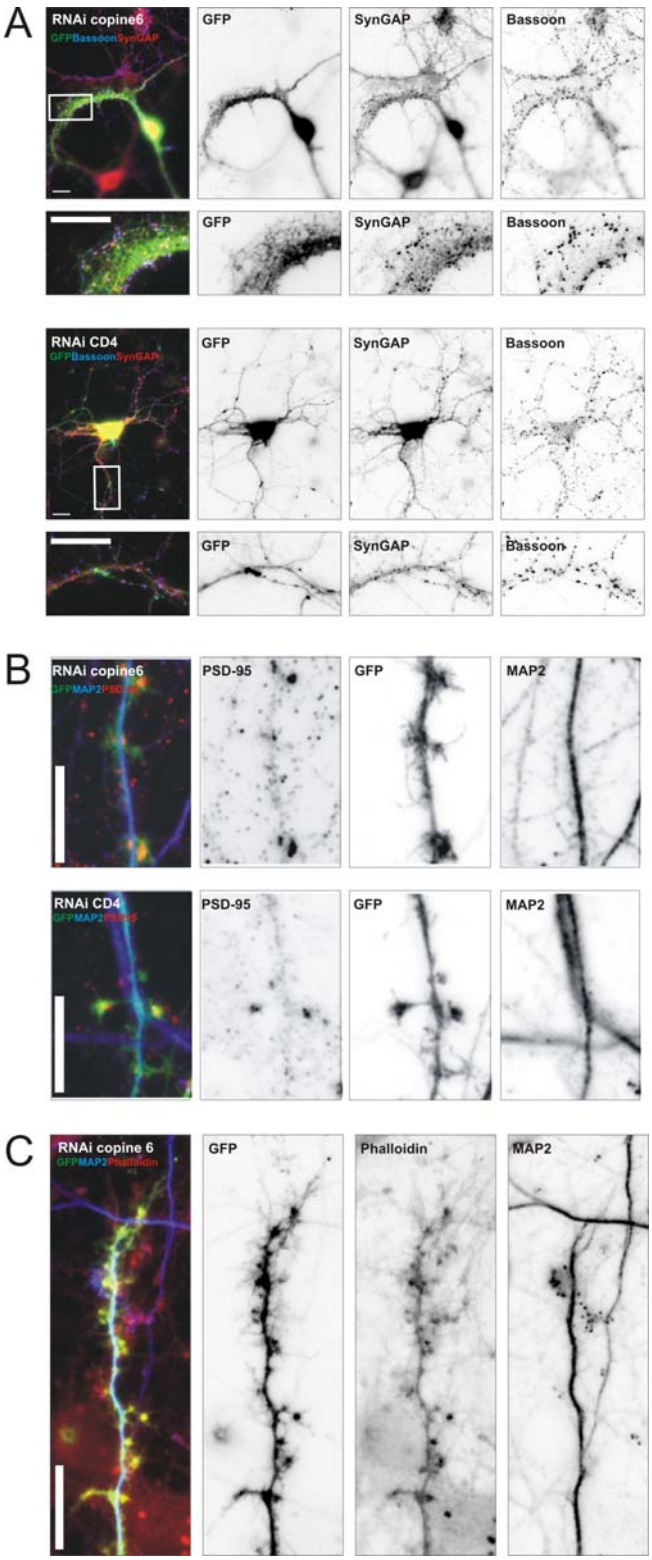
Supplementary 3 Copine 3

(A, B) We generated polyclonal antibodies by constructing a peptide of the 16 C-terminal amino acids of Copine 3 and KLH and raised them in rabbits. These antibodies recognized a single band (indicated by the red arrow) corresponding to the appropriate size of copine 3 in lysates of various brain regions (left) and in lysates derived from primary hippocampal culture at day in vitro 7, 11 and 14. (C) ATP pulldown using lysate from rat adult cortex. From left to right (two lanes each) total lysate, flow trough, ATP pulldown and the first wash of the pulldown was loaded. The pulldown was performed in the presence (left lanes) and absence (right lanes) of calcium. Actin and Tubulin are present in total lysate and in the flow trough but absent in the ATP pulldown and in the fist wash. Copine 3 binds calcium-independently to ATP. Interestingly, copine 6 is enriched in the first wash, suggesting a co-immunoprecipitation with copine 3 or ATP followed by a loss of contact during the subsequent washing steps. (D) Glia-effect of copine 3 knockdown. Cells were transfected with GFP and RNAi against CD4 (left) or copine 3 (right) at DIV 7 and analyzed at DIV14. Pictures show the inversion of the GFP signal. Knockdown of copine 3 in rat primary glial cells causes an ectopic outgrowth of long filopodia structures and an increase of average glia size. Scale bar = 20 µm.



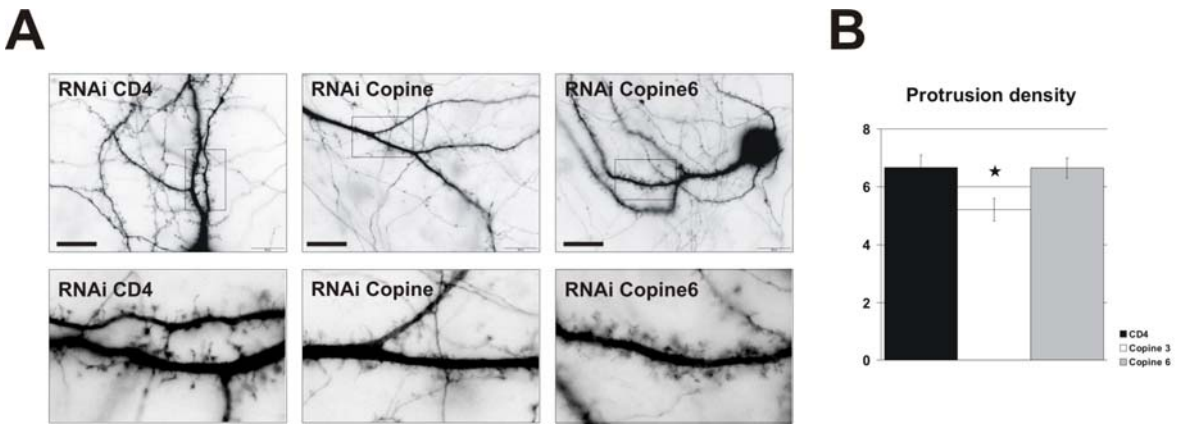
Supplementary 4 Synaptosomal fractions

(A) Preparation of synaptosomal fractions. Adult rat cortex was homogenized using a bead beater and crude membrane fractions were generated according to the flowchart. As indicated, synaptosomes were enriched with sequential centrifugation using 1.2 and 0.8 M sucrose, respectively. The so enriched synaptosomal fraction was then washed in the presence of EDTA or calcium. The samples loaded in (B) are highlighted in red. P = synaptosomal pellet, C1 = first wash, C2 = second wash. (B) Copine 3 and Copine 6 localized in synaptosomes. Note the light band of Copine 6 protein upon first washing with EDTA. This suggests that Copine 6 is at least partially attached transiently to plasma membranes in a calcium dependent manner. No signal was detected for Copine 3 protein



Supplementary 5 High magnification of copine 6 knockdown

(A-C) Knockdown against copine 6 does not affect synapse formation but alters actin cytoskeleton. Cells were transfected at DIV 7 with GFP and RNAi against copine 6 and CD4, respectively. (A) Staining for synaptic contacts as indicated by colocalization of the presynaptic marker Bassoon (blue) and the postsynaptic protein SynGAP (red). As described above, the knockdown of copine 6 cause an increase in the number of Bassoon/SynGAP colocalizations. High magnification pictures (white boxes) show Bassoon/SynGAP costaining at the tips of the ectopic structures growing from the dendrite, suggesting no impairment in synapse formation. (B) High magnification images of spines stained for PSD-95 (red) and MAP2 (blue) indicate that knockdown of copine 6 increases PSD-95 accumulation as mentioned above but does not alter spine structure. Note that ectopic outgrowth orginates directly at the dendrite. No obvious changes can be observed at the spine neck and of the spine head as visualized in the GFP channel. (C) Staining for MAP2 (blue) and Phalloidin (red) indicate that the ectopic outgrowth is due to alteration of the underlying actin cytoskeleton. Scale bar = 10 µm in all pictures.



Supplementary 6 Knockdown of copines after synapse formation

(A) Knockdown of copine 3, copine 6 and CD4 at DIV14 and analysed at DIV18. Note the reduction of copine 3 expression results in a significant loss of GFP positive mushroom-shaped structures, indicating a loss of synapses. In parallel, the number of protrusions becomes reduced. Loss of copine 6 results in ectopic outgrowth along the dendrite. Scale bar = 20 µm (B) Analysis of protrusion density at DIV 18. Knockdown of copine 3 results in a significant reduction whereas knockdown of copine 6 does not alter protrusion density. Spine diameter was not analyzed due to high variations in the level of maturation of the individual neurons. Data represent the analysis of neurons from at least two experiments, n = 15–20 neurons per group, ≥500 clusters per group. Error bars represent mean ± SEM. The asterisk denotes significance values of $p < 0.01$ of copine 3 compared to CD4 and copine 6, respectively.

Supplementary 7 Table of shRNA sequences

Construct	Sequence
shRNAi-cpn 1-1	GGACTGAACGTGTTCGCAACT
shRNAi-cpn 1-2	GGAAGCTAGAAACCTAGATAA
shRNAi-cpn 3-1	GGTTCACCGAACAGAGGTTAT
shRNAi-cpn 3-2	GGAGCTCACCTGTTGAATTTG
shRNAi-cpn 4-1	GCATTCAATGCACGGAAATTG
shRNAi-cpn 4-2	GGGAAAGGGATTAAACCAAA
shRNAi-cpn 5-1	GCACCGAGGTCATTGACAACA
shRNAi-cpn 5-2	GCAGGATGGTTCCCAGTATTC
shRNAi-cpn 6-1	GGAGATCTATAAGACCAATGG
shRNAi-cpn 6-2	GCTTGTCCTCAGAAGTATTCG
shRNAi-cpn 8-1	GCAACCCTCAGAATCCTTACT
shRNAi-cpn 8-2	GGACGGCGTCATCTCAGATAT
shRNAi-Pak 1	GGTTCTATCGATCCATCTTAG
shRNAi-Pak 2	GCATTCAAACCAAGTCATTCA

Two knockdown constructs were designed for every copine family member and tested in primary hippocampal culture. No difference could be observed, suggesting a specific knockdown. In consequence all subsequent experiments were performed with the first RNAi construct.

CHAPTER 3

The role of copine family members in synapse formation

Galic M, Kriz A and Ruegg MA [2006]

In preparation

Abstract

Copines are a scarcely described family of cytosolic proteins that are thought to be involved in a variety of calcium-dependent structural and functional changes in organisms reaching from Paramecium to human. Recent studies suggest a function for copines as a calcium sensor involved in the formation and rearrangement of synapses. In this review, we discuss the role of copines as general sensors for calcium in several phenomena and in controlling structural and functional plasticity of synapses. Moreover, we will also highlight how copines can serve as upstream regulators of the actin cytoskeleton to alter synapse structure. Thus, copines might represent a bridge between activity and structural changes in synaptic plasticity.

Introduction

The protein family of copines was first described as a novel class of cytosolic proteins that bind to plasma membranes in a calcium-dependent manner [143]. It is this biochemical property that gave the family its name. Copine, the French word for “friend”, was selected because of the observation that the protein associates with lipid membranes “like a companion” [143]. Furthermore, copines are capable of interacting with a wide variety of "target" proteins [152]. Copines appear to be absent from the *Sacchromyces cerevisae* genome, while the genomes of *Paramecium*, *Arabidopsis*, *C. elegans*, and human encode two, three, five or nine copine genes, respectively [220, 221]. The biochemical properties of the copines and the fact that copines are expressed in plants, animals, and protozoa suggests that copines participate in conserved pathways important for calcium signalling. In agreement, copines were described to be involved in a wide range of biological activities including growth control, exocytosis, mitosis, apoptosis, gene transcription, and cytoskeletal organization [146, 164].

While our understanding of the function of copines in various aspects of cell function has grown in the past eight years, a great deal of mystery still surrounds the function of copines in synapse formation. In this review, we will discuss recent studies that provide evidence that copines actively participate in synapse formation in the central nervous system by regulating synapse number, function and stability. These new studies provide

strong evidence that copine function in neurons exceeds simple protein shuffling but that copines are regulators of synaptic plasticity. We will begin by reviewing the biological properties of the copine family providing evidence that copines contain all necessary features to transduce increased synaptic calcium concentrations due to receptor activity to structural changes of the synapse, and then summarize and discuss recent studies that provide support for the idea that copines indeed regulate synapse formation.

The biochemical properties of copines

So far 9 copine members were described in rat. Figure 1 shows the dendogram, alignment and sequence homologies between the various members (Figure 1). Recent studies have suggested a possible function for copines as calcium sensors. Copines are cytosolic proteins characterized by two C2 domains at the amino-terminus and an A domain at the C-terminus. C2 domains are calcium-dependent, phospholipid-binding domains that regulate calcium or lipid binding properties on the proteins in which they reside and via the A domain copines are capable of interacting with a wide variety of "target" proteins, that are themselves components of intracellular signalling pathways [152]. It is assumed that copines bring calcium dependently their interacting proteins in the immediate vicinity of the membranes. Thus, proteins that were spatially separated accumulate due to multimerization of copines inter se. Evidence for this assumptions arise form studies showing copine 1 as a monomer with a blocked N terminus at low calcium concentrations. Upon calcium binding copine 1 undergoes conformational changes which then lead to exposure of hydrophobic patches [222]. In consequence, copine 1 forms higher-order multimers and binds phospholipids with preference for negatively charged phospholipids over neutral phospholipids [153]. The C-terminal portion of copine has a distant similarity to the protein-binding domain of certain integrins, named the A domain [143, 220]. Yeast two-hybrid screening and pull-down experiments using the immobilized copine led to the discovery of a variety of interacting proteins [152]. Examination of the sequences and inferred structural features of the target domains revealed that a majority of this proteins included sequences predicted to form α-helical coiled-coils [152].

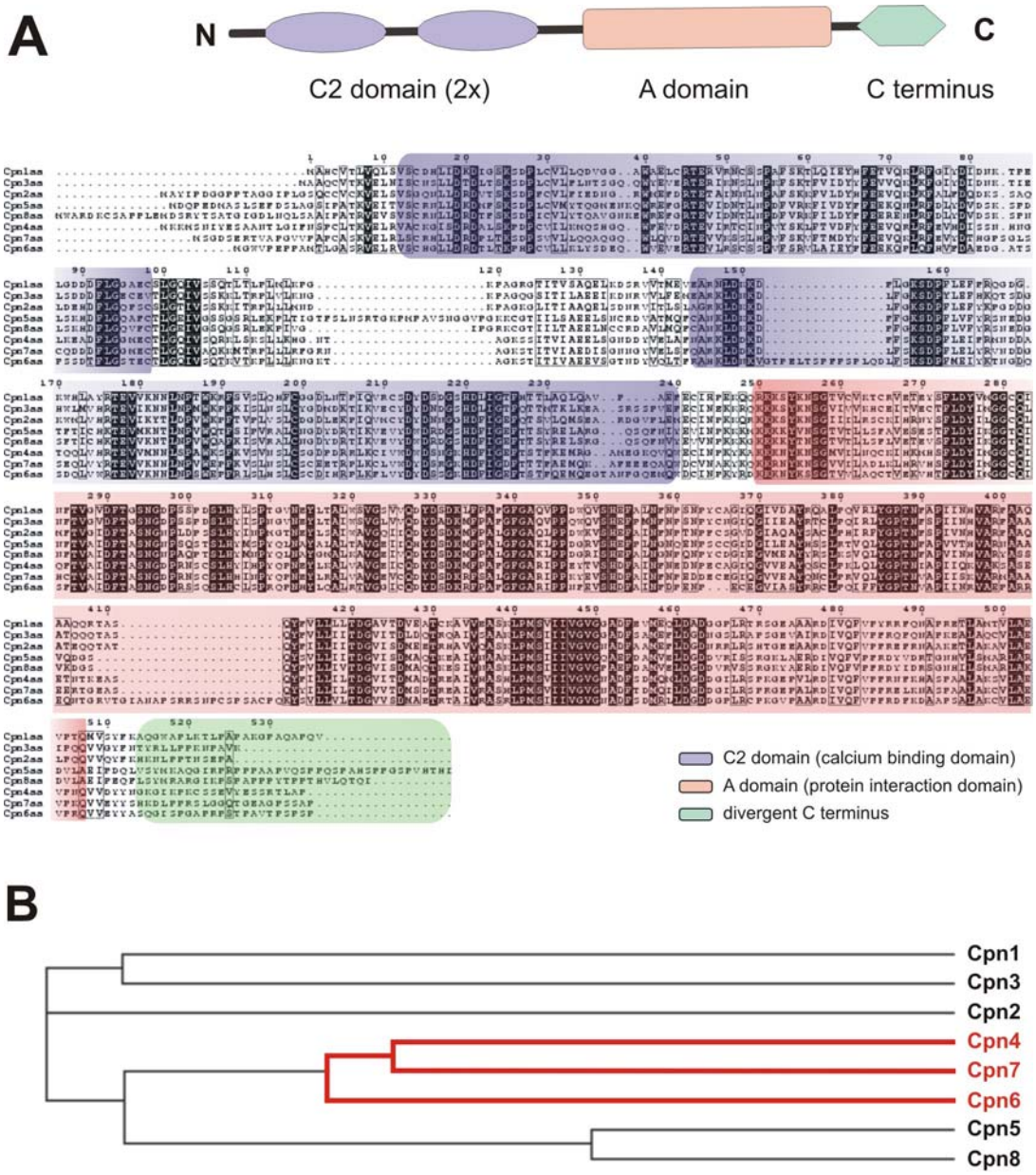


Figure 1 Sequence alignment of the rat copines.

(A) Structure of copine proteins and alignment of the predicted amino acid sequences. Sequences were aligned using ClustalW from the EMBL-EBI homepage. Asterisk mark the consensus homologous in all nine family members, the C2 and A domain are highlighted in purple and red. The sequences are retrieved from the NCBI homepage and double checked with nucleotide blast on the rat genome published on the UCSC Genome Bioinformatics Site. (B) Dendrogram showing the relative homologies between the nine copine family members was made using the ClustalW from the EMBL-EBI homepage. Brainspecific proteins are indicated in red.

The full-length copine 1 was found to recruit these proteins to immobilized phosphatidylserine in a calcium-dependent manner, suggesting that copines may indeed be able to localize their targets to membrane surfaces in the cell in response to calcium fluxes. The recruitment of collagen to the lipid substrate required the presence of magnesium suggesting the requirement of magnesium for proper biochemical function. The copines thus contain all properties for calcium signalling to proteins involved in a wide range of biological activities, and many mechanisms attributed to synaptic plasticity are accompanied or induced by alterations in calcium concentrations.

Copine expression in the brain

Three out of the nine copines are specifically expressed in the brain. These are copine 4, 6 and 7. The remaining copine family members show a more general expression pattern, being present in the brain but also in many other organs at different levels or are not characterized in the case of copine 9.

Copine 4 expression shows highest levels in the olfactory bulb. Lower levels are detected in the amygdala, hippocampus, frontal cortex, cerebral cortex and in dorsal root ganglia (DRG). In primary hippocampal culture, suppression of endogenous copine 4 expression by RNA interference significantly inhibited dendritic development and this inhibitory effect was accompanied by alterations in actin cytoskeleton within the dendrites. Other reports show evidence for an interaction of Cdc42 binding protein MRCKβ and β-actin with the A domain of copine 4 [152]. Interestingly, dominant-negative forms of Cdc42 cause significant reduction in the number of primary dendrites in cortical neurons [211]. Thus, copine 4 might be involved in dendrite initiation and branching thought interaction with the small GTPase Cdc42.

Copine 6 shows highest levels in amygdala, hippocampus, olfactory bulb and DRG. To a lesser extent, copine 6 can be detected in the frontal cortex, preoptic, cerebral cortex, hypothalamus, dorsal root ganglion, trigeminal and in the spinal cord. Within pyramidal hippocampal neurons, copine 6 is present in the somato-dendritic compartment but is absent in axons. Copine 6 is enriched in spines and binds calcium dependent to plasma membranes. Upon kainate injection and electrical stimulation evoking hippocampal CA1 long-term potentiation copine 6 expression becomes up-regulated [154, 166]. These

findings of activity-dependent gene expression were further supported in another study that showed transcriptional upregulation of copine 6 mRNA in vitro and in vivo during initial synapse formation. To test whether copine 6 is able to modify synapse formation, rat hippocampal neurons were raised in the absence of copine 6. These experiments showed that loss of copine 6 profoundly increase synapse number, size and activity in developing and mature synapses. Immunoprecipitation of brain lysate unveiled interaction of copine 6 with Rac 1. Constitutively active Rac 1 increase the number of Purkinje cell spines [129], whereas dominant-negative Rac1 causes a progressive reduction in spine number [125]. It is suggested that copine 6 is a negative regulator of spine density; presumably trough a calcium dependent regulation of the small GTPase Rac 1.

The last family member that is exclusively found in the brain is copine 7. It is most highly expressed in hippocampus, amygdala and olfactory bulb. Sequence analysis identified two alternatively spliced transcript variants that encode different isoforms. Interaction partners and physiological function are unknown [183].

Effects of copine on synaptic plasticity

The ability of single synapses to modulate their strength in an activity-dependent fashion is called plasticity [223]. Synaptic plasticity occurs at single spine level and is regulated by local protein trafficking, synthesis or degradation [117-122]. Knockdown of copine 6 has been show to increase spine size and number, indicating that regulation of copine levels might be involved in the development and plasticity of dendritic spines.

In the adult brain, increased calcium concentrations in spines can be triggered by synaptic activity and it can have opposite effects on spine morphology depending on their magnitude and duration. Moderate and transient elevation of intracellular calcium concentration induces spine elongation [116]. In contrast, large and sustained increases in calcium levels cause spine shortening and in some cases collapse [138]. Thus, cytosolic calcium levels have to be regulated tightly. Calcium concentration in the spine is controlled on one hand by the duration and amount of calcium influx and on the other hand by diffusion of calcium across the spine neck and active removal of calcium from the spine cytoplasm [139]. Generally, calcium ions that enter the cell are rapidly buffered by calcium binding proteins (CBPs) that are distributed throughout the cytoplasm. In

consequence, calcium entry into a cell creates a concentration gradient across the spine, with concentrations, as high as hundreds of μM, near the mouth of the channel [224]. If proteins are concentrated very near these receptors the probability of their activation can be orders of magnitude greater than if they are localized further away. In *Dictyostelium*, changes in calcium causes a very transient membrane localization of a GFP-copine fusion protein [150]. The transient localization of copine at plasma membranes often occurred multiple times within the same cell, suggesting that the translocation from cytosol to membranes and back to the cytosol is a response to fast intracellular calcium spikes or waves [150]. This suggests that copine rather "bind and react" to changing calcium concentrations rather than just "bind and buffer" it. Given that independent copine members bind to specific proteins [152], an increase of calcium orchestrates the relocalization of cytosolic proteins to plasma membranes. As a consequence, copine-interacting proteins accumulate calcium-dependently at plasma membranes in spines. As an extension of this idea, calcium can also cause the assembly of copine heteromers [153]. Each copine binds to independent interacting proteins and a calcium dependent accumulation might promote biochemical reactions by spatial enrichment of interacting partners (discussed below). In the following we will discuss functions attributed to copines that are calcium-dependent and might affect spine formation upon multimerization.

Protein Expression. Copines regulate protein expression. In Arabidopsis, the *copine* family regulates cell death by repressing a number of *R* genes [146]. The biochemical mechanism by which copines regulate gene expression is yet to be determined. One possibility is that copines influence protein expression trough direct interaction with nucleic acids or the proteins responsible for the transcription or translation, respectively. The other possibility is that copines bind to regulators of these events and therefore indirectly regulate protein expression.

Vesicle fusion. AMPA receptor incorporation into synapses is involved in activity-dependent, long-term changes in synaptic strength. Vesicle pools containing AMPA receptors fuse with the plasma membrane and AMPA receptors traffic laterally into the synapses. Little is known about the machinery involved in vesicle storage and fusion or

receptors incorporation into synapses. There is evidence that the incorporation is driven in a calcium dependent manner [156, 225]. In *Arabidopsis*, copine gene function is required for exocytosis [148]. This function could either be due to copines acting catalytically (increasing the fusion of vesicles with the membrane) or structurally (by associating with the plasma membrane to maintain membrane function at low temperature). Copine might be involved in vesicle exocytosis at the presynaptic side. At the time, there is no evidence for a role of copines in vesicle fusion. However, presynaptic proteins organizing the exocytosis machinery at the transmitter release site contain C2 binding domains like copines [156-163]. ,

Kinase. Recent publications suggest that copines might actively participate in the modulation/activation of effector proteins in spine formation. Copine 3 show intrinsic kinase activity [165]. In vitro kinase assays were performed with immunoprecipitated endogenous copine 3, chromatography-purified endogenous copine 3, and recombinant copine 3. The exogenous substrate myelin basic protein was phosphorylated in vitro kinase assays containing copine 3 immunoprecipitate or purified copine 3 [165]. Interestingly, there is no classical kinase catalytic domain in copine 3. Thus, it may represent the first member of a novel kinase family. Phosphorylation can act as a posttranslational modification to rapidly alter protein function, and phosphorylation-mediated activation can produce long-lasting changes in the molecular composition of synapses.

Ubiquitination. Copines seem also to contribute to protein degradation. A possible direct link between copines and ubiquitination pathway is based on the interaction of the NEDD8-conjugating enzyme UBC12 and the copine 1 A domain [152]. NEDD8 is an ubiquitin-like protein that is covalently attached to proteins targeted for degradation through the co-ordinated action of the conjugating enzyme UBC12 and other enzymes. Recent data suggest that copines may regulate NF *κ*B signalling in a calcium dependent way by promoting I*κ*B degradation via an activatory effect on UBC12 [164]. Possibly, endogenous copine binds UBC12 and promotes its association with other components of the signalling pathway on the membrane surface, or regulates its activity directly in a calcium-dependent fashion. Elevating spontaneous activity enhances the ubiquitination

of postsynaptic proteins [226]. A handful of proteins in the postsynaptic density (PSD), namely Shank, GKAP, AKAP79/150, and PSD-95, have been found to undergo activity-dependent ubiquitination [227, 228]. Intriguingly, each of these postsynaptic targets of ubiquitination is a multivalent scaffold protein capable of complexing several postsynaptic proteins through multiple protein interaction motifs [226, 228].

Small Rho GTPases. Copines affect the actin cytoskeleton. Knockdown of copine 6 causes an increase in the number and size of spines, whereas the loss of copine 3 causes a depolymerisation and, consequently, a reduction in the number and length of dendritic protrusion. Spines and their filopodial precursors are rich in filamentous actin [229]. The changing spine head contains a variety of proteins in the postsynaptic density regulating the actin filament. These proteins are regulated by small GTPases of the Rho family. Different Rho GTPases have distinct effects on the actin organization of spines. For example, the extension of filopodia requires actin filament polymerization and elongation, which is likely to be mediated by increased Cdc42 activity and/or decreased RhoA activity. Shaping a rounded spine head likely requires the assembly of branched actin networks, which is likely promoted by both Rac and Cdc42 [180, 230]. Spine retraction is likely mediated by RhoA through an increased contractility of the actin filaments. Interestingly, there is evidence for a biochemical interaction of copine 4 with Cdc42 and copine 6 with Rac 1, respectively [152]. This offers the possibility, that copines influence the activities of Rac1, Cdc42 and eventually RhoA during dendritic spine development and remodelling.

Scaffold. Calcium is not essential for copines to bind to lipid membranes composed of phosphatidic acid [153] or plasma membranes isolated from Arabidopsis cells [148]. Furthermore, copine protein was constitutively localized to the plasma membrane in transfected leaf protoplasts [148] and copine 6 in the brain was not completely removed from plasma membranes upon calcium depletion [154]. Thus, some copine proteins might be constitutively localized to plasma membranes and serve as scaffolds mediating the assembly of receptors and synaptic proteins. The integrity of the postsynaptic density is important for normal spine morphology, in part because the actin filaments that shape dendritic spines are attached to it and in part because a number of proteins in the

postsynaptic density regulate spine morphogenesis. Perturbing the function of scaffolding proteins in dendritic spines affects spine morphology. Scaffolding proteins contribute to the clustering and stabilization of glutamate receptors and the other densely packed components of the postsynaptic density [231, 232]. They also bind to each other, thus contributing to the formation of diverse multiprotein complexes. In *C. elegans*, copine was shown to be required for maintenance of normal levels of nAChRs at synaptic sites [155]. The copine homolog NRA-1 associates with the levamisole receptor [155]. Deletion of copine caused resistance to cholinergic agonists and reduced synaptic levamisole receptor levels; thus, copine appears to play a role in targeting or stabilizing the levamisole receptor at the plasma membrane [155].

Mechanism of copine in synaptic plasticity

Synaptic plasticity due to changes in copine composition. Changes of copine composition can influence the effect of copines. It is not clear whether each copine is responsible for a particular functions, but copines bind to individual interacting partners [152], show a tissue specific expression (discussed above) and becomes transcriptionally upregulated upon synaptic activity [166]. Taking this into account, changes in relative amounts of copines might cause alterations in the composition and function of complexes. The presence or stochiometric changes of individual copines within a complex might therefore cause changes in the protein composition and alter the receptivity to diverse upstream pathways and in consequence the outputs originating from the complex (Figure 2A).

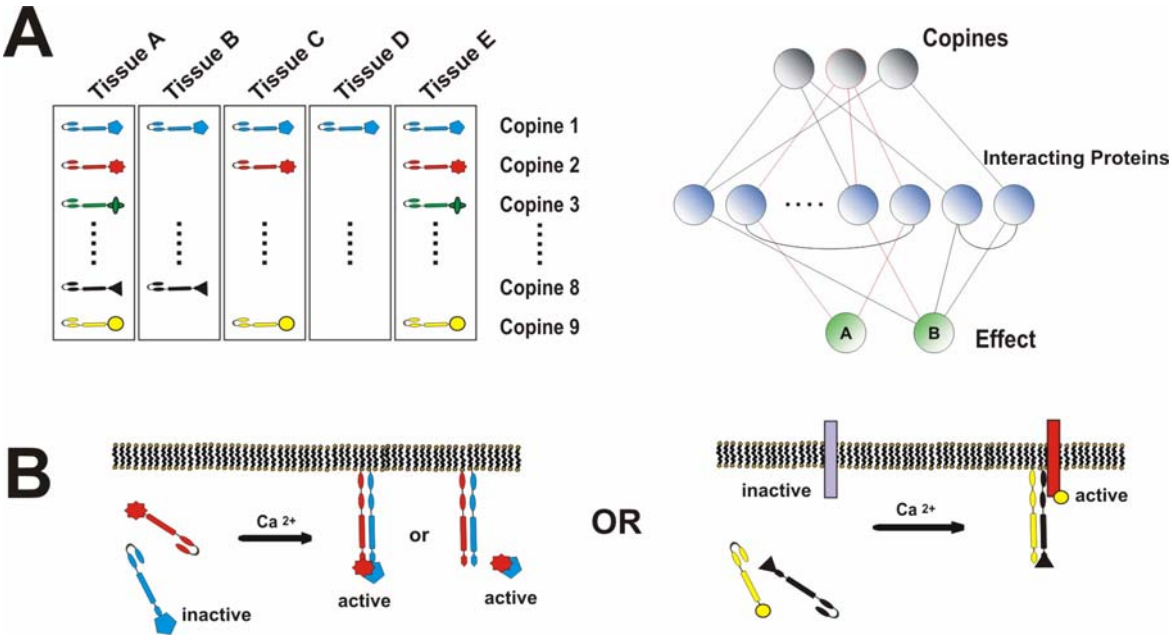


Figure 2 Models of copine function

(A) Copine levels vary in different tissues (left picture) and individual copine family members bind to specific interacting proteins (right picture). The picture to the right illustrates how changing compositions of copines affect the output of individual complexes. Individual copines bind to specific proteins but also share some common interaction partners as indicated by the connections. Changing combinations of individual copines or modulations in the expression level of present copines in a tissue during development or due to a stimulus can change the output of complexes. As indicated in red, modulations of the stochiometry of copine can – dependent on the presence of interacting proteins - cause a new effect (A) or modulating an existing one (B). (B) Calcium dependent relocalization of copines and interacting proteins. For reasons of simplification copine are illustrated to form hetero-dimer instead of hetero-multimer. At low calcium concentrations, individual copines are present as monomers in the cytosol. As a result of increasing calcium concentration, copines undergo a conformational change at the N-terminal part as indicated by the opening of the loop. In consequence, copine multimerize and bind to the plasma membrane. Due to the multimerization of copines *inter se* proteins that bind to individual copines accumulate. As a result, these proteins can interact within the complex (left picture). Alternatively, target proteins can be localized at the plasma membrane (right picture), since the relocalization of the complex to the plasma membrane accumulates copine-interacting proteins in the vicinity of the plasma membrane (right picture).

Synaptic plasticity due to changes of calcium. At low calcium concentrations, copines are present as monomers in the cytosol [152]. Upon stimulation of the synapse, calcium concentration increases in spines. In consequence, copines bind inter se and relocalize to the plasma membrane (Figure 2B). Indeed, binding of copine 1 to plasma membranes is dose-dependent with a saturation at 2mM calcium and a half maximal binding at calcium concentration from 3 µM to10 µM [153]. Cytosolic calcium in spines using the calcium indicator mag-Fura 5 have demonstrated calcium accumulations of 20-40 µM in dendritic spines in response to depolarization or synaptic stimulation of hippocampal CA1 pyramidal neurons. Thus, copine relocalization occurs in a calcium range achieved during synaptic activity. Relocalization, in turn, brings copine binding proteins together which then can lead to multiple effects: Proteins can be phosphorylated or degraded by copines directly or by copine binding proteins. As consequence, we observe calcium-dependent modifications leading to structural changes (Figure 2B). Modifications might involve alteration in protein level (transcription, relocalization, degradation) or protein activity (binding, degradation, phosphorylation). One possible target are members of the small Rho GTPases family. In their GTP-bound state, Rho GTPases Rac 1 and Cdc42 bind to their downstream effector Pak 1 (p21-activated kinase) which leads to the activation of LIMK (LIM-domain-containing protein kinase) [197] and myosin light chain kinase (MLCK), which causes increased actin filament turnover and cell motility [233]. Copine 6 might acts as a negative regulator of spine formation, presumably trough inhibition of the GIT1/PIX/Rac1/Pak1 signalling module (Figure 3A). However, there exist alternative explanations as summarized in Figure 3B. The interaction of copine 6 with Rac 1 does not necessary mean that the binding contributes to the observed phenomenon. Interestingly, knockdown of copine 6 increase in the number of functional synaptic contacts on a single neuron without affecting synaptic size. Thus, copine 6 might increase the concentration of synaptic AMPA-R and, consequently, synaptic activity. In that case, spine remodelling would be the effect of a copine 6 dependent increase in synaptic activity (Figure 3B).

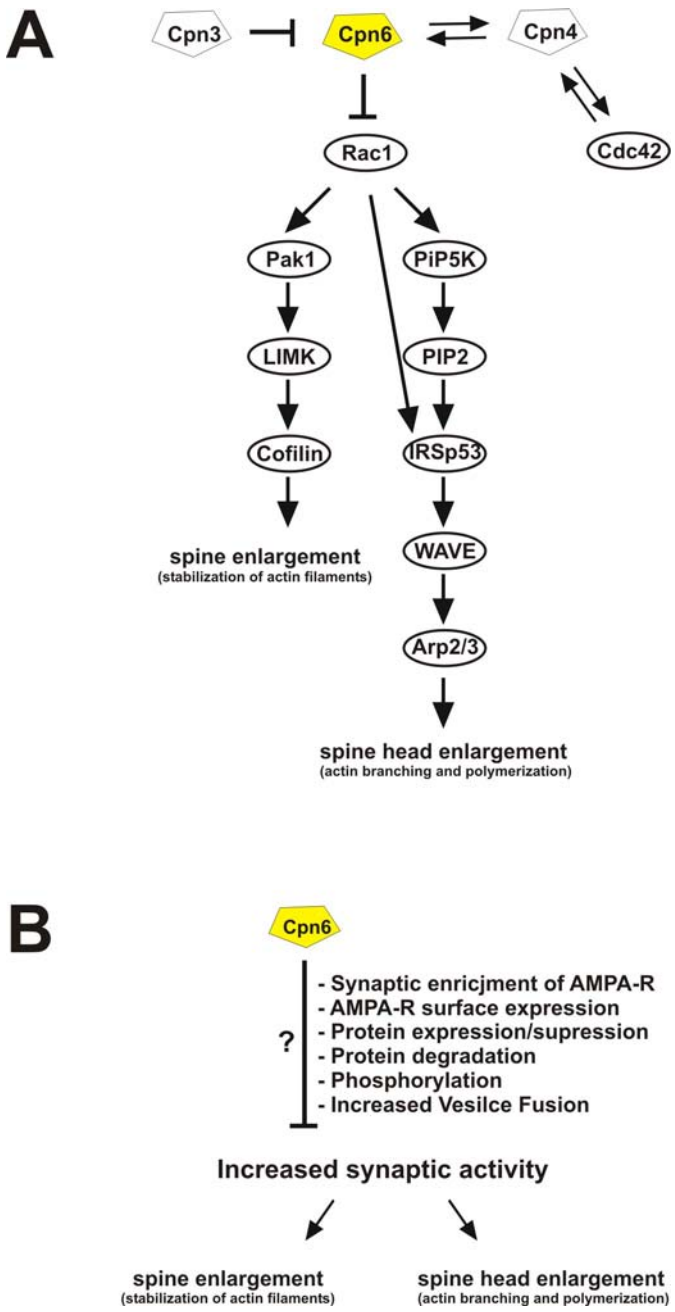


Figure 3 Copines affecting the actin cytoskeleton

(A) Model of copine 6 directly affecting the actin cytoskeleton. Rac 1 and Cdc42 activate Pak 1, a serine– threonine kinase that phosphorylates and activates LIM kinases 1 and 2. The LIM kinases, which are also serine– threonine kinases, in turn phosphorylate and inhibit the actin depolymerizing proteins ADF and cofilin to reduce actin filament turnover and cell motility. Another pathway by which Rac 1 and Cdc42 likely promotes actin nucleation and branching in the dendritic spine head is by Arp2/3. Activated Arp2/3 complex nucleates actin polymerization and branching, which may be the mechanism leading to spine head enlargement. Rac 1 and Cdc42 can also promote actin polymerization by binding to the adaptor insulin receptor substrate p53 (IRSp53). Copine 6 might act as a negative regulator of spine formation, presumably trough inhibition of the GIT1/PIX/Rac1/Pak1 signalling module. Activation of Rac 1 causes a reduction in the size of the dendritic spines but increases their number [125, 127], and inhibition of Rac 1, drastically decreases the number of both spines and synapses in cultured hippocampal slices and dissociated hippocampal neurons [125, 128]. (B) Summary of possibilities how copines enhance synaptic activity. Receptors become enriched at synaptic sites which in turn lead to increased activity and in consequence to actin remodelling within the spines.

Copines and neurodevelopmental diseases

Since copines affect spine morphology, changes in copine expression could contribute or be involved in neurodevelopmental disorders. Indeed, deformed dendritic spines and changes in spine density are a hallmark of many neurological conditions, notably in virtually every disease in which cognitive performance is impaired [132]. Substantial decreases in dendritic spine density in pyramidal cells of the neocortex and hippocampus can be observed in human tissue from Alzheimer's patients [130]. Dendritic spine loss is reported in other non-Alzheimer's type dementias, and may represent a pathological acceleration of the normal decrease in dendritic spine density observed in senescence [131]. Furthermore, pyramidal cells in several different forms of mental retardation have a lower than normal density of spines, including Down's syndrome and fragile X syndrome [132, 133]. Decreases in spine density and structural synaptic abnormalities are also common in human tissue from psychotic schizophrenic patients [134], and in hippocampi from patients suffering from uncontrolled epileptic seizures [135]. In many of these diseases, the involved proteins and underlying mechanisms and are not known yet.

Conclusions

In summary, recent studies have shown that copines are necessary for the formation, function and stability of CNS synapses in vitro. Whether copines have any other functions during neuronal development remains elusive. Further work, particularly using live imaging of developing spines and the analysis of knockout and transgenic mice, will provide information about the real-time dynamic behavior of copines and/or of how changes of individual copine-multimer complexes affect the actin cytoskeleton and the molecular composition of the spine.

CHAPTER 4

General discussion and perspectives

The aim of this doctoral thesis was to detect novel genes involved in synapse formation and modulation. What we found is an entire gene family that has so far not been described to be involved in synapse formation. The central findings of this work are summarized in the following.

Novel screening approach in primary hippocampal culture

In order to detect genes involved in synapse formation of the central nervous system, we proposed a novel system to screen for genes during initial synapse formation in primary rat hippocampal culture. We anticipate that genes identified in our screen encode components involved in synapse formation and maturation. Based on our initial success, we assume that a systematic exploration of this system by microarray would lead to the identification of numberous already known but also novel genes and thus could lead to a characterization of novel synaptic players. Furthermore, in complement and extend to such a study mass spectrometry analysis using the same system combined with purification of synaptosomal fractions would accomplish the analysis, and could define further new targets to study. The advantages of our system are multiple, and such a parallel approach would lead to numberous possibilities to obtain knowledge at different hierarchical levels. In addition, the output could be fast and straight forward tested for its functional impact in the same system using RNAi transfection. In summary, a screening assay is definitively a promising next step.

Molecular mechanisms of copines in synapse formation

The role of Copine family members during synapse formation are starting to unfold and future work will shed light on how and which Copine will affect spine formation. The so far obtained results and possible future implementations were discussed in detail in the previous chapter. Thus, we will here just give a brief summary of the main findings.

(1) Copine family members are transcriptionally and translationally upregulated during synapse formation in primary hippocampal cultures and in whole cortex of newborn rats during the period of initial synapse formation.

(2) Misregulation of copine family members causes changes in neurite morphology. Reduction in the level of copine 4 caused dendrite retraction and ectopic lamelipodial outgrowth. Interestingly a knockdown of copine 3 during initial synapse formation showed aspiny dendrites whereas a knockdown against copine 6 show the opposing effect, namely an increase in spine size, number and activity. Double knockdown of copine 3 and 6 revert the aspiny effect of copine 3.

(3) Spatial expression profiles of copine 3 and copine 6. Copine 6 is expressed exclusively in the brain. Within the brain, the protein is mainly expressed in the hippocampus and dentate gyrus and at lower levels in the cortex. On the cellular level, copine 6 is present in the somato-dendritic compartment and enriched in spines. Copine 3, by contrast, is expressed widely throughout most of the body. Highest expression in the brain occurs in the cortex, dentate gyrus and hippocampus. On cell level, no enrichment can be observed.

(4) Molecular model of copine function in neuronal cells. Our experiments suggest that copines act as regulators of spine formation by direct interaction with small Rho GTPases. We have shown that copine 6 negatively regulates spines formation. Interestingly it also binds to copine 3, Pak 1, Rac 1 and plasma membranes at the postsynaptic site in a calcium dependent manner. In our model, activity dependent changes in postsynaptic calcium levels might contribute to the relocalization of copine 6 and its binding partners to plasma membranes within spines and so regulate spine morphogenesis.

(5) Spine formation and neurodevelopmental disorders. Subtle dysfunctions of the cytoskeletal dynamics have been associated with in deficiencies in neuronal connectivity and function, which in turn lead to defects in cognitive function and behaviour. Dendritic spines are irregularly shaped and have abnormal densities in a number of neurodevelopmental disorders. Thus malfunction of copine might be involved in some of these diseases.

In the following we will discuss different strategies to further analyze the biochemical and functional properties of copines.

(1) Biochemical properties of the multimolecular copine complex. Copines bare some core properties. However, it remains elusive whether all copines share these properties. Thus one approach would be to confirm these data for all subsequently discovered copine family members. Experiments to be done would involve a proper description of all copine interacting proteins [152]. Given that a specific antibody is available, the interaction of copines with unknown target proteins should be tested upon Co-immunoprecipitation in the presence of changing calcium concentrations from tissue-lysates and analyzed by mass spectrometry. These findings are crucial towards the final model of copines working in a multimolecular complex. In consequence following questions could be addressed:

- Multimerization of copines *inter se*. As a key follow-up experiment, the copines-interaction has to be conformed by co-overexpressed in COS cells. The proteomics approach will give a broad overview of available complexes but will not answer whether the complex composition is based on the ability of an interaction of copines *inter se*.

- Copine and receptor trafficking [155]. There is accumulating evidence for an involvement of copines in receptor trafficking, thus the proteomic approach will under circumstances shed light on a role of copines in receptor trafficking or scaffolding.

(2) Biochemical aspects of individual copines. This assay is focussed to dissect the role of individual copines in multimer complexes. A proper understanding of the biochemical properties of the domains on individual copines would be a needed complementation to understand the function of copines in multimolecular complexes. Following questions could be addressed:

- Relocalization and plasma membrane-binding assays. In this experiment, cloned copine family members (and deletion mutants) are sedimented in the presence of various phospholipids at different calcium concentrations in vitro

[143]. As readout we receive the binding affinity of the individual copine family members in dependence of changing calcium concentrations to various phospholipids types. These findings are of particular interest under the assumption that copines act as multimers. Thus, changes in copine type composition could also change the responsiveness to alternations in calcium concentrations.

- Copine, a novel kinase family. The role of copines as a kinase could be analyzed in a straight forward assay [165]. ATP pulldown experiments would easily and fast give a first hint whether the specific copine binds to ATP – a pre-exquisite for a kinase activity. Deletion mutants of copines in cell culture and subsequent in vitro kinase assay of the deletion mutants will show if there is a kinase domain and whether all copines share this particular kinase domain.

(3) The role of copine 6 in various aspects of neuronal development. Knockdown of copine 6 affects spine shape. Hence, little is known of its role in other aspects of neuronal development or what a loss of copine might causes in vivo. The role of copine 6 function should be further analysed at different hierarchical levels. Note that the experiments listed in the following might also be performed with all other copines that show an effect in neuronal development or changes in spine morphology.

- Copine 6 affects actin cytoskeleton. However, it remains unclear how exactly copine 6 accomplishes its function Copine 6 could function trough protein interaction but might also affect protein phosphorylation, protein synthesis or protein degradation [164, 165]. Primary hippocampal cultures, transfected with an overexpression and/or knockdown construct and a subsequent proteomic and microarray analysis would unveil whether this is the case. However, this would require a lentivirus since only high transfection efficiencies would lead to detectable changes.

- Two photon live imaging in acute hippocampal slices: Copine 6 appears to be enriched in dendritic spines and calcium appears to cause plasma membrane binding of copines. However, a depolarization-dependent relocalization of copine 6 has so far not been studied. As a further permutation of the same

aspect, the function of copine 6 or its misregulation could be studied by life-imaging.

- Copine 6 knockdown mice. The existing results have to be confirmed *in vivo*. Thus, a Copine 6 knock-out or knock in mouse is absolutely required. In addition such a mouse would open the gates to a pleiotropy of new experiments. Behaviour studies would give a hint what cognitive function are affected to what extend.

(4) Copines in axonal pathfinding. Given this evidence that copines affect actin cytoskeleton, one could conclude that other aspects of actin reorganization like axon pathfinding, which also shows calcium dependent growth cone extension and steering, might also depend on copines. Thus, further studies on copine 6 function with emphasis on axonal development represent another promising aspect of copine function in neuronal development.

(5) The role of copines at the neuromuscular junction. Copines were initially described to be upregulated during synapse formation at the neuromuscular junction. Interestingly, copine 1 shows a MyoD binding site in its promoter. It would be worth a try to challenge the role of all copines in synapse formation at the NMJ. A fast approach would involve the knockdown of individual copines in myoblasts.

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Acknowledgements

I would like to thank Professor Markus A. Rüegg for the opportunity to perform this work in his Laboratory in the Department of Neurobiology and Pharmacology, Biozentrum, University of Basel, Switzerland between October 2002 and September 2006.

I am grateful to Markus for his support, trust and the thereof resulting liberty I experienced the last years. He gave me the freedom to explore my project by my own as well as the guidance not to loose focus– an opportunity to gather valuable experience not many doctoral students can gain in that extend.

Also I would like to thank the people who were involved in my project or helpful when I had questions and problems. Furthermore, I want to thank my labmates, former and present and the people on the floor.

In the end I want to thank my parents and my friends for their invaluable support.

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Erklärung

Ich erkläre, dass ich die Dissertation „Regulation of dendritic spine morphogenesis and synapse formation by copines" nur mit der darin angegebenen Hilfe verfasst habe und bei keiner anderen Universität und keiner anderen Fakultät der Universität Basel eingereicht habe.

Milos Galic

Basel, 4. September 2006