Molecular mechanisms of collybistin-dependent gephyrin clustering at inhibitory synapses

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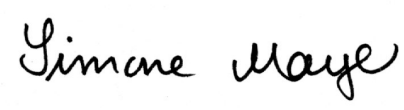
Herewith I declare, that I prepared the PhD Thesis "Molecular mechanisms of collybistin-dependent gephyrin clustering at inhibitory synapses" on my own and with no other sources and aids than quoted.



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Quod superest, perge,
et iam non cum aliis,
sed tecum ipse certa.

Marcus Tullius Cicero

List of Publications

Parts of this thesis have been published:

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Abstract

Information processing and transmission in neuronal networks in the mammalian brain occurs through intercellular communication between neurons at synapses. Inhibitory synapses play a key role, for example to maintain network homeostasis, and their malfunction results in various neurodevelopmental diseases. At inhibitory postsynapses, neurotransmitter receptors are anchored in apposition to presynaptic neurotransmitter release sites by the scaffold protein gephyrin, whose recruitment is dependent on the presence of collybistin (Cb) in various brain areas, such as the hippocampus. Most Cb isoforms contain three domains, an autoinhibitory src homology 3 (SH3) domain, a Dbl homology (DH) domain, which catalyzes the nucleotide exchange on the small Rho-like GTPase Cdc42, and a pleckstrin homology (PH) domain, which binds to phosphatidylinositol 3-phosphate. The notion of an involvement of Rho family GTPases in the regulation of Cb-dependent gephyrin clustering at synaptic sites is controversial. In this study, we have investigated the involvement of Cdc42 and its closest homolog TC10 in inhibitory postsynapse assembly. We show that both GTPases are able to relieve the autoinhibition of Cb and thereby allow Cb to trigger gephyrin microcluster formation at the plasma membrane in non-neuronal cells. This TC10-triggered Cb-dependent gephyrin microcluster formation requires GTP-bound TC10 and the ability of Cb to bind to phosphoinositides. While Cb can activate TC10 in cells, this is not essential for gephyrin microcluster formation. Furthermore, we identify two distinct binding sites for TC10 on Cb - a GDP-specific one in the DH domain, and a GTP-specific one in the PH domain. In neurons, overexpression of TC10 in its GTP-bound state increases gephyrin clustering and inhibitory neurotransmission, whereas GDP-TC10 has opposite effects. TC10 is membrane-anchored through prenylation, basic residues, and palmitoylation in its carboxy terminus and the former two are required for TC10-triggered Cb-dependent gephyrin microcluster formation. In conclusion, we provide evidence for a dual role of small Rho family GTPases in Cb-dependent gephyrin clustering. Binding in the GTP-bound state to the PH domain relieves the autoinhibition exerted by the SH3 domain and provides a second membrane anchor to target Cb to specific subcellular compartments.

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List of Abbreviations

Abbreviation	Term
CA	Constitutively active
Cb	Collybistin
CHAPS	3-((3-Cholamidopropyl) dimethylammonio)-1-propanesulfonate
DGC	Dystrophin-associated glycoprotein complex
DH	Dbl homology
DN	Dominant negative
ER	Endoplasmic reticulum
$GABA_AR$	γ-amino butyric acid type A receptor
GABA	γ-amino butyric acid
GAP	GTPase-activating proteins
GDI	Guanine nucleotide dissociation inhibitors
GDP / GTP	Guanosine-5'-diphosphate / Guanosine-5'-triphosphate
GEF	Guanine nucleotide exchange factor
GlyR	Glycine receptor
GST	Glutathione S-transferase
hPEM-2	Human posterior end mark 2
IP	Immunoprecipitation
(m)IPSC	(Miniature) Inhibitory postsynaptic current
IPTG	Isopropyl-a-D-thiogalactopyranosid
KO	Knockout
mant-GDP	Methylanthraniloyl-GDP
MDGA	MAM domain-containing GPI anchor proteins
Mena/Vasp	Mammalian enabled / vasodilator stimulated phosphoprotein
Moco	Molybdenum-cofactor
NL	Neuroligin

Abbreviation	**Term**
PCR	Polymerase chain reaction
PDZ	PSD-95/disk-large/zona-occludens-1
PH	Pleckstrin homology
PI(3)P	Phosphatidylinositol 3-phosphate
PI(3,5)P_2	Phophatidylinositol 3,5-biphosphate
PI(4,5)P_2	Phophatidylinositol 4,5-biphosphate
PSD	Postsynaptic density
SH3	Src homology 3
S-SCAM	Synaptic scaffolding molecule
VIAAT	Vesicular inhibitory amino acid transporter
WASP	Wiskott-Aldrich syndrome protein
WT	Wildtype

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Introduction

1.1 Neuronal networks in the mammalian brain depend on synaptic transmission

The nervous system is responsible for sensing the environment, processing this information and producing motor outputs that maximize the chances of survival of the organism. While the sensory input and motor output are mainly executed by the peripheral nervous system, the central nervous system, composed of the brain and the spinal cord, is the processing unit. The brain is composed of millions of cells, most of which are glial cells and mainly serve a supportive function, while information processing, storage and transmission is performed by neurons. Neurons are connected with each other in different networks through specialised cell-cell contacts called synapses, which allow fast and reliable information transmission. Each neuron in the mammalian brain connects to other neurons via an estimated 10,000 synapses (Sheng et al., 2012). Thus synapses are one of the basic building blocks of the mammalian brain and understanding the details of synaptic transmission and its plastic changes is a prerequisite for understanding all higher brain functions, from locomotion to emotions, the abnormalities of which lead to various neurological and psychiatric diseases.

1.1.1 Principles of synaptic transmission

Neurons use electrical signals for communication. At rest, the permeability of the plasma membrane and the distribution of ions, which is maintained predominantly by the Na^+/K^+-ATPase, together lead to a negative membrane potential of circa -60 to -70 mV. Over long distances, signals are transmitted by action potentials, transient depolarisations of the membrane, that propagate through the axon of a neuron and are then transmitted to the next neuron at a synapse. Two types of synapses are

distinguished: electrical and chemical synapses.

At electrical synapses, gap junctions connect the two cells making their cytoplasm continuous. Signal transmission occurs when ions pass between the cells; it is thus bidirectional, fast and cannot be subject to complex regulatory mechanisms. These synapses are widespread in the mammalian brain and allow synchronisation, for example between inhibitory interneurons in the neocortex, hippocampus and thalamus (Connors and Long, 2004).

At the more intensely studied chemical synapses, however, information transmission occurs across two membranes. As an action potential propagates through the axon and reaches a presynaptic terminal, the depolarisation of the membrane potential triggers voltage-gated Ca^{2+} channels to open. The ensuing Ca^{2+} influx is sensed by the protein synaptotagmin, which is localised on vesicles containing neurotransmitters. A cascade of molecular events is then triggered that ultimately leads to the fusion of synaptic vesicles with the plasma membrane and hence the release of neurotransmitter into the synaptic cleft. The neurotransmitter diffuses in the extracellular space, across the approximately 20 nm wide synaptic cleft. Signal transmission occurs when it binds to neurotransmitter receptors on the plasma membrane of the postsynaptic neuron. Signal termination is achieved as neurotransmitter molecules are taken up by glial cells and the presynaptic neuron.

There are two types of neurotransmitter receptors. Ionotropic neurotransmitter receptors are ion channels, which, upon binding of the ligand, open their pore and selectively allow ions to follow their electrochemical gradient. This causes a change in the membrane potential of the postsynaptic neuron. Upon spatiotemporal integration of all synaptic inputs of an individual neuron at the axon hillock, if a certain threshold value is reached, an action potential is generated and propagates down the axon. Therefore synaptic inputs on the dendrites and the soma determine the firing behaviour of a neuron and thus its activity in a neuronal network.

A second class of neurotransmitter receptors are metabotropic, G-protein coupled receptors. Upon binding of a ligand on the extracellular side, the conformation of the receptor on the intracellular side changes, allowing it to initiate signalling cascades that can have long-lasting consequences on cell function, for example by influencing transcription. Metabotropic neurotransmitter receptors thus function as modulators and affect the intracellular signaling on a longer time scale, while signaling through ionotropic receptor activation leads to immediate electrical changes in the cell.

1.1.2 Inhibitory synapse function in neuronal networks

In the mammalian cerebral cortex, most neurons belong to the relatively uniform class of excitatory pyramidal neurons, which use glutamate as a neurotransmitter (Markram et al., 2004). Upon binding of glutamate to cognate ionotropic receptors, Na^+ enters the cell, leading to a depolarisation of the membrane potential. The activity of pyramidal neurons is controlled by a diverse class of inhibitory interneurons, which release γ-amino butyric acid (GABA) from their presynaptic terminals in the mammalian brain and glycine in the spinal cord (Markram et al., 2004). The ionotropic receptors for GABA and glycine, the $GABA_A$ receptors ($GABA_A$Rs) and the glycine receptor (GlyRs), respectively, are ligand-gated Cl^- channels. During development the opening of these channels leads to a depolarisation of the membrane potential with an ensuing increase in intracellular Ca^{2+} concentration, which through calcium-mediated intracellular signaling cascades ultimately influences neuronal network maturation (Cellot and Cherubini, 2013). As the brain matures, a change in expression of Cl^- transporters causes a change in the equilibrium potential of Cl^-, so that Cl^- enters the cell upon channel opening, which leads to a decrease in the membrane potential and thus causes an inhibitory effect on the firing probability of the postsynaptic neuron.

It is the dynamic interplay between excitation and inhibition that allows complex cognitive functions to be performed. For example, inhibitory interneurons play an important role since they allow a precise temporal control of spike timing of excitatory pyramidal neurons. Moreover, they are essential for synchronizing neuronal activity to allow the generation of network oscillations (Klausberger and Somogyi, 2008). Gamma oscillations (30-80 Hz), for instance, contribute to cognitive functions such as spatial navigation. The importance of inhibitory transmission in the generation of these oscillations is demonstrated in experiments where evoked oscillations are completely blocked by $GABA_A$R antagonists, but not antagonists of ionotropic glutamate receptors (Bartos et al., 2007). In conclusion, for homeostasis in a neuronal network, an overall balance between excitation and inhibition (E/I balance) needs to be maintained. This balance is established during development, when neurons are born and migrate to the appropriate location in the brain, where they then form connections, which are later refined in an experience-dependent manner (Ramamoorthi and Lin, 2011).

Perturbations in neuronal development that lead to malfunctional neuronal circuits owing to an imbalance between excitation and inhibition are considered to be causative for many neurological and psychiatric disorders, such as schizophrenia, epilepsy and autism spectrum disorders (Ramamoorthi and Lin, 2011). Such an imbalance may be due to malfunctional excitatory or inhibitory neurotransmission and may arise in any developmental stage (Ramamoorthi and Lin, 2011). As outlined below (1.2), muta-

tions in many components of inhibitory postsynapses have been identified in patients with different neurodevelopmental disorders. These findings highlight the central importance of inhibitory synapses for normal brain function.

1.1.3 Differences between excitatory and inhibitory synapses

Initial electron microscopic studies of rat cerebrocortical synapses led to the distinction of two types of synapses by E. G. Gray. Type I synapses are found mostly on dendrites and dendritic protrusions called spines, and are characterized by a thickening especially at the postsynaptic membrane. Type II synapses predominate on axosomatic areas and do not show an asymmetric thickening of the membranes (Gray, 1959).

Later on, it was discovered that Gray's type I synapses are excitatory, while type II synapses are inhibitory (see Figure 1). There is a third type of synapses, the neuromodulatory synapses, which do not have ionotropic receptors.

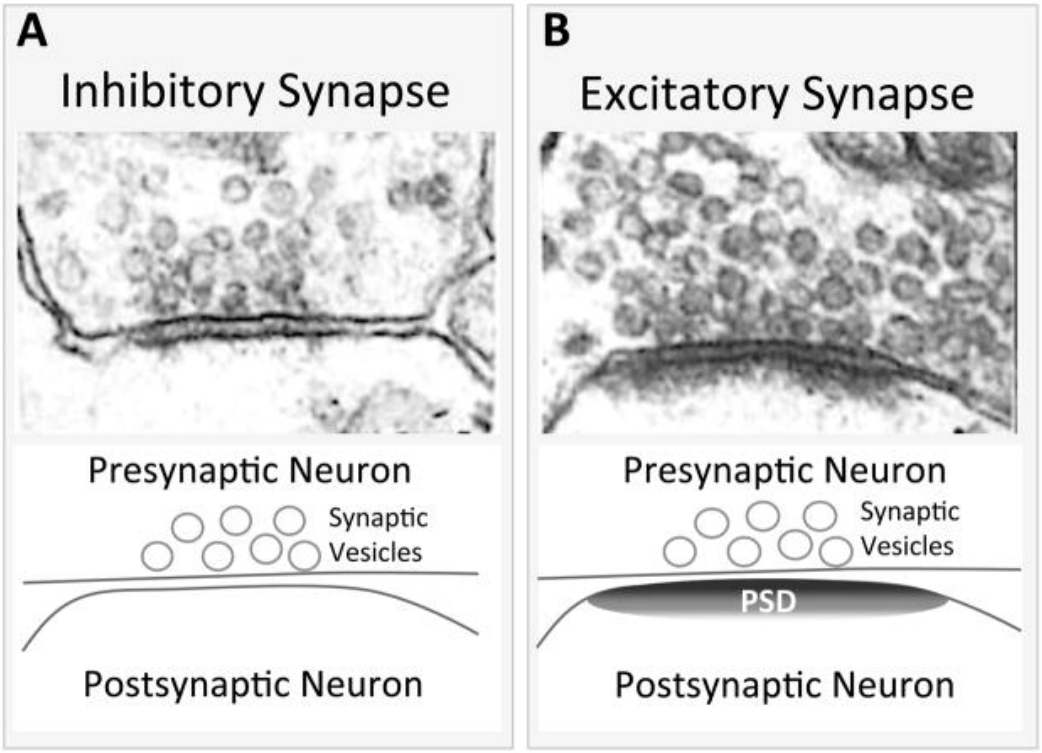

Figure 1: Electron microscopic images of inhibitory and excitatory cortical synapses. Electron micrograph of an inhibitory (A) and excitatory (B) synapse (top) with schematics (bottom) showing the symmetric and asymmetric arrangement of pre- and postsynaptic specialisations, respectively. Postsynaptic density, PSD. Source: Kuzirian and Paradis (2011), original images from Colonnier (1968).

At a molecular level, it seems that there are only few differences between inhibitory and excitatory synapses presynaptically (Boyken et al., 2013). However, the molecular

composition of postsynapses is clearly distinguishable despite sharing the same evolutionarily conserved organisational principles (Figure 2). In both types of synapses, neurotransmitter receptors and cell adhesion proteins are located at the plasma membrane to establish a tight coupling between pre- and postsynaptic neurons, which allows efficient signal transmission. Intracellularly, a proteinaceous scaffold links the transmembrane proteins to different cytoskeletal elements as well as interacting with enzymes, which can carry out signaling functions (Emes and Grant, 2012).

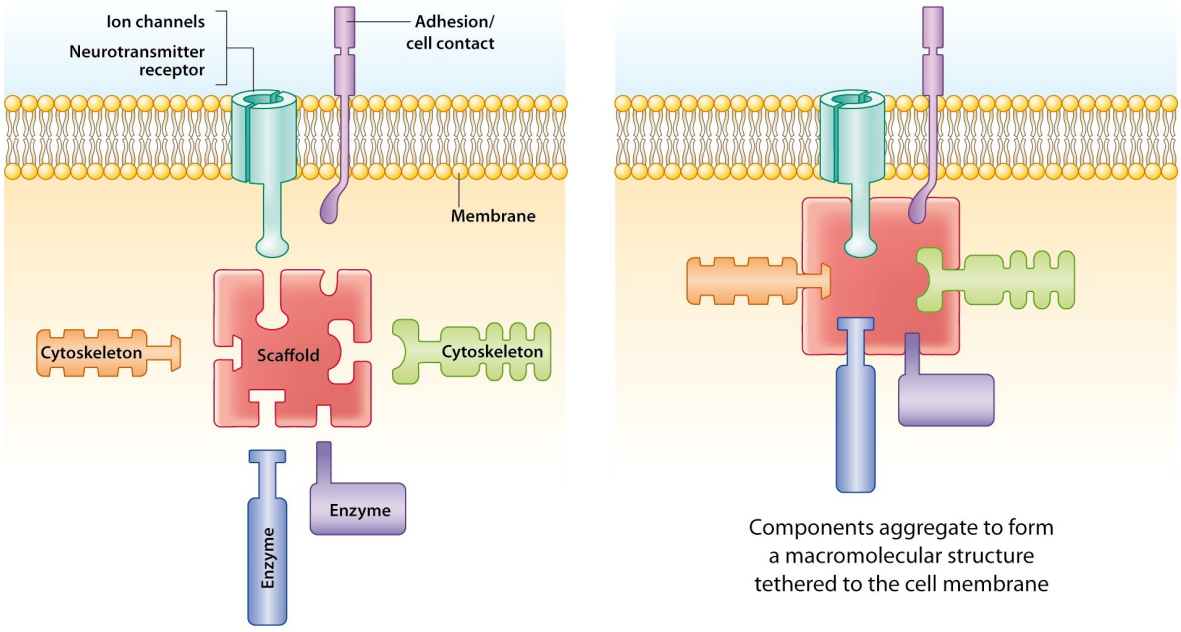

Figure 2: Organising principles at the postsynapse The postsynaptic apparatus is comprised of five key classes of proteins, which allow signal reception from the environment and communication to intracellular signaling pathways. Neurotransmitter receptors and cell adhesion proteins are inserted in the membrane and interact with scaffold proteins, which in turn interact with the cytoskeleton to limit the lateral movement of transmembrane proteins and enzymes subserving signaling functions. On the left, the components with the binding sites for the scaffold protein are introduced, on the right, the formation of macromolecular complexes through the assembly of the components is depicted. Source: Emes and Grant (2012).

More than 40 years ago, protocols were developed to biochemically purify the electron-dense area underneath the postsynaptic membrane at excitatory synapses, called the postsynaptic density (PSD) (Carlin et al., 1980; Davis and Bloom, 1973; Fiszer and Robertis, 1967). Since then this has allowed the identification of many proteins of the PSD, as for example in a recent study, where almost 1,500 different proteins and their relative abundances were identified through mass spectrometric analysis (Bayes et al., 2011). Interestingly, less than 10% of these were ion channels and recep-

tors, emphasizing the importance of intracellular scaffold and signalling components for postsynaptic function (Grant, 2013). To allow dynamic changes in signalling features, the composition of the PSD is dynamic, for example the translocation of a major neuronal kinase, calcium-calmodulin-dependent protein kinase II (CaMKII), is dynamically controlled by synaptic activity (Shen and Meyer, 1999).

Inhibitory synapses are much harder to characterize biochemically, since the lack of an electron-dense PSD makes their isolation difficult. Additionally, they are much more sparse in the brain than excitatory synapses (Kuzirian and Paradis, 2011). For this reason, the understanding of the molecular architecture of inhibitory postsynapses and the dynamic mechanisms that allow activity-dependent remodeling has long been lagging behind the one of excitatory synapses.

1.2 The molecular architecture of the inhibitory postsynapse

Despite the lack of an understanding as detailed as the one we have for the excitatory PSD (see 1.1.3), research in the last decade has identified many components of the inhibitory synapse as well as some of the molecular mechanisms underlying inhibitory postsynapse formation (Figure 3). At synaptic sites, $GABA_A$Rs and cell adhesion molecules are clustered in apposition to the presynaptic release site to minimize the diffusion distance for neurotransmitters and thereby allow fast and efficient neurotransmission. Beneath the synaptic plasma membrane, a scaffold formed by the proteins gephyrin or synaptic scaffolding molecule (S-SCAM) restricts the diffusion of synaptic proteins by interacting with the subsynaptic cytoskeleton. Many other proteins with roles in intracellular signaling and activity-dependent remodeling are also enriched in the inhibitory postsynapse. The best characterized regulator of gephyrin function at inhibitory postsynapses is the guanine nucleotide exchange factor (GEF) collybistin (Cb), which is necessary for the synaptic localisation of gephyrin and $GABA_A$Rs in many areas of the mammalian brain.

1.2.1 GABA receptors

$GABA_A$Rs belong to the family of pentameric Cys-loop ligand-gated ion channels and mediate fast inhibitory neurotransmission. They are a very diverse receptor class, with 19 different subunits encoded in the mammalian genome (Luscher et al., 2011). Further diversity is generated through alternative splicing, variable combinations of receptor subunits being assembled into complexes in the endoplasmic reticulum (ER) (Luscher

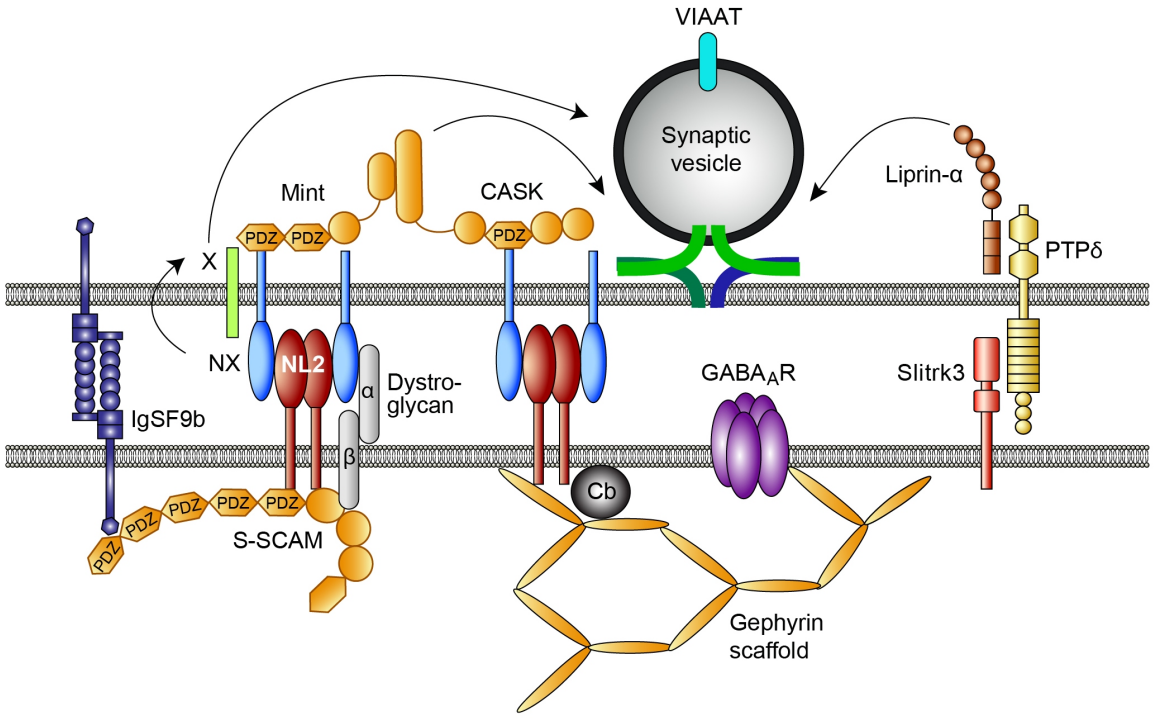

Figure 3: Molecular architecture of the inhibitory synapse. All transsynaptic interactions promoting specifically inhibitory synapse assembly are shown, together with some key proteins in the presynapse and the scaffold proteins and intracellular signalling proteins known to be involved in regulating inhibitory postsynapse assembly. Mechanistic details are explained in the text. CASK, calcium/calmodulin-dependent serine protein kinase; Cb, Collybistin; $GABA_AR$, $GABA_A$ receptor; MINT, Munc18 interacting protein; NL, Neuroligin; NX, Neurexin; PDZ, PSD-95/disk-large/zona-occludens-1 domain; PTP, protein tyrosine phosphatase; S-SCAM, synaptic scaffolding molecule; Slitrk, Slit- and Trk-like; VIAAT, vesicular inhibitory amino acid transporter; X, unidentified protein potentially interacting with NX extracellularly and transducing the synaptogenic signal intracellularly. Adapted from Brose (2013).

et al., 2011) and posttranslational modifications, such as phosphorylation (Fritschy and Panzanelli, 2014). The different $GABA_AR$s are expressed differentially in development in different brain regions and are located at different types of synaptic and extrasynaptic sites (Luscher et al., 2011). At synapses, receptors composed of one γ, two α and two β subunits prevail. Furthermore, different subunits of the same subclass have different localisations: for example, α1 subunits are mostly incorporated into receptor complexes that localise at dendritic and somatic synapses, while α2 subunits are predominantly found in receptor complexes at the axon initial segment (Nusser et al., 1996) and α4-6 subunit-containing receptors are mostly localised extrasynaptically (Luscher et al., 2011). The γ2 subunit also regulates the subcellular localisation of the entire receptor complex, since it is necessary for postsynaptic $GABA_AR$ clustering

despite being dispensable for the transport of $GABA_A$Rs to the plasma membrane (Essrich et al., 1998). The molecular composition of the receptor complexes does not only affect their subcellular localisation, but also their pharmacological and biophysical properties and hence has important consequences for the properties of inhibitory neurotransmission (Luscher et al., 2011). Clinically most important is the differential regulation of different $GABA_A$Rs by benzodiazepines, which are used for anxiolytic, sedative, anti-convulsant and muscle relaxant treatments (Fritschy and Panzanelli, 2014).

Since GABA saturates its synaptic receptors when released by the presynapse, the fidelity of transmission depends directly on the number of postsynaptic $GABA_A$Rs (Luscher et al., 2011). Hence, even small changes in $GABA_A$R expression lead to behavioural consequences (Crestani et al., 1999) and mechanisms affecting the trafficking of $GABA_A$Rs to synapses have direct physiological effects. Understanding the molecular mechanisms that regulate $GABA_A$R clustering at inhibitory synapses is therefore crucial to understand the dynamics of GABAergic neurotransmission.

GlyRs are ionotropic neurotransmitter receptors for glycine and are predominantly mediating inhibitory synaptic transmission in the spinal cord, the brain stem and caudal regions of the brain (Dutertre et al., 2012). Since their synaptic localisation does not depend on the presence of Cb (Papadopoulos et al., 2007), they will not be further discussed here. However, since GlyRs are also anchored at the plasma membrane by the scaffold protein gephyrin, drawing parallels in the mechanism of clustering and trafficking is helpful to understand $GABA_A$R clustering in the forebrain.

$GABA_B$ receptors are metabotropic, G-protein coupled GABA receptors, which are predominantly located extrasynaptically and mediate slow, modulatory effects when stimulated (Luscher et al., 2011). Because of their predominant localisation at extrasynaptic sites as well as presynaptic release sites, they are not further discussed here in the context of inhibitory synaptic transmission.

1.2.2 Cell adhesion proteins

Synapse formation, maturation and maintenance require the coordinated development and adjustment of pre- and postsynaptic specialisations to allow faithful information transmission across the synaptic cleft. This is ensured by transsynaptic signalling through secreted molecules (Terauchi et al., 2010) and cell adhesion proteins. The latter are localised on both sides of the synapse and interact strongly transsynaptically so that pre- and postsynaptic compartments are biochemically copurified even in the presence of detergent (Fiszer and Robertis, 1967).

1.2.2.1 Neurexin and neuroligin

The best studied, synapse-specific cell adhesion proteins are the presynaptically localised neurexins and the postsynaptically localised neuroligins (NLs). They are thought to interact transsynaptically and thereby instruct the differentiation of pre- and postsynaptic specialisations (Krueger et al., 2012).

Neurexins are expressed from two different promoters of one of three genes and are extensively alternatively spliced, which led to the notion that they might be involved in a synapse-specific recognition code (Südhof, 2008). Their intracellular domain has a binding site for PSD-95/disk-large/zona-occludens-1 domain (PDZ) domains and it has been proposed that the interaction with PDZ domain-containing proteins that promote neurotransmitter release, such as calcium/calmodulin-dependent serine protein kinase (CASK) and Munc18 interacting protein (Mint), is essential for the synaptogenic effect of neurexins (Krueger et al., 2012). However, a recent study indicates that neurexins interact with other, yet unidentified proteins in the synaptic cleft through their extracellular Laminin A, neurexin, and sex hormone-binding protein (LNS) domains to induce presynaptic differentiation (Gokce and Südhof, 2013).

NLs are a family of postsynaptically localised cell adhesion proteins with four different isoforms in rodents (NL1-4) and five in humans. They have an extracellular catalytically inactive acetylcholinesterase (AChE)-homology domain, which mediates dimerisation and the interaction with neurexins, a transmembrane domain, and a variable intracellular domain, which interacts with different proteins in the postsynaptic density (Südhof, 2008). All NLs bind to PDZ domain-containing proteins, which organize excitatory postsynapses, and gephyrin, the major scaffold protein at inhibitory postsynapses (Poulopoulos et al., 2009). Despite this feature, NLs are partially localised in a synapse-specific manner. NL1 is found exclusively at excitatory synapses (Song et al., 1999), whereas NL3 and NL4 can be localised to both excitatory and inhibitory synapses, but the mechanisms controlling their recruitment to different synapse types is unknown (Budreck and Scheiffele, 2007; Baudouin et al., 2012; Hoon et al., 2011; Graf et al., 2004). Interestingly, NL function is further diversified through the differential dimerisation properties of the different isoforms (Poulopoulos et al., 2012).

NL2 is the only NL isoform exclusively localised at inhibitory synapses (Varoqueaux et al., 2004). When expressed in non-neuronal cells, NL2 induces presynaptic differentiation by interacting with neurexins on co-cultured neurons (Scheiffele et al., 2000). Conversely, neurexins cause clustering of NL2 and gephyrin (Graf et al., 2004). Overexpression of NL2 leads to an increase in the number of inhibitory synapses on dendritic shafts and increases inhibitory postsynaptic currents (IPSCs) in an activity-dependent manner (Chubykin et al., 2007). Animals overexpressing NL2 show several different

behavioural abnormalities such as impaired social interactions (Hines et al., 2008). Conversely, genetic deletion of NL2 reduces IPSC amplitudes in the neocortex (Chubykin et al., 2007). In the hippocampus of NL2 knockout (KO) animals, a specific decrease in GABAergic transmission and a loss of gephyrin and $GABA_AR\gamma2$ immunoreactivity was found at perisomatic but not dendritic sites on CA1 pyramidal neurons in the *Stratum pyramidale* (Poulopoulos et al., 2009). On a network level, loss of NL2 results in an increase in granule cell activity in the dentate gyrus of the hippocampus (Jedlicka et al., 2011). Interestingly, in the somatosensory cortex, loss of NL2 differentially affects synapses of different inhibitory interneuron subtypes onto excitatory neurons: whereas IPSCs evoked by the stimulation of somatostatin-positive neurons were not changed in the absence of NL2, cell-to-cell IPSC amplitudes evoked from fast-spiking, parvalbumin-positive neurons were decreased (Gibson et al., 2009). Furthermore, loss of NL2 function may be partially compensated by NL4 as indicated by an increase in NL4 immunoreactivity in the retina of NL2 KO mice (Hoon et al., 2011).

In summary, the conserved function of the neurexin-NL interaction is to couple the organisation of inhibitory and excitatory specialisations (Missler et al., 2012). Deletion of NL1-3 (Varoqueaux et al., 2006) or all α-neurexins (Missler et al., 2003) significantly hampers synaptic transmission leading to perinatal death, but does not interfere with synaptogenesis. In line with their essential function in organising synapses, mutations in the genes encoding NL2-4 have been identified in patients with different cognitive disorders. Specifically, malfunctional NL2 has been reported in a schizophrenia patient (Sun et al., 2011), and many mutations in the genes encoding NL3 and NL4 occur in ASD patients (Südhof, 2008). A NL4 mutation has also been reported in a patient with mental retardation (Laumonnier et al., 2004).

Apart from the transsynaptic interaction with NLs, neurexins also bind other postsynaptic proteins. Furthermore, neurexins can even be localised postsynaptically where they inhibit the function of NL1 and NL2 in *cis* (Taniguchi et al., 2007; Lee et al., 2013). At excitatory synapses, presynaptic neurexins interact with a plethora of other proteins, such as leucine-rich repeat transmembrane neuronal proteins (LRRTMs) (Linhoff et al., 2009; Krueger et al., 2012), whereas at inhibitory synapses, the only other interactions known so far are with $GABA_AR$ and dystroglycan (Südhof, 2008). The direct interaction with $GABA_AR$ inhibits the maturation of inhibitory synapses (Zhang et al., 2010).

1.2.2.2 Dystroglycan

The transmembrane protein dystroglycan is expressed ubiquitously and processed to form a dimer of α- and β-dystroglycan. In most tissues, the extracellular domains bind

to extracellular matrix proteins, but since these are absent from the brain, they interact with neurexins instead (Sugita et al., 2001). The intracellular domains interact with dystrophin, which in turn interacts with the actin cytoskeleton. Dystroglycan has been found to be localised at approximately a third of all inhibitory synapses in mature cultured hippocampal neurons (Levi et al., 2002). However, loss of dystroglycan does not affect gephyrin and $GABA_AR$ clustering (Levi et al., 2002). Dystroglycan is required to cluster dystrophin (Levi et al., 2002), which partially colocalises with $GABA_ARs$ and whose loss causes $GABA_AR$ mislocalisation without affecting gephyrin localisation (Knuesel et al., 1999). Together, these findings indicate that the dystrophin-associated glycoprotein complex (DGC) contributes to the maturation of a subset of GABAergic synapses but acts independently of $GABA_AR$ clustering by gephyrin.

1.2.2.3 Slitrk3 and protein tyrosine phosphatase

Slitrk3 is one of six members of a family of transmembrane proteins that are characterized by an intracellular homology to Trk family proteins and extracellular leucine-rich repeat domains. Slitrk3 has recently been shown to be specifically localised at inhibitory postsynapses and to induce inhibitory presynaptic differentiation through interaction with protein tyrosine phosphatase (PTP)-δ (Takahashi et al., 2012; Yim et al., 2013). Genetic ablation of Slitrk3 leads to a decrease in inhibitory synaptic transmission and a loss of inhibitory presynapses in specific lamina of the CA1 area of the hippocampus (Takahashi et al., 2012).

1.2.2.4 IgSF9b

Woo and colleagues have identified the first homophilic cell adhesion molecules at inhibitory synapses: the immunoglobulin superfamily protein IgSF9b (Woo et al., 2013), which has been associated with a depressive disorder (Shyn et al., 2011). The authors show that while IgSF9b cannot trigger synapse formation and is expressed at later stages in development than NL2, it promotes the development of inhibitory synapses on inhibitory interneurons. Conversely, knockdown of IgSF9b reduces synaptic gephyrin clusters and diminishes inhibitory synaptic transmission (Woo et al., 2013). The localisation of IgSF9b and gephyrin was examined by super-resolution imaging and found to be only minimally overlapping (Woo et al., 2013). This may be due to the molecular spacer that is formed by the scaffold protein S-SCAM, which interacts with both IgSF9b and NL2 and thus links these two cell adhesion systems.

1.2.2.5 Cooperation between different cell adhesion systems

So far, five different synaptic cell adhesion molecules are known to act specifically at inhibitory synapses: NL2, NL4, dystroglycan, Slitrk3 and IgSF9b. Their mode of action may be either distinct, partially overlapping or cooperative. For example, NL2 and IgSF9b have been shown to coexist at the same synapses in different sub-synaptic domains (Woo et al., 2013), and genetic deletion of either Slitrk3 or NL2 causes a loss of inhibitory synapses in the *Stratum pyramidale* of the CA1 region of the hippocampus, indicating that both may be required for synapse formation in this area (Takahashi and Craig, 2013).

In addition to these specific positive regulators of inhibitory synaptic differentiation, Lee and colleagues have identified MAM domain-containing GPI anchor proteins (MDGAs), members of the Ig superfamily of cell adhesion proteins, as the first negative regulator of inhibitory synapse formation (Lee et al., 2013). MDGAs interact with NL2 in *cis* and thus prevent binding of β-neurexin to NL2 and consequently reduce inhibitory synapse number and inhibitory synaptic transmission (Lee et al., 2013). As with many of the synaptic adhesion molecules, the relevance of MDGA function for normal brain function is illustrated by the different psychiatric disorders that are associated with defects in genes encoding MDGAs (Bucan et al., 2009; Li et al., 2011).

1.2.3 The scaffold: Gephyrin and S-SCAM

Neurotransmitter receptors are confined to the postsynaptic compartment through their interaction with a proteinaceous scaffold that interacts with both cell adhesion proteins and cytoskeletal elements. The scaffold is essential for providing stability to the synapse.

1.2.3.1 Gephyrin

Gephyrin is the most prominent scaffold protein of the inhibitory postsynaptic density (Luscher et al., 2011). Originally identified through its interaction with GlyR, it also associates with $GABA_A$Rs by binding the α1-3 and β2-3 subunits, albeit with a much lower affinity (Tretter et al., 2008; Saiepour et al., 2010; Tretter et al., 2011; Maric et al., 2011; Kowalczyk et al., 2013). At the same time, gephyrin interacts with cytoskeletal proteins, such as Kinesin superfamily protein 5 (KIF5) and mammalian enabled / vasodilator stimulated phosphoprotein (Mena/Vasp), and with synaptic cell adhesion proteins such as NL2 (Luscher et al., 2011). Thereby gephyrin links neurotransmitter receptors to the subsynaptic cytoskeleton, confining their localisation to synaptic sites. Gephyrin function is further regulated by proteins such as Cb (see 1.2.4) and heat

shock protein 70 (Hsc70) (Machado et al., 2011).

The central role of gephyrin in inhibitory synaptic transmission has been demonstrated by the decrease of α2- and γ2-subunit containing $GABA_A$Rs at inhibitory postsynapses in gephyrin KO mice or when gephyrin expression is reduced through RNAi (Kneussel et al., 1999; Levi et al., 2004; Essrich et al., 1998). Conversely, the loss of different $GABA_A$R subunits, such as the γ2-subunit, also results in the loss of gephyrin from synaptic sites, so there seems to be an interdependence between gephyrin and $GABA_A$R clustering (Essrich et al., 1998).

Several different deletions and a point mutation of the human gene encoding gephyrin, GPHN, as well as irregular splicing of gephyrin mRNA, have been identified in patients diagnosed with epileptic seizures (Förstera et al., 2010; Lionel et al., 2013), autism spectrum disorders (Lionel et al., 2013; Prasad et al., 2012), schizophrenia (Lionel et al., 2013) and hyperekplexia (Rees et al., 2003). Apart from this synaptic function, gephyrin is also essential for molybdenum-cofactor (Moco) biosynthesis in non-neuronal tissues (Feng et al., 1998) and homozygous genetic defects in GPHN lead to pathologies associated with Moco deficiency (Lionel et al., 2013).

Gephyrin is highly conserved in vertebrates. Tissue-specific alternative splicing generates proteins with different subcellular localisations (Nawrotzki et al., 2012) and different posttranslational modifications may further provide functional diversity (Tyagarajan and Fritschy, 2014). The 93 kDa protein consists of three domains, an amino-terminal G-domain, which resembles the bacterial MogA protein, a central linker domain, and a carboxy-terminal E-domain, which resembles the bacterial MoeA domain (Luscher et al., 2011). MogA and MoeA are involved in Moco biosynthesis in bacteria. In isolation, the G-domain dimerizes and the E-domain trimerizes. These oligomerisation properties are essential for synaptic clustering (Saiyed et al., 2007) and have led to the suggestion that gephyrin may form a hexagonal scaffold underneath the plasma membrane. However, a structural analysis of holo-gephyrin through atomic force microscopy and small-angle X-ray scattering indicates that E-domain dimerisation is prevented in full-length gephyrin (Sander et al., 2013), calling this model into question. Instead, Sander et al. (2013) found that gephyrin exists in different compact and extended states, which depend on the flexible linker region. Using different single-molecule based imaging techniques, Specht et al. (2013) have recently gained a better understanding of the three-dimensional organization of gephyrin at inhibitory synapses. They found that in agreement with previous observations, gephyrin forms a two-dimensional lattice at a constant distance underneath the synaptic plasma membrane. Different packing densities of gephyrin at different synapses were observed (Specht et al., 2013), which may be explained by the differences in conformational

states described by Sander et al. (2013).

1.2.3.2 S-SCAM

Several lines of evidence indicate that scaffolding proteins other than gephyrin must exist since genetic deletion of gephyrin does not lead to a complete loss of $GABA_ARs$ and inhibitory neurotransmission in hippocampal neurons (Levi et al., 2004). S-SCAM (also called membrane-associated guanylate kinase inverted-2 or atrophin interacting protein-1) is a large protein (141 kDa) that can have a scaffold function at inhibitory and excitatory postsynapses (Hirao et al., 1998; Sumita et al., 2007). The domain architecture is similar to that of the main scaffold protein at excitatory synapses, PSD-95: S-SCAM consists of five or six PDZ domains, one guanylate kinase-like domain, and two WW domains (Hirao et al., 1998). At inhibitory synapses S-SCAM interacts with the cell adhesion proteins β-dystroglycan, NL2 and IgSF9b (Sumita et al., 2007; Woo et al., 2013). Since the binding sites are non-overlapping – it binds to β-dystroglycan through its WW domains, to NL2 through its WW domains and the second PDZ domain, and to IgSF9b through its PDZ domains 4 and 5 (Woo et al., 2013; Sumita et al., 2007)– S-SCAM can act as a bridge between these different cell adhesion complexes. S-SCAM also interacts with the intracellular signalling protein SynARFGEF (1.2.5), which links it to the actin cytoskeleton (Fukaya et al., 2011).

Mutations in the gene encoding S-SCAM, MAGI2, have been identified in patients diagnosed with epilepsy (Marshall et al., 2008) and schizophrenia (Karlsson et al., 2012). Since S-SCAM is localised at both excitatory and inhibitory synapses, a direct effect of the mutations on inhibitory synaptic transmission cannot be inferred. Instead, it underlines the notion that many of these psychiatric disorders are due to an imbalance of excitation and inhibition.

1.2.4 Collybistin – an intracellular signalling protein

Cb was first identified in 1997 in ascidian embryos and, due to its polarised localisation, called posterior end mark 2 (PEM-2) (Satou and Satoh, 1997). Two years later, the human homolog, hPEM-2 was found on the X chromosome through a homology search aimed at identifying further Dbl family GEFs for small Rho GTPases (Reid et al., 1999). The protein was characterized to have an N-terminal src homology 3 (SH3) domain and a tandem of a Dbl homology (DH) and a pleckstrin homology (PH) domain, which is characteristic for all Dbl-family GEFs (Figure 4). Cdc42 is specifically activated by hPEM-2 in cells, as shown biochemically and morphologically (Reid et al., 1999).

In 2000, Kins and colleagues showed for the first time a link between Cb function

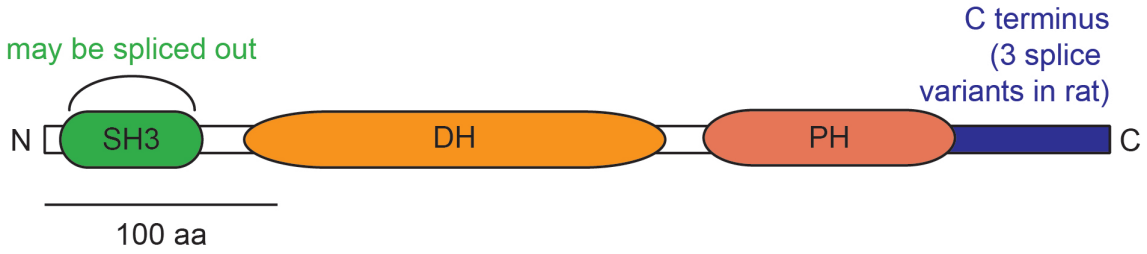

Figure 4: Domains of Cb. N-terminal src homology 3 (SH3) domain, Dbl homology (DH) domain and pleckstrin homology (PH) domain.

and synaptic gephyrin clustering (Kins et al., 2000). Through a yeast two-hybrid screen aimed at detecting new gephyrin binding proteins, the authors identified two different transcripts of the same gene in a cDNA library from a newborn rat brain (Kins et al., 2000). The protein sequence showed 93% identity with the hPEM-2 sequence. Due to the suspected function as a GEF, the authors called these proteins "collybistin", from the ancient Greek word for "exchange" (Kins et al., 2000). The two thus identified splice variants differ in their C-termini and only one contains an SH3 domain (Kins et al., 2000). Intriguingly, coexpression of CbI, the longer variant containing the SH3 domain, with gephyrin in non-neuronal cells resulted in the accumulation of both proteins in cytoplasmic aggregates, which had been previously observed for gephyrin, while the coexpression of the shorter variant led to the clustering of both proteins at the plasma membrane in a significant proportion of cells (Kins et al., 2000). Upon co-expression of the GlyR β-subunit, which binds gephyrin, all three components localised at submembrane microclusters (Kins et al., 2000). This seminal paper was the first to show the potential role of Cb in regulating gephyrin and hence neurotransmitter receptor targeting to the plasma membrane.

1.2.4.1 Cb expression

The expression, alternative splicing and localisation of Cb has been analysed in many studies. Different lines of evidence indicate that Cb is mainly expressed in the brain, with only small amounts being detected in the heart and skeletal muscle (Kins et al., 2000; Reid et al., 1999). By using *in situ* hybridisation, it has been shown that Cb mRNA is expressed in postmitotic neurons in different regions of the central nervous system at the time of neuronal differentiation and synaptogenesis (Kneussel et al., 2001). Subcellularly, recombinantly expressed and endogenous Cb is present both at inhibitory synaptic sites and in the cytoplasm (Harvey et al., 2004; Chiou et al., 2011). The selective localisation of Cb at different types of synapses was studied using an antibody against the C-terminal domain of CbIII, the longest splice variant (see

below) (Patrizi et al., 2012). The authors describe that CbIII colocalises with 40-80% of gephyrin-positive synapses in different regions of the brain, but also localises at gephyrin-deficient synapses in the cerebellum that are α-dystroglycan-positive (Patrizi et al., 2012). Moreover, Cb colocalisation with all synaptic $GABA_AR$ α subunits (α1-3) and GlyR was observed in the spinal cord (Patrizi et al., 2012).

Reverse transcription polymerase chain reaction (RT-PCR) experiments from brain and spinal cord samples showed that apart from the previously identified CbI and II isoforms (Kins et al., 2000), there is also a longer isoform, called CbIII with a C-terminus that is almost identical to the one of hPEM-2 (Harvey et al., 2004). Of these isoforms, the mRNAs of SH3(+)CbII and CbIII are the most abundant in the adult rat brain (Harvey et al., 2004). In HEK293 cells, coexpression of SH3(+)CbII does not mediate the formation of submembrane gephyrin microclusters, like CbI (Harvey et al., 2004). Expression of CbII and SH3(+)CbII in hippocampal neurons has differential effects on inhibitory postsynapses: CbII expression significantly increases gephyrin and $GABA_AR$ γ2-subunit cluster size as well as the amplitudes of miniature IPSCs (mIPSCs), while SH3(+)CbII expression increases gephyrin and $GABA_AR$ γ2-subunit cluster density in dendrites (Chiou et al., 2011). Interestingly, in both cases, this is not accompanied by an increase in NL2 clusters (Chiou et al., 2011). Similar results were obtained in a different study, with the interesting observation that CbII induced mostly synaptic clusters as judged by the colocalisation with the α2-subunit as compared to SH3(+)CbII, which induced extrasynaptic gephyrin clusters (Tyagarajan et al., 2011). According to a recent study, in which all different Cb isoforms could rescue the effects of Cb knockdown on gephyrin clustering and inhibitory synaptic transmission, functional differences between C-termini and even the presence or absence of the SH3 domain are less pronounced than anticipated from overexpression studies (Körber et al., 2012).

1.2.4.2 Cb loss affects inhibitory synapse formation

The relevance of Cb in inhibitory synaptogenesis was demonstrated in Cb KO mice, which displayed a specific loss of synaptic gephyrin and γ2-subunit-containing $GABA_ARs$ in the hippocampus, the cerebellum and the amygdala, together with an accumulation of gephyrin in cytoplasmic aggregates (Papadopoulos et al., 2007). This matches the high expression of Cb observed in these areas (Patrizi et al., 2012). Using electrophysiology, it was shown that most prominently dendritic inhibition and synaptic plasticity in the hippocampus was reduced (Papadopoulos et al., 2007). On a behavioural level, mice displayed increased anxiety, as expected since the amygdala and the septohippocampal network control this behaviour, as well as a deficiency in hippocampus-dependent memory formation (Papadopoulos et al., 2007). Inactivation

of Cb at various time points in development showed that in the hippocampus Cb is essential for the initial formation of synapses in the first weeks of postnatal life, as well as for the maintenance of synapses in adulthood (Papadopoulos et al., 2008). Electrophysiological analysis of the network activity in the dentate gyrus of anesthetized Cb KO mice revealed that loss of Cb increases network excitability and plasticity through a decrease of predominantly dendritic inhibitory inputs (Jedlicka et al., 2009). Surprisingly, despite its localisation in the spinal cord and its ability to cause GlyR clustering together with gephyrin in non-neuronal cells, Cb KO did not affect gephyrin or GlyR clustering in the spinal cord and KO mice did not show neuromotor deficits (Papadopoulos et al., 2007). This indicates that other intracellular signalling proteins may compensate for the function of Cb at these synapses.

1.2.4.3 Molecular mechanisms of Cb-dependent gephyrin clustering

Despite several studies aiming at deciphering the molecular mechanism by which Cb potentially recruits gephyrin to the plasma membrane in the brain, a detailed mechanistic understanding is still lacking.

A current model postulates that Cb can exist in different conformations. First evidence for this was provided from crystallographic data, where CbII lacking the SH3 domain was found to exist in two states due to a 37° movement of the PH domain with respect to the DH domain (Xiang et al., 2006). Interestingly, the more compacted conformation was suggested to be thermodynamically favoured (Xiang et al., 2006). Due to the strong similarities with its closest homolog, APC-stimulated guanine nucleotide exchange factor (ASEF, also called ARHGEF4) (Mitin et al., 2007), an activation model for the more compacted Cb in the presence of the SH3 domain was suggested (Poulopoulos et al., 2009). Since SH3(+)Cb isoforms cannot mediate gephyrin clustering in non-neuronal cells (Kins et al., 2000; Harvey et al., 2004) and most isoforms expressed in the brain contain an SH3 domain (Harvey et al., 2004), mechanisms must exist to relieve this autoinhibition.

The first protein identified to relieve the autoinhibition of the SH3 domain was NL2 (Poulopoulos et al., 2009). Later the same function was shown also for NL4 (Hoon et al., 2011) and potentially the α2-subunit-containing $GABA_A$Rs (Saiepour et al., 2010). Interestingly, a mutation in the SH3 domain (G55A) of a patient suffering from hyperekplexia and seizures was identified and shown to reduce the number of dendritic gephyrin clustering and abolish binding to both NL2 and $GABA_A$R α2 (Harvey et al., 2004; Poulopoulos et al., 2009; Saiepour et al., 2010). However, the loss of binding may be due to misfolding of the SH3 domain induced by this mutation (Harvey et al., 2004).

The DH domain is the catalytic domain of a GEF. A point mutation in the DH domain (R290H) of hPEM-2 adjacent to the site of Cdc42 binding was identified in patients diagnosed with seizures and mental retardation (Lemke et al., 2012; Xiang et al., 2006). Owing to this observation and the finding that Cdc42 is the only small GTPase activated by Cb (Jaiswal et al., 2013), one hypothesis is that the GEF activity of Cb towards Cdc42 is crucial for gephyrin clustering. However, several lines of evidence contradict this hypothesis.

First, a DH domain mutant that can no longer activate Cdc42, CbII NE232-233AA, can still trigger recombinant or endogenous gephyrin clustering in HEK293 cells or wild-type dissociated hippocampal neurons, respectively (Reddy-Alla et al., 2010). Second, coexpression of a dominant negative mutant of Cdc42 or the Cdc42 binding domain of an effector of Cdc42 (see 1.3.1) did also not prevent CbII-mediated gephyrin microcluster formation in non-neuronal cells, indicating that active Cdc42 is not required for this process (Reddy-Alla et al., 2010). Third, even the conditional inactivation of Cdc42 in the forebrain did not result in any loss of gephyrin or $GABA_AR$ clustering as observed in the hippocampus of Cb KO mice (Reddy-Alla et al., 2010). Fourth, both CbII and SH3(+)CbII can mediate the Cdc42 nucleotide exchange in HEK293 cells, so their differential ability to induce gephyrin clustering cannot be explained by the GEF activity towards Cdc42 (Tyagarajan et al., 2011).

However, the binding site of gephyrin was suggested to be located in the linker region between the SH3 domain and the DH domain (Grosskreutz et al., 2001), even though this result may also be explained by domain misfolding in the mutants generated to identify the binding site (Xiang et al., 2006). Therefore the DH domain mutation in the patients diagnosed with seizures and mental retardation (Lemke et al., 2012; Xiang et al., 2006) and the loss of synaptic gephyrin clusters in neurons transfected with Cb constructs lacking the DH domain (Harvey et al., 2004; Tyagarajan et al., 2011) may be due to a lack of gephyrin binding or interaction with small GTPases (Xiang et al., 2006).

The PH domain generally regulates the membrane attachment of Dbl family GEFs. Expression of a Cb mutant lacking the PH domain led to a loss of membrane targeting and the accumulation of both gephyrin and Cb in cytoplasmic aggregates in HEK293 cells as well as to a loss of dendritic gephyrin clusters in neurons (Harvey et al., 2004). The loss of membrane targeting may be explained by the loss of binding of two arginine residues (RR303-304) in the β3-β4 loop of the PH domain to phosphatidylinositol 3-phosphate (PI(3)P) (Kalscheuer et al., 2009; Reddy-Alla et al., 2010). Since PI(3)P is constitutively present at endosomes and its generation is only induced at the plasma membrane upon stimulation, for example by insulin (Falasca and Maffucci, 2009), it

is not clear in which subcellular compartment the interaction with PI(3)P occurs (Papadopoulos and Soykan, 2011). The relevance of PH domain function is also illustrated by the observation that several patients with mutations that render the Cb PH domain non-functional suffer from various symptoms associated with epilepsy, anxiety, aggression and mental retardation (Kalscheuer et al., 2009; de Ligt et al., 2012).

1.2.4.4 Potential new mechanisms of Cb regulation

Several studies have shown interactions of Cb with various other proteins, but, how they might impact on Cb-dependent gephyrin clustering is unclear so far. First, Cb has been described to bind to the E3 ubiquitin ligase Smurf1 (Yamaguchi et al., 2008) and this may be important for regulating proteasomal degradation and turnover of Cb. Second, Cb and gephyrin have been shown to bind to the translation initiation factor eIF3H and this may have a potential link to mTOR signalling, which is important in inhibitory synapse formation (Sertie et al., 2010; Wuchter et al., 2012). Third, hPEM-2 has been identified as a downstream effector of heterotrimeric G-protein signalling, which may be relevant for the regulation of GABAergic synapses (Nagae et al., 2011; Saba et al., 2011). Finally, Cb has been found to be essential for the phosphorylation of gephyrin through cyclin-dependent kinases and may thus have an additional important regulatory effect on gephyrin function (Kuhse et al., 2012). Further work is needed to obtain a mechanistic understanding of the potential contribution of these protein interactions to gephyrin clustering.

1.2.4.5 Cb malfunction leads to various psychiatric disorders

Genetic defects in the gene encoding Collybistin, ARHGEF9, have been identified in patients with different psychiatric disorders such as epilepsy, X-linked mental retardation, aggressive behaviour, anxiety and hyperekplexia (Harvey et al., 2004; Marco et al., 2009; Kalscheuer et al., 2009; Lesca et al., 2011; Shimojima et al., 2011; Lemke et al., 2012; de Ligt et al., 2012). Some point mutations are particularly interesting with regard to understanding Cb function as outlined above (1.2.4.3).

1.2.5 Other intracellular signalling proteins

Since Cb KO does not lead to a complete loss of GABAergic synapses in the brain (1.2.4.2), other regulators of gephyrin clustering at GABAergic synapses must exist. Recently, a different GEF was identified to be expressed in many brain areas and localised in the somatodendritic compartment at gephyrin-positive synapses: SynARFGEF (Fukaya et al., 2011). SynARFGEF is a GEF for the small GTPase Arf6, which

regulates trafficking between the plasma membrane and endosomes and remodeling of the actin cytoskeleton. Moreover, SynARFGEF binds to dystrophin, which is part of the DGC complex, and the scaffolding protein S-SCAM (Fukaya et al., 2011). It may therefore be an alternative GEF to Cb in the regulation of inhibitory postsynaptic assembly.

1.2.6 The regulation of the actin cytoskeleton is important for inhibitory synapse formation and function

Synaptic function depends on dynamic changes in the composition of the PSD since the strength of synaptic transmission needs to be adjusted homeostatically in neuronal networks. Synaptic strength is influenced by many factors; on the postsynaptic side, the number and motility of receptors is an important determinant of the efficacy of synaptic transmission (Choquet and Triller, 2013). Inversely, activity also controls the diffusion dynamics of receptors, such as $GABA_A$Rs (Bannai et al., 2009). The receptor motility is influenced through variable interactions between the receptor and the subsynaptic cytoskeleton, which is mediated by synaptic scaffold proteins. In line with this concept, synaptic gephyrin domains have been shown to be highly motile, altering their shapes and positions within minutes (Specht et al., 2013). In order to understand the dynamic remodeling of synaptic strength, it is therefore of prime importance to identify modulators of the interaction between the scaffold proteins and the subsynaptic cytoskeleton.

Both the micotubule network and the actin cytoskeleton play an important role at inhibitory postsynapses by regulating the lateral diffusion of gephyrin and neurotransmitter receptors (Kirsch and Betz, 1995; Charrier et al., 2006). The importance of the actin cytoskeleton has been demonstrated initially by Kirsch and Betz, who showed that actin depolymerising drugs induce the formation of smaller gephyrin clusters in spinal cord neurons (Kirsch and Betz, 1995). The same was observed in immature hippocampal neurons, but not in mature hippocampal neurons (Bausen et al., 2006; Allison et al., 2000). The recycling of $GABA_A$Rs also depends on the actin cytoskeleton directly at the plasma membrane (Heisler et al., 2011). Interestingly, when HEK293 cells expressing recombinant gephyrin were treated with actin filament depolymerizing drugs, the formation of actin patches led to the redistribution of gephyrin to submembrane microclusters (Bausen et al., 2006). These effects may be due to the interaction of gephyrin with various proteins that regulate the actin cytoskelton. Gephyrin interacts with polymerized tubulin (Kirsch et al., 1991), the actin-binding protein profilin (Mammoto et al., 1998), and the actin-binding protein Mena/Vasp (Giesemann et al., 2003).

Since the actin cytoskeleton is regulated by small GTPases, it is not surprising that there is evidence for the involvement of these in inhibitory synapse formation. One example is the small GTPase Arf6, which is activated by the SynArfGEF, a protein specifically localised at inhibitory synapses (1.2.5) (Fukaya et al., 2011). Furthermore, a GTPase-activating protein (GAP) for the small GTPase Rac, Srgap2, has recently been identified to regulate gephyrin clustering (Okada et al., 2011). As described previously (1.2.4.3), Cb is essential for gephyrin clustering in some areas of the brain and also a GEF for Cdc42, a Rho family GTPase that regulates the actin cytoskeleton (1.3.2.1). Furthermore, Cdc42 might even bind gephyrin in the absence of Cb (Tyagarajan et al., 2011). Interestingly, genetic abnormalities in genes encoding Rho GTPases or their regulators, which were previously mostly identified in cancer, have also recently been associated with autism spectrum disorders and intellectual disability (Najmabadi et al., 2011; Pinto et al., 2010).

1.3 Rho-like GTPases are molecular switches regulating the actin cytoskeleton

Small GTPases are small proteins (approximately 20-30 kDa) that classically function as binary molecular switches – they exist in an "ON" state when bound to guanosine-5'-triphosphate (GTP) and in an "OFF" state when bound to guanosine-5'-diphosphate (GDP). The binding of GDP or GTP changes their three-dimensional structure dramatically so that they can specifically interact with other proteins in one of the two states only. Consequently, the state of nucleotide binding is very important for their function and requires fine control mechanisms (Figure 5).

1.3.1 Regulation of small GTPases

In the GDP-bound state, small GTPases are very stable. The switch to the GTP-bound state occurs through the exchange of the GDP nucleotide for a GTP nucleotide via a transient unstable transition state. This requires the catalysis by an enzyme, the GEF, which binds the small GTPase in the GDP-bound state with low affinity and destabilizes the GDP-GTPase binding (Figure 5). Upon GDP dissociation, the GEF stabilizes the energetically unfavourable transition state through a high affinity binding. Since in the cytoplasm GTP is in excess compared to GDP, this likely leads to the binding of GTP to the small GTPase. The GTP-bound GTPase has a low affinity for the GEF, so it dissociates from the GEF and interacts with its effectors to regulate cell function (Cherfils and Chardin, 1999). Sometimes the GEF can additionally be

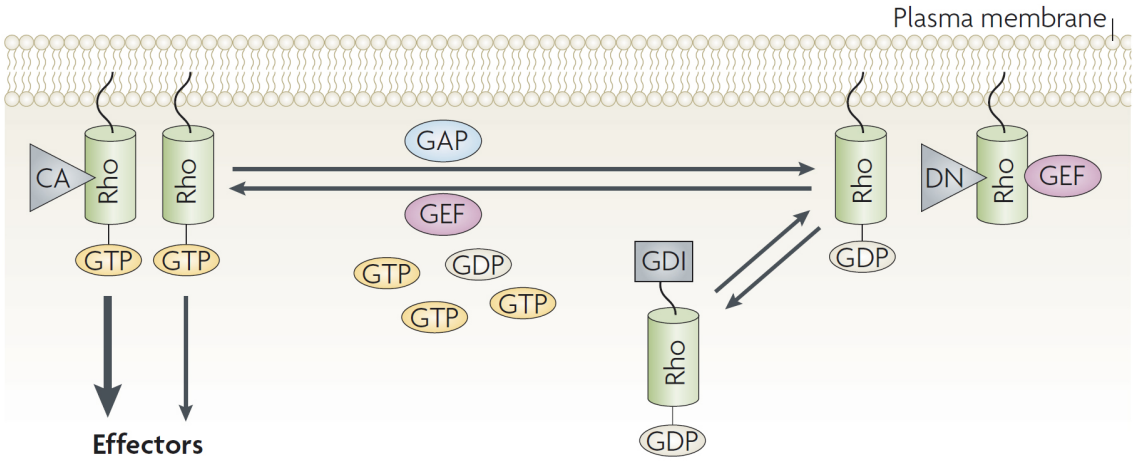

Figure 5: GTPase cycle. Regulation of small GTPases by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), guanine nucleotide dissociation inhibitors (GDI) and posttranslational prenylation, which anchor GTPases at membranes. In the GTP-bound state, GTPases interact specifically with effectors. A mutation that renders the protein GTP-bound is called constitutively active (CA), a mutation that renders is permanently GDP-bound is called dominant negative (DN). Guanosine-5'-triphosphate, GTP; guanosine-5'-diphosphate, GDP. Source: Heasman and Ridley (2008)

an effector and the GTPase can bind to the GEF on a different domain in a GTP-dependent manner (e.g. Cohen et al. (2007)).

The GTPase has an intrinsic enzymatic activity that hydrolyses the bond between the β- and γ-phosphate of the nucleotide. The activity of this intrinsic GTPase is, however, generally very low and can be enhanced by the interaction with GAPs (Figure 5).

Furthermore, small GTPase function is regulated by controlling the subcellular localisation since GTPases have unique functions at specific compartments (Liu et al., 2012). An important determinant of the intracellular distribution of GTPases is the C-terminal hypervariable region, which can contain diverse types of subcellular localisation signals. Most importantly, the C-terminus is generally prenylated irreversibly, leading to the localisation of the GTPase at a membrane. In the GDP-bound state, however, the binding of guanine nucleotide dissociation inhibitors (GDI) to the prenyl group can lead to a cytoplasmic localisation of the GTPase (Figure 5).

In order to study the cellular function of small GTPases, mutant forms of GTPases that are trapped in one of the two states are employed (Heasman and Ridley, 2008). Dominant negative (DN) mutations, trap GTPases in their GDP-bound state, where they can still interact with their GEFs but no exchange occurs (Figure 5). Constitutively active (CA) mutations, trap the GTPase in the GTP-bound state where they can constitutively bind to their effectors (Figure 5).

In addition to the classical regulation of GTPase function by GEFs, GAPs and GDIs, further levels of control through posttranslational modifications such as transglutamination, phosphorylation and AMPylation have also been described (Liu et al., 2012).

1.3.2 Rho family GTPases and cytoskeletal regulation

Small GTPases are divided into several families on the basis of sequence similarity. The Rho family is a member of the Ras-superfamily of GTPases and controls many cellular processes, the most well-known one being the regulation of the actin cytoskeleton. More recently, Rho GTPases have also been shown to regulate membrane and vesicle trafficking (Heasman and Ridley, 2008). There are 20 different Rho-family GTPases in higher eukaryotes that are highly conserved. Further diversity is created through alternative splicing in some cases (Figure 6). Twelve Rho-like GTPases are classically activated as described above (1.3.1), while the others are predominantly GTP-bound and regulated by other mechanisms, for example posttranslational modifications (Heasman and Ridley, 2008). The twelve classical Rho GTPases predominantly differ from each other in the N- and C-terminal extensions of their central GTPase domain and hence can exert different biological functions. Furthermore, there is a plethora of regulators of Rho GTPase function: there are more than 80 different GEFs, more than 60 different GAPs and three GDIs. The specificity of the different regulatory proteins for specific GTPases is variable and there is also an overlap of effectors between some Rho GTPases, of which more than 100 have been described (Hall, 2012). The regulatory network is thus highly complex and not fully understood so far.

The carboxy terminus of Rho GTPases is particularly important for their function since it determines their subcellular localisation, among other properties. Most Rho family GTPases terminate in a CaaX motif, where C is a cysteine, that is separated from the terminal amino acid (X), which dictates the identity of the prenyl group, by two alipathic amino acids (aa). The lipid modification through a thioether linkage can be either a farnesylation, when a 15-carbon lipid anchor is attached, or a geranyl-geranylation when a 20-carbon lipid anchor is attached. Prenylation occurs posttranslationally in the cytoplasm, followed by cleavage of the three terminal amino acids (aaX) at the ER by endoprotease Ras-converting enzyme 1 (Rce1) and carboxymethylation by isoprenylcysteine-carboxyl-methyltransferase (Icmt). A second membrane targeting motif in the C-terminus is required to allow a specific subcellular localisation. Reversible palmitoylation of further Cys residues through a thioester linkage may cause the localisation of the protein in lipid rafts and prevent the association with GDIs, while basic amino acids can interact with negatively charged membrane

lipid head groups (see (Ahearn et al., 2012) for a review).

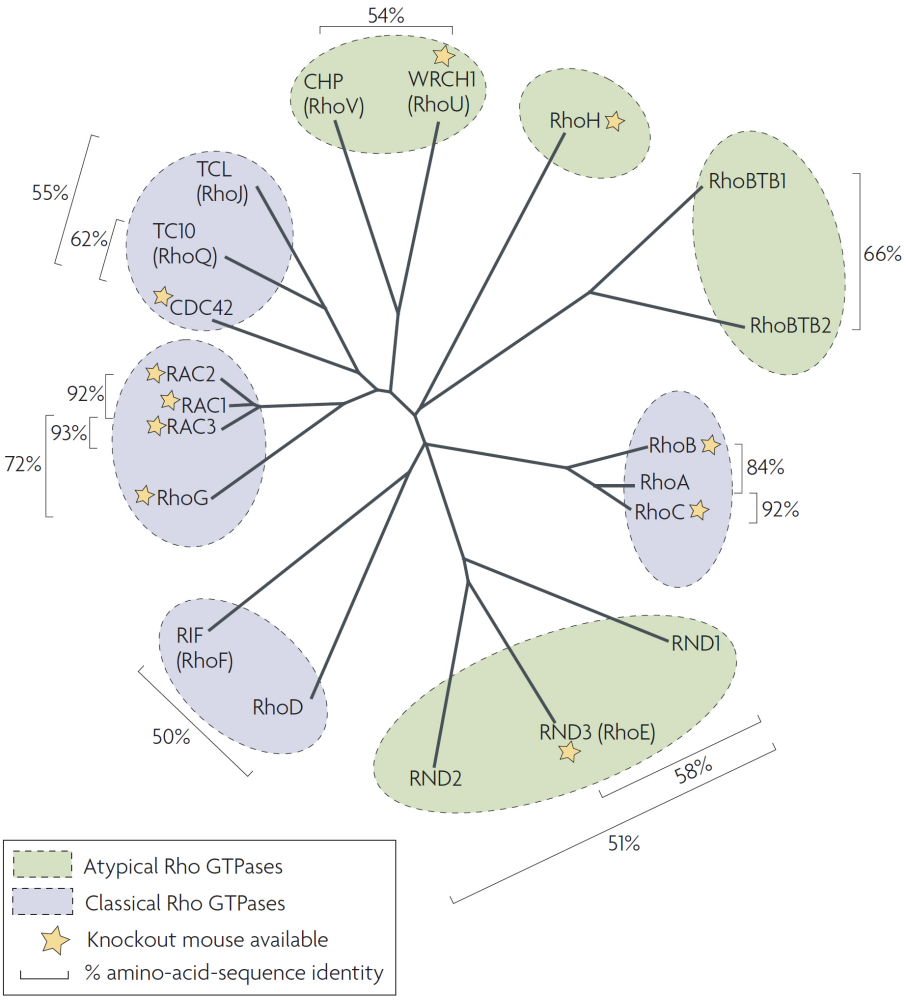

Figure 6: Rho-like GTPase family. Unrooted phylogenetic tree based on the alignment of 20 Rho GTPase proteins, which form eight subfamilies. A pairwise alignment of protein sequences was used to calculate the amino acid sequence identities as indicated. Classical Rho GTPases (blue) undergo GDP-GTP cycling, while atypical Rho GTPases (green) are constitutively GTP-bound. As indicated, knockout mice are only available for 10 of these GTPases. Adapted from (Heasman and Ridley, 2008)

1.3.2.1 Cdc42

Together with RhoA and Rac, Cdc42 is one of the most conserved Rho-family GTPases. Cdc42 is expressed ubiquitously and has been described to have various functions in different cell types, most of which are due to its ability to control the actin cytoskeleton. Cdc42 is efficient in inducing the formation of filopodia by stimulating the formation of bundles of filamentous actin through its interaction with proteins such as Wiskott-Aldrich syndrome protein (WASP), which in turn activates the actin-related

protein-2/3 (ARP2/3) complex, a nucleator of actin filament formation. This ability is harnessed in yeast cells to allow cellular polarisation prior to budding. In neurons, Cdc42 is particularly important for axon generation and likely also involves the inhibition of the actin filament severing protein cofilin. Furthermore, Cdc42 may also direct the subcellular targeting of recycling endosomes (Heasman and Ridley, 2008). Cdc42 has also been shown to regulate vesicle fusion through its interaction with Exo70, a component of the exocyst complex (Wu et al., 2010). Apart from the canonical isoform, a splice variant that differs in the last ten amino acids may be particularly important in the brain, where its palmitoylation state and hence subcellular localisation is regulated by neuronal activity (Kang et al., 2008) (Figure 7). Furthermore, a di-lysine motif in the C-terminus of Cdc42 has been shown to bind to the γCOP subunit of the coatomer protein complex COPI, which mediates vesicle transport in the Golgi apparatus, whereas a di-arginine motif binds specifically to phophatidylinositol 4,5-biphosphate (PI(4,5)P_2) and phophatidylinositol 3,5-biphosphate (PI(3,5)P_2) (Figure 7) (Johnson et al., 2012; Wu et al., 2000). Another recent study showed that an interaction of the positively charged Cdc42 C-terminus with negatively charged membrane lipids prevents binding to a GDI (Das et al., 2012).

1.3.2.2 TC10

The closest homolog of Cdc42 is TC10, which shares sequence (62% amino acid identity with Cdc42) and structural similarities with Cdc42 (Figures 6 and 7) (Neudauer et al., 1998; Hemsath et al., 2005).

Subcellularly, TC10 colocalises with filamentous actin and with lipid raft markers at the plasma membrane, and in vesicular structures it colocalises with markers of the endosomal system and the Golgi apparatus (Neudauer et al., 1998; Murphy et al., 1999; Michaelson et al., 2001; Watson et al., 2001, 2003). On a tissue level, TC10 is expressed predominantly in the heart and skeletal muscle and at low levels in other tissues (Neudauer et al., 1998; Murphy et al., 1999). In the brain, TC10 is most highly expressed in the CA1 region of the hippocampus, in the midbrain and brainstem, and the expression increases throughout embryonic development (Tanabe et al., 2000).

Due to the extensive structural similarities with Cdc42, the two proteins share some effectors and subserve similar biological functions. For example, TC10 also interacts with WASP, albeit with much lower affinity than Cdc42, and induces filopodia like Cdc42 (Neudauer et al., 1998; Murphy et al., 1999; Hemsath et al., 2005). The differences are, however, physiologically relevant, since TC10 does not rescue a cdc42-deficient yeast strain (Murphy et al., 2001).

The most prominent differences between Cdc42 and TC10 are in the N- and C-

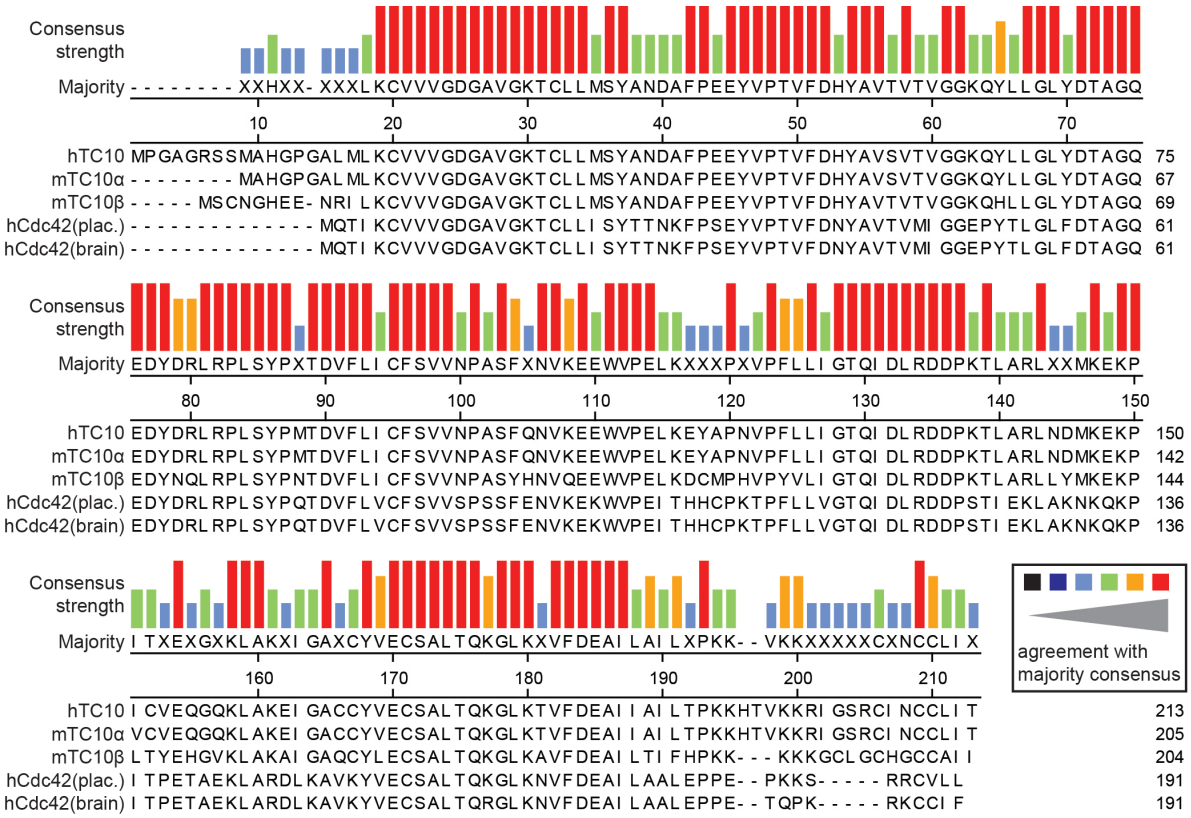

Figure 7: Alignment of TC10 and Cdc42 isoforms. Amino acid sequence alignment of human TC10, mouse TC10α and mouse TC10β, and human placental (plac.) and brain Cdc42 isoforms. The majority consensus sequence is shown (majority). On top a histogram of agreement between the majority consensus and each column of aligned residues is shown. As denoted, the strength of agreement is indicated by height and color, with red being strongest.

termini (Figure 7). The N-terminal extension of TC10, which is absent in Cdc42, is important for the disruption of cortical actin (Kanzaki et al., 2002; Chunqiu Hou and Pessin, 2003). The C-terminus of TC10 has very distinct features compared to Cdc42. While the CaaX Cys of Cdc42 is geranyl-geranylated, the one of TC10 is farnesylated (Roberts et al., 2008). However, these differences are unlikely to be functionally important (Michaelson et al., 2001; Roberts et al., 2008). Furthermore, there are two more Cys residues in the C-terminus of TC10 that can be palmitoylated and prevent the solubilisation of membrane-bound TC10 by RhoGDI, as well as assuring the localisation in lipid rafts (Michaelson et al., 2001; Murphy et al., 2001; Watson et al., 2003). The localisation of TC10 is further controlled by a polybasic stretch in the C-terminus that prevents sorting to lysosomes and can interact with negatively charged membrane lipids (Valero et al., 2010).

TC10 regulates membrane trafficking in different cellular contexts. The best understood role of TC10 is in the regulation of the exocytosis of vesicles containing the

glucose transporter GLUT4 in response to insulin stimulation in adipocytes (Chiang et al., 2001). Here, the interaction of TC10 with two different effector proteins, the exocyst complex subunit Exo70 and Cdc42 interacting protein 4 (CIP4), controls GLUT4 vesicle trafficking to the plasma membrane (Leto and Saltiel, 2012). In neurons, TC10 is involved in the regulation of neurite outgrowth and axon regeneration and its interaction with the exocyst complex has also been shown to be implicated in this process (Tanabe et al., 2000; Dupraz et al., 2009). Furthermore, trafficking of different other transmembrane proteins may also be regulated by TC10 through its interaction with the effector PIST (Neudauer et al., 2001; Wente et al., 2005; Cheng et al., 2005).

1.4 Aim of the study

The accumulation of inhibitory neurotransmitter receptors at postsynaptic sites in the mammalian brain depends mostly on the scaffold protein gephyrin. In specific areas of the forebrain, the GEF Cb is essential for clustering gephyrin at inhibitory postsynaptic sites. However, Cb is autoinhibited and needs to be activated by interaction with other proteins such as NL2. The phenotype of NL2 KO animals does, however, not mirror the phenotype of Cb KO animals. Therefore other proteins that can activate Cb must exist (Brose, 2013). So far, the molecular mechanisms through which Cb is activated and subsequently mediates gephyrin clustering are unclear. Since Cb is a GEF for the small GTPase Cdc42, a potential function of Cdc42 activation was suspected. However, conditional deletion of Cdc42 in the mouse forebrain did not affect gephyrin or $GABA_AR$ clusters at inhibitory synapses, showing that the interaction with Cdc42 is not essential for mediating gephyrin clustering. However, there is ample evidence for the involvement of the actin cytoskeleton in inhibitory synapse formation and actin cytoskeletal dynamics are regulated by small Rho family GTPases. The aim of the present study was therefore to analyze the importance of the interaction of Cb with small Rho family GTPases in mediating gephyrin clustering focusing on TC10, the closest homolog of Cdc42.

2

Materials and Methods

Materials and methods are largely taken from our recent publication (Mayer et al., 2013) and modified for better clarity and completeness. Materials and methods for data shown in the appendix can be found in the publication only (Mayer et al., 2013). Standard molecular biology techniques such as polymerase chain reaction (PCR) were performed as described (Sambrook and Russell, 2001), following recommendations by the suppliers of the reagents. All standard laboratory chemicals were obtained from established commercial suppliers.

2.1 Expression vectors

cDNA constructs were mostly available, see Mayer et al. (2013) for details. TC10-H-Ras and TC10-K-Ras cDNA was provided by Jeffrey E. Pessin (Albert Einstein College of Medicine, New York).

Six different TC10-specific miRNAs and a negative control miRNA for use with the BLOCK-iT Pol II miRNA RNAi Expression Vector Kit (Invitrogen) were purchased and cloned into pcDNA 6.2-GW/EmGFP according to instructions by the manufacturer (by Suneel Reddy-Alla). The miRNA with the best knockdown efficiency, miRNA 4D had the following sequence: ATTCCTTTAGCTCTGGTACCC.

Four TC10-specific shRNAs for the rat sequence and a negative control shRNA in a psi-H1 vector were obtained from Genecopoeia (RSH051709-CH1).

2.1.1 Mutagenesis of TC10 and Cdc42 plasmids

Oligonucleotide-directed mutagenesis was performed using the QuikChange mutagenesis kit (Stratagene) according to the manufacturers instructions. In brief, cloned Pfu AD (Agilent) and the associated buffer, dNTPs (Bioline), 100 ng DNA template, and

7 pmol of each primer in a 50 μL reaction were used. The following thermocycling protocol was followed: initial denaturation at 95°C for 3 min, followed by 18 cycles of 95°C 30 s, 55°C 1 min, 72°C 14 min, with a final elongation at 72°C for 16 min. Afterwards template DNA was digested using DpnI (NEB) for 1.5 h at 37°C and the result was checked by gel electrophoresis. PCR products were cloned in TOP10 chemocompetent cells (Invitrogen). Clones were grown in antibiotic-containing medium and plasmid DNA was purified by using a miniprep kit (Invitrogen). Sequences of oligonucleotide primers are indicated in tables 1, 2 and 3. Oligonucleotides were generated by an inhouse facility (AGCT lab) and sequences of mutagenized cDNAs were confirmed by DNA sequencing.

Table 1: Oligonucleotide primers used for TC10 mutagenesis (except C-terminus). direction, dir; forward, fw; reverse, rv. If the primer overlaps with the vector, the plasmid is given in brackets

cDNA	**mutation**	**dir.**	**sequence (5'-3')**
TC10 (pKH)	DeltaN	fw	GATTACGCTGGATCCAAGTGCGTGGTGGTC
TC10 (pKH)	DeltaN	rv	GACCACCACGCACTTGGATCCAGCGTAATC
TC10 (pKH)	GAG3-5ASA	fw	CGCTGGATCCCCCGCATCCGCCCGCAGCAGCATGG
TC10 (pKH)	GAG3-5ASA	rv	CCATGCTGCTGCGGGCGGATGCGGGGGATCCAGCG
TC10	GPG12-14ASA	fw	GCAGCATGGCTCACGCGTCCGCCGCGCTGATGCTC
TC10	GPG12-14ASA	rv	GAGCATCAGCGCGGCGGACGCGTGAGCCATGCTGC
TC10	D52A	fw	CCCACCGTCTTCGCCCACTACGCAGTC
TC10	D52A	rv	GACTGCGTAGTGGGCGAAGACGGTGGG
TC10	Y54A	fw	CACCGTCTTCGACCACGCCGCAGTCAGCGTCACC
TC10	Y54A	rv	GGTGACGCTGACTGCGGCGTGGTCGAAGACGGTG
TC10	DY52+54AA	fw	CACCGTCTTCGCCCACGCCGCAGTCAGCGTCAC
TC10	DY52+54AA	rv	GTGACGCTGACTGCGGCGTGGGCGAAGACGGTG
TC10	hum-rat-miRNA-3C	fw	GGGGCAAGCAGTACCTCTTGG GACTCTATGACACGGC
TC10	hum-rat-miRNA-3C	rv	GCCGTGTCATAGAGTCCCAAGAGGT ACTGCTTGCCCC
TC10	hum-rat-miRNA-4D	fw	GAAAGAGGAGTGGGTACCAGAACTAAA GGAATACGCACCAAATG
TC10	hum-rat-miRNA-4D	rv	CATTTGGTGCGTATTCCTTTAGTTCT GGTACCCACTCCTCTTTC
TC10	hum-rat-miRNA-2B	fw	CTTTATCTTACCCAATGACTGACGTC TTCCTCATATGCTTCTCGGTGG
TC10	hum-rat-miRNA-2B	rv	CCACCGAGAAGCATATGAGGAAGACG TCAGTCATTGGGTAAGATAAAG

Table 2: Oligonucleotide primers used for TC10 C-terminus mutagenesis. direction, dir; forward, fw; reverse, rv. If the primer overlaps with the vector, the plasmid is given in brackets

cDNA	mutation	dir.	sequence (5'-3')
TC10	KKH-NQN	fw	CATAGCCATTTTAACTCCAAACCAAAA CACTGTAAAAAAAAGAATAGG
TC10	KKH-NQN	rv	CCTATTCTTTTTTTTACAGTGTTTTGGTTT GGAGTTAAAATGGCTATG
TC10	KK199/200SS	fw	CTCCAAAGAAACACACTGTAAGCAGCA GAATAGGATCAAGATGTATAAAC
TC10	KK199/200SS	rv	GTTTATACATCTTGATCCTATTCTGCTG CTTACAGTGTGTTTCTTTGGAG
TC10	RR201-5QQ	fw	CCAAAGAAACACACTGTAAAAAAACAGATAGGAT CACAGTGTATAAACTGTTGTTTAATTACG
TC10	RR201-5QQ	rv	CGTAATTAAACAACAGTTTATACACTGTGATCC TATCTGTTTTTTTACAGTGTGTTTCTTTGG
TC10	RR201-5AA	fw	CCAAAGAAACACACTGTAAAAAAAGCCATAGG ATCAGCCTGTATAAACTGTTGTTTAATTACG
TC10	RR201-5AA	rv	CGTAATTAAACAACAGTTTATACAGGCTGATCC TATGGCTTTTTTTACAGTGTGTTTCTTTGG
TC10	DeltaKR	fw	CTATCATAGCCATTTTAACTCCAGCGGCAGC CACTGTAGCAGCAGCAATAGGATCA GCATGTATAAACTGTTGTTTAATTACG
TC10	DeltaKR	rv	CGTAATTAAACAACAGTTTATACATGCT GATCCTATTGCTGCTGCTACAGTGGCT GCCGCTGGAGTTAAAATGGCTATGATAG
TC10	C206S	fw	GTAAAAAAAAGAATAGGATCAAGATCT ATAAACTGTTGTTTAATTACGTG
TC10	C206S	rv	CACGTAATTAAACAACAGTTTATAGAT CTTGATCCTATTCTTTTTTTTAC
TC10 (pKH)	C209S	fw	GAATAGGATCAAGATGTATAAACTC TTGTTTAATTACGTGAGAATTC
TC10 (pKH)	C209S	rv	GAATTCTCACGTAATTAAACAAGAGTT TATACATCTTGATCCTATTC
TC10 (pKH)	C206S-C209S	fw	GTAAAAAAAAGAATAGGATCAAGATCT ATAAACTCTTGTTTAATTACGTGAG
TC10 (pKH)	C206S-C209S	rv	CTCACGTAATTAAACAAGAGTTTATAG ATCTTGATCCTATTCTTTTTTTTAC
TC10 (pKH)	CCC206/09/10SSS	fw	GTAAAAAAAAGAATAGGATCAAGAAGTATAAA CAGTAGTTTAATTACGTGAGAATTCAATC
TC10 (pKH)	CCC206/09/10SSS	rv	GATTGAATTCTCACGTAATTAAACTACTGTTT ATACTTCTTGATCCTATTCTTTTTTTTAC
TC10 (pKH)	C210S	fw	GGATCAAGATGTATAAACTGTAGTTT AATTACGTGAGAATTCAATCG
TC10 (pKH)	C210S	rv	CGATTGAATTCTCACGTAATTAAA CTACAGTTTATACATCTTGATCC
TC10 (pKH)	LIT-VLS	fw	GATCAAGATGTATAAACTGTTGTGTACTTT CGTGAGAATTCAATCGATGGC
TC10 (pKH)	LIT-VLS	rv	GCCATCGATTGAATTCTCACGAAA GTACACAACAGTTTATACATCTTGATC

2.1.2 Cloning of HA-Cdc42 (placental)

Placental Cdc42 was cloned from a pKMyc vector to a pKH vector. In order to do so, a BamHI restriction site was added upstream of the placental Cdc42 coding sequence by site-directed mutagenesis (see 2.1.1, primers as in indicated in Table 3), the PCR product was isolated by gel purification (Invitrogen kit), then overhangs were generated using Redtaq polymerase for the ensuing TOPO cloning (Invitrogen kit). Cdc42 was excised from the TOPO vector by restrictrion digestion using the 5' BamHI restriction site and a 3' EcoRI site, the target vector pKH-TC10 was digested in the same way using NEB restriction enzymes and protocols. Ligation (T4 DNA ligase, Invitrogen) was performed overnight, then plasmids were cloned as described before (2.1.1).

Table 3: Oligonucleotide primers used for Cdc42 mutagenesis. direction, dir; forward, fw; reverse, rv. If the primer overlaps with the vector, the plasmid is given in brackets

cDNA	**mutation**	**dir.**	**sequence (5'-3')**
Cdc42	RR186-187AA	fw	CCAGAACCGAAGAAGAGCGCCGCCTG TGTGCTGCTATGAGAATTC
Cdc42	RR186-187AA	rv	GAATTCTCATAGCAGCACACAGGCGG CGCTCTTCTTCGGTTCTGG
Cdc42	RR186-187QQ	fw	CCAGAACCGAAGAAGAGCCAGCA GTGTGTGCTGCTATGAG
Cdc42	RR186-187QQ	rv	CTCATAGCAGCACACACTGCTGG CTCTTCTTCGGTTCTGG
Cdc42	KK183-184SS	fw	GGAGCCTCCAGAACCGAGCAGC AGCCGCAGGTGTGTGC
Cdc42	KK183-184SS	rv	GCACACACCTGCGGCTGCTG CTCGGTTCTGGAGGCTCC
Cdc42	Myc-toHA	fw	GGACCTGGGATCCATGCAGACAATTAAG
Cdc42	Myc-toHA	rv	CGATTGAATTCTCATAGCAGCACACACCTG

2.2 Antibodies

Antibodies were used for immunocytochemistry, Western blotting and immunoprecipitation as indicated in Tables 4 and 5.

Table 4: Primary antibodies. * designates antibodies used for data shown in appendix, but listed here for completeness, immunocytochemistry: ICC, Western Blot: WB, Immunoprecipitation: IP

Epitope	Species	Company	Dilution	Application
*gephyrin, mAb7a, monoclonal	mouse	Connex	1:3,000	ICC
*VIAAT (vesicular inhibitory amino acid transporter), polyclonal	guinea-pig	Synaptic Systems	1:2,000	
collybistin, monoclonal	mouse	BD Biosciences	1:500	ICC, WB)
collybistin, polyclonal	rabbit	Synaptic Systems	0.7μg per mg protein	IP)
*TC10, polyclonal	mouse	Abcam (ab168645)	1:100	ICC)
TC10, polyclonal	rabbit	Abcam (ab107573)	1:100	WB)
HA, polyclonal	rabbit	Zymed Laboratories, Invitrogen	1:2,000	ICC)
*HA, monoclonal	mouse	Covance	1:2,000	ICC
*c-Myc polyclonal	rabbit	Sigma-Aldrich (C3956)	1:1,000	ICC
c-Myc clone 9E10 monoclonal	mouse	Sigma-Aldrich	1:1,000	ICC
TC10, polyclonal	rabbit	Abcam (ab107573)	1:250	WB
TC10, polyclonal	rabbit	Sigma-Aldrich(T8950)	1:4,000	WB
*gephyrin 3B11, monoclonal	mouse	Synaptic Systems	1:3,000	WB
*HA, conjugated with peroxidase, monoclonal	rat	Roche	1:10,000	WB
*c-Myc polyclonal	rabbit	Sigma-Aldrich (C3956)	1:2,000	WB
beta-tubulin	mouse	Sigma-Aldrich (T4026)	1:2000	WB
anti-GST-HRP conjugate		GE Healthcare (RPN1236V)	1:10,000	WB
ChromoPure IgG	rabbit	Jackson ImmunoResearch		IP

2.3 Expression and purification of proteins

2.3.1 Glutathione S-transferase-tagged proteins

Glutathione S-transferase (GST)-CbII (SH3(+) or ΔSH3) fusion proteins were expressed in the Rosetta2 *E. coli* strain. Bacteria were cultured in Terrific Broth at 37°C to an OD_{600} of 0.6-0.8, and transferred to 23°C to induce protein expression with 0.2 mM isopropyl-a-D-thiogalactopyranosid (IPTG). After 6h, cells were harvested by centrifugation (4,550 $\times$ g, 20 min) and resuspended in cold PBS (140 mM NaCl, 2.5 mM KCl, 1.8 mM KH_2PO_4, and 1 mM Na_2HPO_4, pH 7.5; 30 mL per 500 mL culture) containing protease inhibitors (1 μg/mL aprotinin, 0.5 μg/mL leupeptin, 1.74

Table 5: Secondary antibodies. * designates antibodies used for data shown in appendix, but listed here for completeness, immunocytochemistry: ICC, Western Blot: WB, Immunoprecipitation: IP

Epitope	Species	Company	Dilution	Application
Alexa Fluor 488 or 555 anti-mouse or anti-rabbit IgG	goat	Invitrogen	1:2,000	ICC
Alexa Fluor 555 or 633 anti-guinea pig IgG	goat	Invitrogen	1:2,000	ICC
Anti-mouse IgG Cy5 conjugate	goat	Millipore	1:1,000	ICC
peroxidase conjugated AffiniPure anti-mouse or anti-rabbit IgGs	goat	Jackson ImmunoResearch Laboratories	1:10,000	WB
IRDye800 anti-mouse IgG conjugate	goat	Rockland	1:5000	WB

μg/mL PMSF) and 10 mM EDTA. After addition of lysozyme, DNase I and 1 mM $MgCl_2$, cells were lysed by sonication (VS-70, Sonoplus, Bandelin, 2 times 1 min, 100% intensity, 40% cycle) and incubated in the presence of 1% Triton-X 100 (vol/vol) for 30 min at 4°C. Cellular debris was removed by centrifugation (10,000 × g, 20 min, 4°C). To increase protein stability, 3-((3-Cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS) was added to the supernatant to a final concentration of 0.5% (wt/vol). The lysate was cleared by ultracentrifugation (148,000 × g, 60 min, 4°C), and the supernatant was loaded onto glutathione Sepharose 4B beads (100-200 μL per 500 mL culture, GE Healthcare) and incubated for 2 h at 4°C on a rotator. Beads were washed twice with at least 50 bead volumes of PBS containing 0.5% (wt/vol) CHAPS and protease inhibitors (as above), and three times with at least 50 bead volumes of 50 mM Tris/HCl (pH 8.0), 500 mM NaCl, 0.5% (wt/vol) CHAPS, and protease inhibitors. Beads were resuspended in approximately 5 bead volumes of elution buffer (50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 10 mM EDTA, 0.5% (wt/vol) CHAPS, and 1 mM DTT). For kinetic measurements, the GST tag was cleaved off by incubating the beads in elution buffer with 5 U thrombin (GE Healthcare) per 200 μL of beads for 16 h at room temperature under agitation. Supernatants were collected and concentrated in Centriprep YM-3 centrifugal filter devices (Amicon, Millipore).

TC10 for kinetic measurements was expressed and purified by Mamta Jaiswal (Institute for Biochemistry and Molecular Biology II, Medical Faculty, Heinrich-Heine-University, Düsseldorf, Germany) as described previously (Jaiswal et al., 2013).

2.3.2 His-TC10

His-TC10 was expressed from a pRSET-A plasmid in BL21 DE3 *E. coli*, grown to an OD_{600} of 0.6-0.8, cooled to 25°C, and induced with 200 μM IPTG. Cells were

harvested after 20 h as described (2.3.1), resuspended in 10 mL lysis buffer (50 mM Tris/HCl, pH 8.0, 300 mM NaCl, 10 mM imidazole, and protease inhibitors) and lysed as described (2.3.1). After ultracentrifugation (148,000 × g, 1 h, 4°C), 2 mL of a 50% Ni-NTA slurry (Qiagen) and GDP (final concentration 100 μM, Millipore) were added to the supernatant and incubated for 60 min at 4°C. After three washes with 50 mM Tris/HCl, pH 8.0, 300 mM NaCl, 20 mM imidazole, 1% (vol/vol) Triton-X-100, and protease inhibitors, and two washes with 50 mM Tris/HCl, pH 8.0, 300 mM NaCl, 50 mM imidazole, 1% (vol/vol) Triton-X-100, and protease inhibitors, elution was performed in two steps, first with 50 mM Tris/HCl, pH 8.0, 300 mM NaCl, 250 mM imidazole, and protease inhibitors and then with 50 mM Tris/HCl, pH 8.0, 300 mM NaCl, 1 M imidazole, and protease inhibitors. Eluates were pooled and dialyzed against a buffer containing 25 mM Tris/HCl, pH 8.0, 200 mM NaCl, and protease inhibitors to remove imidazole in a Slide-A-Lyser Dialysis Cassette, 10,000 molecular weight cutoff (Thermo Scientific) overnight in at least 500 volumes.

2.4 *In vitro* guanine nucleotide exchange assays

The kinetic assays were performed as described previously for the measurement of GEF-catalyzed nucleotide exchange reactions (Jaiswal et al., 2013). In brief, 0.1 μM methylanthraniloyl-GDP (mant-GDP) loaded Cdc42 (amino acids 1-181, human sequence) or TC10 (amino acids 2-193, human sequence) and 2 μM Cb (SH3(+) or ΔSH3) were preincubated in degassed GEF buffer (30 mM Tris/HCl, pH 7.5, 50 mM $MgCl_2$, 3 mM DTT, 10 mM K_2HPO_4/KH_2PO_4, pH 7.4, and 0.05% (wt/vol) CHAPS) in a fluorescence cuvette at 25°C for 5 min. The mant fluorescence signal was recorded in a Luminescence Spectrometer LS50B (Perkin-Elmer) using an excitation wavelength of 366 nm and an emission wavelength of 450 nm. After recording a stable baseline fluorescence signal, GDP was added to a final concentration of 20 μM and mixed in rapidly to start the reaction. An exponential decrease in fluorescence was observed in the following 20 h owing to a slow release of mant-GDP into the aqueous solution. The data were fitted exponentially using Grafit software (Erithacus Software Ltd.) to determine the dissociation rates of mantGDP-Cdc42 and mantGDP-TC10 in the presence and absence of Cb. These experiments were performed jointly by Mamta Jaiswal (Institute for Biochemistry and Molecular Biology II, Medical Faculty, Heinrich-Heine-University, Düsseldorf, Germany) and myself.

2.5 *In vitro* binding assays

His-TC10 was loaded with GDP or GTPγS (Millipore) at 120 μM in 50 mM Tris/HCl pH 7.5, 1 mM DTT, 5 mM EDTA and protease inhibitors (2.3.1) for 20 min at 30°C. To terminate the exchange, $MgCl_2$ was added to a concentration of 65 mM. The nucleotide-free state was achieved by incubating TC10 with the same buffer but without the addition of any nucleotide. *In vitro* binding assays were performed by incubating purified GST-tagged proteins coupled to glutathione-Sepharose beads with His-TC10 in the different nucleotide-loaded of nucleotide-free states for 2 h at 4°C in 50 mM Tris/HCl, pH 7.5, 1 mM DTT, 0.5% (vol/vol) Triton X-100, 200 mM NaCl, and 5 mM EDTA (for nucleotide-free conditions only) or 5 mM $MgCl_2$ (for nucleotide-bound conditions). After washing four times with at least 20 bead volumes, the beads were resuspended in 50 μL Lämmli buffer, heated at 60°C for 30 min, and analyzed by Western blotting. MemCode staining (MemCode Reversible Protein Stain Kit for Nitrocellulose Membrane, Thermo Scientific) was used to visualize GST-tagged proteins, and TC10 was detected by immunoblotting using the polyclonal rabbit anti TC10 T8950 antibody (see Table 4 and 5).

2.6 Cell culture and immunocytochemistry

COS-7 cells were cultured in DMEM (Gibco, Life Technologies), 10% (vol/vol) FCS (Gibco, Life Technologies), 50 U/mL penicillin, and 50 U/mL streptomycin (Gibco, Life Technologies) at 37°C in 5% CO_2. For transfection, cells were plated in 24-well plates on 12-mm coverslips. Sterile coverslips were coated with poly-L-lysine (Sigma, 0.001% (wt/vol), diluted from 0.01% stock in Dulbecco's PBS, PAA Laboratories) for at least 2 h and washed three times with Dulbecco's PBS before plating. Medium was exchanged to DMEM devoid of supplements before transfection, at approximately 80% confluency. For transfections, 200 ng GFP-gephyrin, 100 ng HA-TC10 (wildtype (WT) or different mutant versions) or HA-Cdc42 (WT or different mutant versions), and/or 100 ng Myc-Cb DNA were used per well; pcDNA 3.1 was used to equalize the total amount of DNA per transfection to 400 ng. Cells were transfected using Lipofectamine2000 (Invitrogen) following the manufacturer's protocol. DMEM containing 10% (vol/vol) FCS and antibiotics (as above) was added 4 h after transfection. Cells were fixed 10 h after transfection in 4% (wt/vol) paraformaldehyde in PBS for 10 min. After three washes in PBS, cells were permeabilized using 40 μg/ mL digitonin or 1% (vol/vol) Triton X-100 in PBS. Blocking was performed in 10% (vol/vol) goat serum in PBS for at least 60 min. For immunostaining, antibodies were diluted as indicated above (see Table 4 and 5) in 10% (vol/vol) goat serum in PBS. Cells were incubated for 1.5 h with primary

and 45 min with secondary antibodies. Cells were washed three times with PBS before mounting with Aqua Poly/Mount (Polysciences) and inspected under an AxioImager Z1 equipped with a Zeiss apochromat 63 objective (Zeiss). For quantifications of cluster sizes, exposure times for GFP-gephyrin were kept constant at 170 ms. Quantifications were performed using the ImageJ software package (http://rsb.info.nih.gov/ij) and standardized intensity thresholding.

2.7 Immunoprecipitation from rat brain lysates

One adult rat was anaesthetized with isoflurane and decapitated according to institutional guidelines. The brain was removed and homogenized in 10 volumes of 320 mM sucrose in ice-cold water containing protease inhibitors (2.3.1) using a Dounce homogenizer at 900 rpm. Nuclei were removed by centrifugation (800 × g, 10 min, 4°C). Proteins in the supernatant were solubilized by diluting the solution to a final protein concentration of 2-3 mg/ml and a final concentration of 1% CHAPS, 150 mM NaCl, 50 mM Tris/HCl, pH 7.5. After ultracentrifugation (142,000 × g, 1h, 4°C), the supernatant was loaded with GTPγS (100 μM) or GDP (1 mM) in 10 mM EDTA by incubating at 30°C for 30 min. To terminate the exchange, $MgCl_2$ was added to a concentration of 65 mM. Rabbit anti-Cb polyclonal antibody (Synaptic Systems) or ChromoPure rabbit IgG (Jackson ImmunoResearch) was added at equivalent concentrations (approximately 0.7 μg antibody per mg protein) and incubated overnight at 4°C. 30 μL of protein A sepharose CL4B (GE Healthcare) suspension were added and incubated for 2-3h at 4°C. Beads were washed four times with 1 mL wash buffer (1% CHAPS, 150 mM NaCl, 50 mM Tris/HCl pH 7.5) and eluted in 40 μL Lämmli buffer and heated 5 min at 95°C. All solutions were supplemented with protease inhibitors (2.3.1) and filtered. Western Blotting onto polyvinylidene difluoride (PVDF) membranes followed by immunodetection of proteins was performed using the following antibodies: monoclonal mouse anti-Cb and polyclonal rabbit anti-TC10 (ab107573) (see Table 4 and 5 for details).

2.8 Testing TC10 knockdown efficiency

To test the knockdown efficiencies of various TC10 knockdown constructs, we transfected Rat2 cells, a rat fibroblast-like cell line, with expression vectors containing miRNAs and shRNAs using Lipofectamine in a 10 cm dish (see 2.6). Cells were grown in medium containing antibiotics (10 μg/mL blasticidine for miRNA constructs, 1.5 μg/mL puromycin for shRNA constructs) to select for transfected clones. Individual

clones were isolated and transferred to a well of a 96-well plate. Clones were expanded in medium containing the appropriate antibiotics until confluency in a 10 cm-dish and lysed in cold buffer (1 % Tx-100, 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, protease inhibitors (2.3.1) and 2 μL benzoase nuclease (Sigma)). Lysates were analysed by Western Blotting on a PVDF membrane, followed by staining using mouse anti-beta tubulin and rabbit anti-TC10 primary antibodies (see table 4) and goat anti mouse / rabbit IR800 secondary antibodies, respectively (according to Licor protocol). Blots were analyzed using the LiCor Odyssey Infrared Imager.

2.9 Statistical analysis

Statistical analysis was performed using the one-way ANOVA variance test followed by a Tukey multiple comparison test, using 95% confidence intervals or Student's t-test (only 3.5). All values represent means ± SEM. Asterisks indicate significant differences (*P <0.05,**P <0.01, and ***P <0.001); n.s. indicates non-significant differences.

3

Results

3.1 Gephyrin microcluster formation by Cb and small GTPases in cells

At synaptic sites, gephyrin forms clusters to immobilize neurotransmitter receptors. In order to study the mechanisms by which gephyrin is recruited to such clusters at the plasma membrane, the subcellular distribution of recombinant gephyrin can be studied in non-neuronal cells when expressed together with other proteins, as first established by Kins and colleagues (Kins et al., 2000). Expression of recombinant GFP-gephyrin in, for example HEK293 cells, results in the formation of intracellular aggregates, while coexpression of the constitutively active Cb isoform ΔSH3CbII (Figure 8) leads to the formation of microclusters at the plasma membrane (Kins et al., 2000). However, the coexpression of SH3 domain-containing variants does not lead to the redistribution of gephyrin from cytoplasmic aggregates to submembranous microclusters (Figure 8) (Kins et al., 2000; Harvey et al., 2004). As described previously, different synaptic proteins, such a NL2, interact with the SH3 domain of Cb and thus relieve the autoinhibition (1.2.4.3). In this cellular assay, the coexpression of these activators with SH3(+)CbII and GFP-gephyrin, leads to the formation of submembranous gephyrin clusters (Poulopoulos et al., 2009; Hoon et al., 2011; Saiepour et al., 2010).

3.1.1 TC10 triggers Cb-mediated gephyrin microcluster formation

We used the microcluster formation assay described above in COS7 cells, a monkey kidney cell line, to test whether TC10 can function as a positive regulator of Cb function and trigger the formation of GFP-gephyrin microclusters in the presence of SH3(+)CbII. We therefore transfected HA-TC10 along with GFP-gephyrin and Myc-

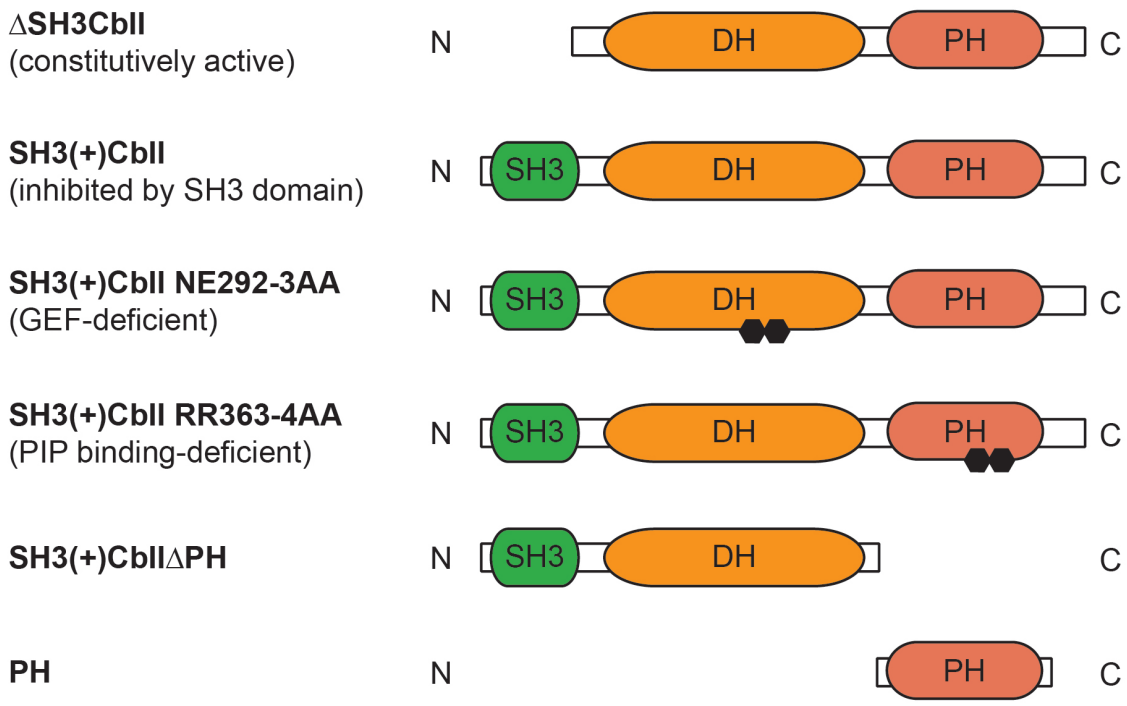

Figure 8: Schematic representation of Cb variants and mutants. N-terminal src homology 3 (SH3) domain, Dbl homology (DH) domain and pleckstrin homology (PH) domain

SH3(+)CbII in COS7 cells for ten hours. Cells were then fixed and immunocytochemistry was performed in order to visualize TC10 and Cb expression using HA- and Myc-tag-specific antibodies, respectively. As expected based on previous publications (Kins et al., 2000; Harvey et al., 2004), expression of GFP-gephyrin alone (Figure 9 A) or together with SH3(+)CbII (Figure 9 C) led to the accumulation gephyrin in intracellular aggregates, whereas GFP-gephyrin coexpression with ΔSH3CbII (Figure 9 B) led to the formation of submembraneous microclusters. Coexpression of TC10 and GFP-gephyrin also led to the formation of gephyrin aggregates (Figure 9 D), however, coexpression of GFP-gephyrin, SH3(+)CbII and TC10 led to the formation of submembraneous microclusters in the majority of the cells (Figure 9 E).

In order to verify that GFP-gephyrin microclusters induced by TC10 and SH3(+)CbII were indeed at the plasma membrane, three-dimensional reconstructions of image stacks of exemplary transfected cells were performed. Confirming our assumption, GFP-gephyrin was found in intracellular aggregates upon coexpression of HA-TC10 (Figure 10 A), while GFP-gephyrin microclusters triggered by the coexpression of HA-TC10 and Myc-SH3(+)CbII were located at the cell periphery (Figure 10 B).

We quantified the formation of GFP-gephyrin microclusters in COS7 cells in the presence of HA-TC10 and Myc-SH3(+)CbII in several ways. First, the proportion

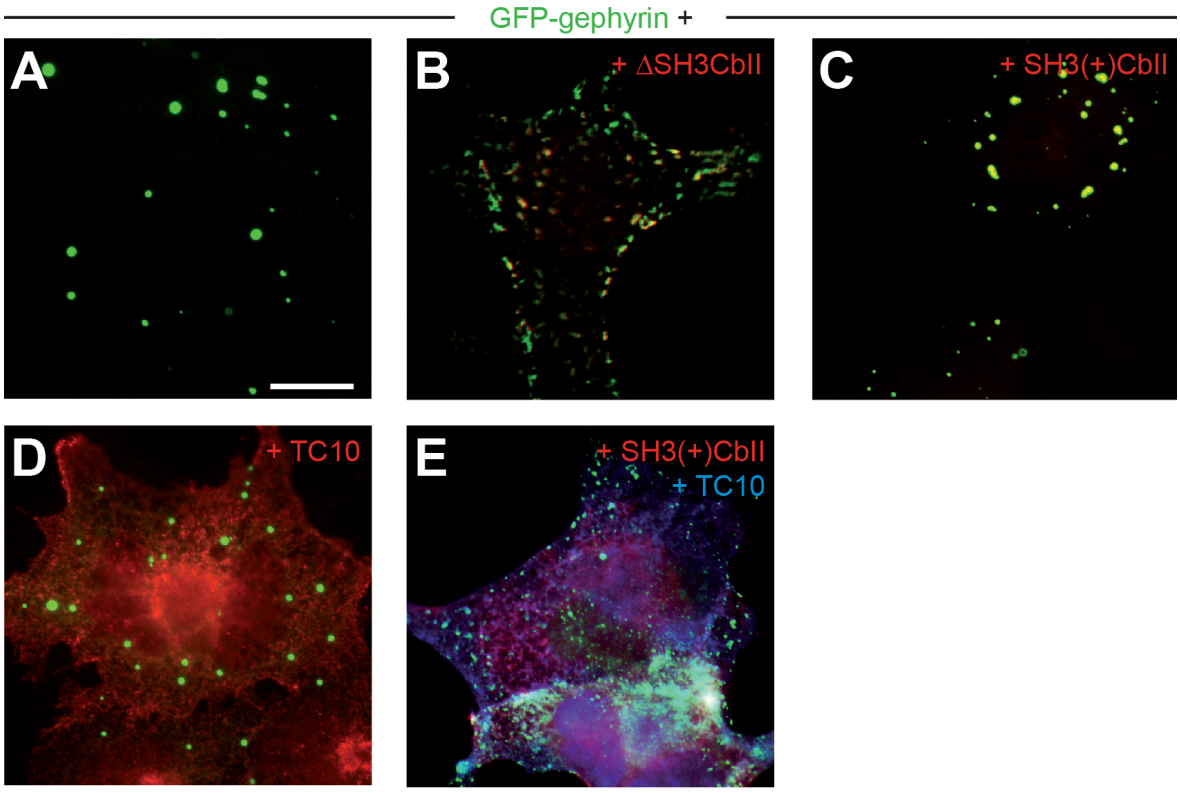

Figure 9: TC10 triggers Cb-mediated redistribution of GFP-gephyrin into submembraneous microclusters. Epifluorescent images of COS7 cells transfected with GFP-gephyrin and Myc-Cb and HA-TC10 as indicated. Scale bar: 10 μm. Experiments were performed by myself, images were prepared by Theofilos Papadopoulos.

of cells per transfection in which GFP-gephyrin was distributed in submembraneous microclusters (>50 GFP-gephyrin puncta per cell) was counted, with the experimenter blind to the condition. A significant increase in GFP-gephyrin microcluster formation when TC10 was coexpressed with SH3(+)CbII was observed: 64.8 $\pm$ 2.7 % of cells expressing GFP-gephyrin, Myc-SH3(+)CbII and HA-TC10 were characterized by GFP-gephyrin microclusters, compared to 1.1 $\pm$ 0.5 % of cells expressing GFP-gephyrin only, 0.6 $\pm$ 0.3 % of cells expressing GFP-gephyrin and HA-TC10, and 26.2 $\pm$ 1.3 % of cell expressing GFP-gephyrin and Myc-SH3(+)CbII (Figure 11 A). As expected, microclusters were observed in 94.3 $\pm$ 0.7 % of cells expressing GFP-gephyrin and Myc-ΔSH3CbII (Figure 11 A). The difference between ΔSH3CbII-induced gephyrin microclusters, and SH3(+)CbII and TC10-induced gephyrin microclusters may be explained by the lower degree of co-transfection of the three constructs as compared to the two constructs. Cotransfection of all constructs was not verified in this quantification, since the experimenter was solely analyzing GFP-gephyrin distribution, blind to the experimental condition.

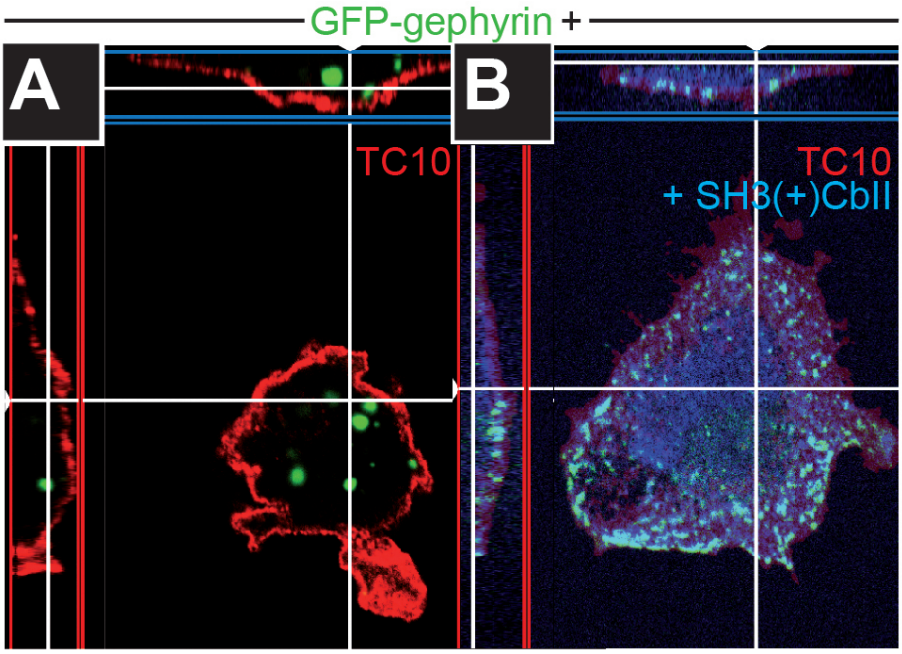

Figure 10: GFP-gephyrin microclusters induced by TC10 and SH3(+)CbII are located at the cell periphery. 2-D compressed Z-stacks of overlaid epifluorescent images of COS-7 cells expressing GFP-gephyrin and HA-TC10 (A) or GFP-gephyrin, HA-TC10 and Myc-SH3(+)CbII (B). Images were processed with the CutView function of the AxioImager software (Zeiss) using a maximum intensity projection algorithm. The CutView panels depict two perpendicular transverse sections per 2-D compressed image as indicated by the white lines. Experiments were performed by myself, images were prepared by Theofilos Papadopoulos.

Second, we took exemplary images of transfected cells, in which expression of all cDNAs was confirmed (examples are shown in Figure 9), and analyzed the cluster numbers and sizes automatically. We found that in agreement with the previous quantification, the total number of gephyrin clusters was significantly increased when GFP-gephyrin was coexpressed with either Myc-ΔSH3CbII (234.6 ± 20.9) or with both Myc-SH3(+)CbII and HA-TC10 (192.4 ± 30.2) compared to cells expressing GFP-gephyrin and Myc-SH3(+)CbII (58.2 ± 13.1), GFP-gephyrin and HA-TC10 (12.9 ± 2.6) or GFP-gephyrin only (22.2 ± 3.3) (Figure 11 B). Interestingly, the total number of microclusters did not differ significantly between ΔSH3-CbII-induced gephyrin microclusters, and SH3(+)CbII and TC10-induced gephyrin microclusters, showing that the differences observed on a population level (Figure 11 A) were likely indeed due to the different cotransfection efficiencies.

Furthermore, we analyzed the size distribution of clusters in the different transfection conditions. In line with the previous analyses, the distribution of cluster sizes per cell was similar in cells double-transfected with GFP-gephyrin and Myc-ΔSH3-CbII,

and cells triple-transfected with GFP-gephyrin, Myc-SH3(+)CbII and HA-TC10, with most clusters smaller than 0.4 μm^2 (Figure 11 E). Interestingly, TC10 and SH3(+)CbII co-induced microclusters were smaller than ΔSH3CbII induced microclusters, as shown by the larger proportion of clusters with sizes between 0.05 and 0.2 μm^2 (Figure 11 C). In contrast, the percentage of clusters larger than 1 μm^2 was significantly smaller when GFP-gephyrin was coexpressed with ΔSH3CbII or TC10 and SH3(+)CbII as compared to cell expressing GFP-gephyrin alone, or together with either Myc-SH3(+)CbII or HA-TC10 (Figure 11 D).

In conclusion, we show here that TC10 can activate SH3(+)CbII in the same way as previously shown activators, for example NL2 (1.2.4.3), such that GFP-gephyrin is redistributed from intracellular aggregates to submembraneous microclusters in non-neuronal cells upon coexpression with TC10 and SH3(+)CbII.

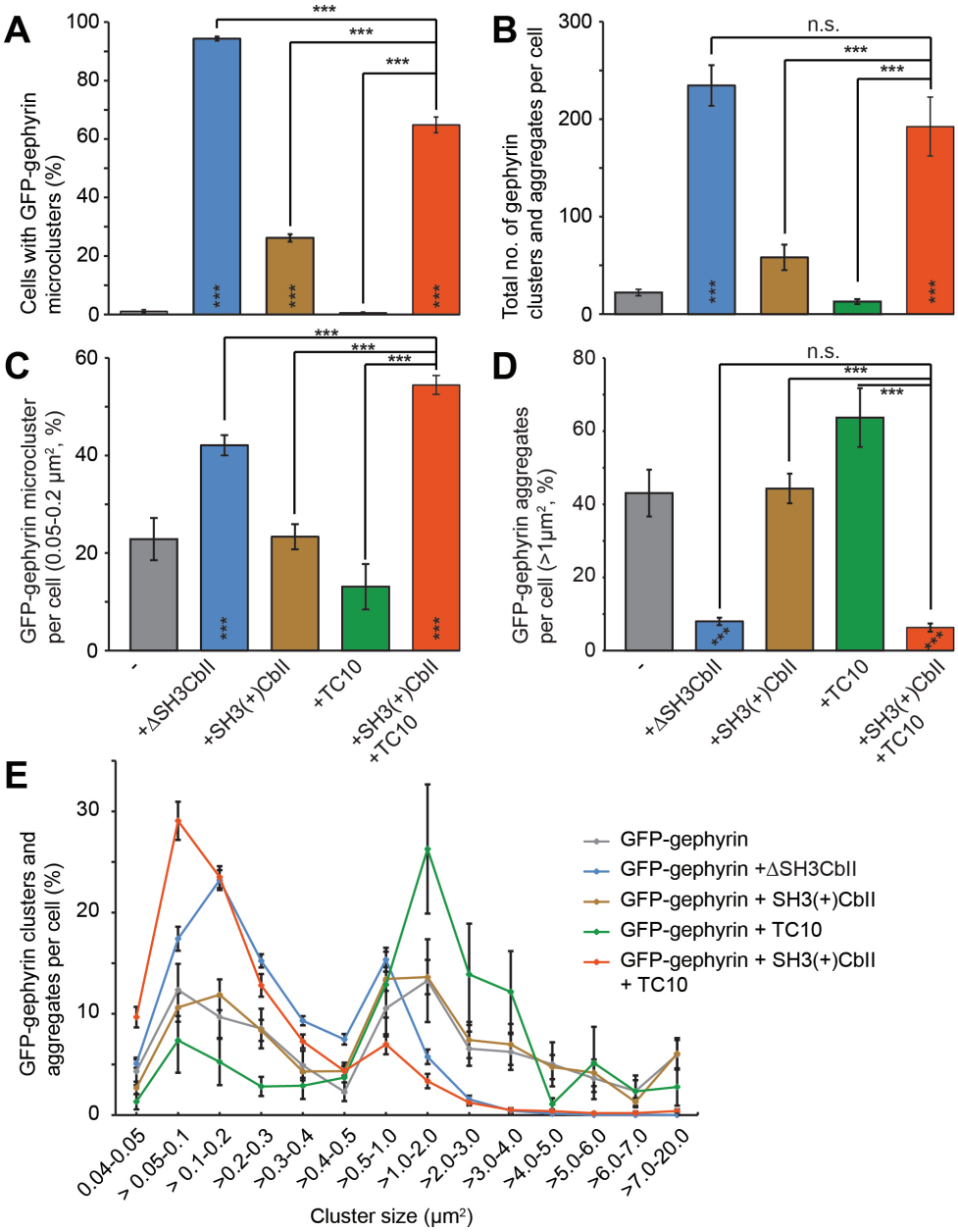

Figure 11: Quantification of TC10-triggered Cb-dependent GFP-gephyrin microclusters. (A) Percentages of GFP-gephyrin (co)transfected cells classified as displaying GFP-gephyrin microclusters (>50 puncta per cell) with co-transfection of HA-TC10 or Myc-CbII as indicated (N=3 independent transfections, n=321-428 cells). (B-E) GFP-gephyrin clusters and aggregates were quantified from images of COS7 cells transfected with GFP-gephyrin, HA-TC10 and Myc-CbII as indicated (n=14-34 transfected cells per transfection condition). (B) Total number (no) of gephyrin puncta per cell. (C) Percentage of GFP-gephyrin puncta between 0.05 and 0.2 μm^2 per cell. (D) Percentage of GFP-gephyrin puncta >1 μm^2 per cell. (E) Gephyrin puncta were binned according to their size. Significance levels compared to cells transfected with GFP-gephyrin only (grey bar) are shown within the bars, significance levels compared to cells transfected with GFP-gephyrin, HA-TC10 and Myc-SH3(+)CbII are indicated on top of the bars. Results are means ± SEM. Experiments were performed by myself, image analysis was performed conjointly with Theofilos Papadopoulos.

3.1.2 Binding of Cb to lipids is required for TC10-triggered gephyrin microcluster formation

Having established that TC10 can trigger Cb-dependent gephyrin microcluster formation in COS7 cells, we sought to test whether the GEF activity of Cb was important in this process. We therefore tested whether a previously described (Reddy-Alla et al., 2010) mutation in the DH domain that impairs the GEF activity of Cb, SH3(+)CbII NE292-293AA (abbreviated as NE/AA from here on, Figure 8) could mediate gephyrin clustering in the presence of TC10. Coexpression of Myc-SH3(+)CbII NE/AA, HA-TC10 and GFP-gephyrin in COS7 cells led to the formation of GFP-gephyrin microclusters (Figure 12 B), which were predominantly smaller than 0.4 μm^2 (Figure 12 E). There was a significant increase in the number of microclusters in this size range (77.0 $\pm$ 1.9 %) compared to cells not expressing TC10 (48.6 $\pm$ 3.9 %)(Figures 12 A and 14).

Therefore, the GEF activity of Cb is not essential for TC10-triggered gephyrin microcluster formation in COS7 cells. This observation is in agreement with previously published results (Reddy-Alla et al., 2010).

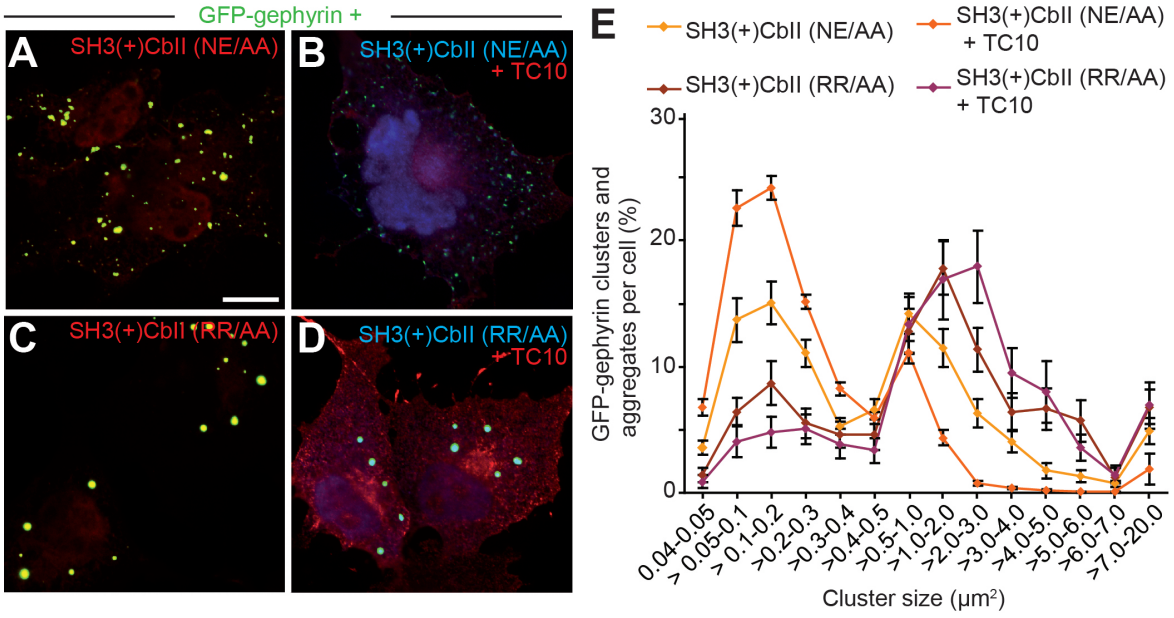

Figure 12: Cb lipid binding but not GEF activity is required for gephyrin redistribution in COS7 cells. (A-D) Epifluorescent images of COS7 cells transfected with GFP-gephyrin, Myc-Cb and HA-TC10 as indicated. Scale bar: 10 μm. (E) GFP-gephyrin clusters and aggregates were quantified from images of transfected COS7 cells and binned according to their size, results are means $\pm$ SEM (n=29-31 transfected cells per transfection condition). Experiments were performed by myself, images were prepared by Theofilos Papadopoulos, image analysis was performed conjointly with Theofilos Papadopoulos.

A previous study reported that two residues in the PH domain of Cb, which are

required for the binding of Cb to PI(3)P, are crucial for mediating gephyrin clustering (Figure 8)(Reddy-Alla et al., 2010). We therefore tested whether the interaction between the PH domain and membrane lipids was necessary for TC10-mediated gephyrin redistribution by cotransfecting COS7 cells with this mutant (Myc-SH3(+)CbII RR363-364AA, abbreviated as RR/AA from here on) together with GFP-gephyrin and HA-TC10. In this condition, GFP-gephyrin accumulated in large cytoplasmic aggregates similar to cells, which express GFP-gephyrin and Myc-SH3(+)CbII RR/AA only (Figures 12 C-E and 14).

These results show that TC10 does not relieve the requirement for lipid binding by the PH domain of Cb and reinforce previous studies which have demonstrated a crucial role of lipid binding for Cb-mediated gephyrin microcluster formation (Kalscheuer et al., 2009; Reddy-Alla et al., 2010).

3.1.3 GTP-TC10 is required for Cb-dependent gephyrin microcluster formation

Next, we aimed at analyzing whether the switch function of TC10 was required for mediating Cb-dependent gephyrin microcluster formation. We therefore coexpressed TC10 mutants, TC10 CA, a constitutively active mutation (Q75L), and TC10 DN, a dominant negative mutation (T23N) (see also 1.3.1) with GFP-gephyrin and SH3(+)CbII (Murphy et al., 2001). While coexpression of GFP-gephyrin with HA-TC10 CA (Figure 13 A) or HA-TC10 DN (Figure 13 C), as well as coexpression of GFP-gephyrin, HA-TC10 DN and Myc-SH3(+)CbII (Figure 13 D) led to the accumulation of GFP-gephyrin in aggregates, HA-TC10 CA coexpression with GFP-gephyrin and Myc-SH3(+)CbII triggered gephyrin microcluster formation (Figure 13 B). Analysis of cluster sizes revealed that TC10 CA significantly increased the percentage of microclusters ($<4\ \mu m^2$) per cell (87.5 ± 1.2 %), whereas TC10 DN had no effect (35.7 ± 3.6 %) as compared to control cells expressing only GFP-gephyrin and SH3(+)CbII (37.9 ± 3.8 %, Figure 14).

Coexpression of TC10 CA or DN with either the GEF-deficient or PI(3)P binding-deficient mutant of Cb (Figure 8) corroborate these findings, showing that TC10 CA triggers GFP-gephyrin microcluster formation in the presence of SH3(+)CbII NE/AA, but not in the presence of SH3(+)CbII RR/AA, while coexpression of TC10 DN never induced gephyrin microcluster formation (Figure 14).

These results indicate that the switching ability of TC10 is not required for gephyrin clustering, instead only GTP-bound TC10 enhances Cb-dependent gephyrin recruitment to the plasma membrane.

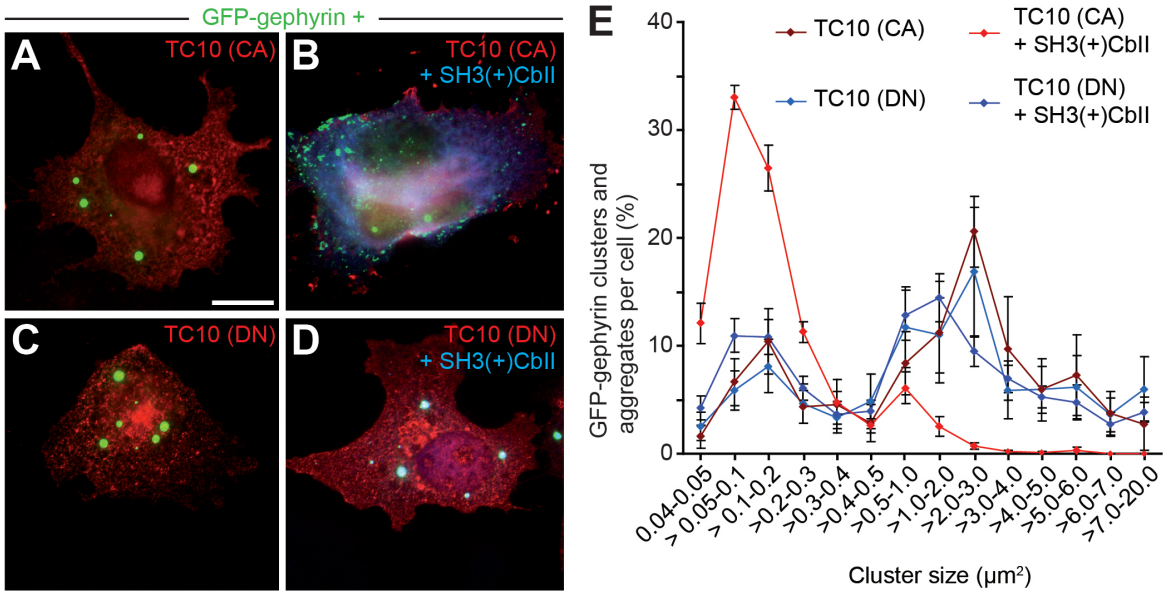

Figure 13: GTP-TC10 is required for Cb-dependent gephyrin redistribution. (A-D) Epifluorescent images of COS7 cells transfected with GFP-gephyrin and Myc-Cb and HA-TC10 as indicated. Scale bar: 10 μm. (E) GFP-gephyrin clusters and aggregates were quantified from images of transfected COS-7 cells and binned according to their size, results are means ± SEM (n=14-34 transfected cells per transfection condition). Experiments were performed by myself, images were prepared by Theofilos Papadopoulos, image analysis was performed conjointly with Theofilos Papadopoulos.

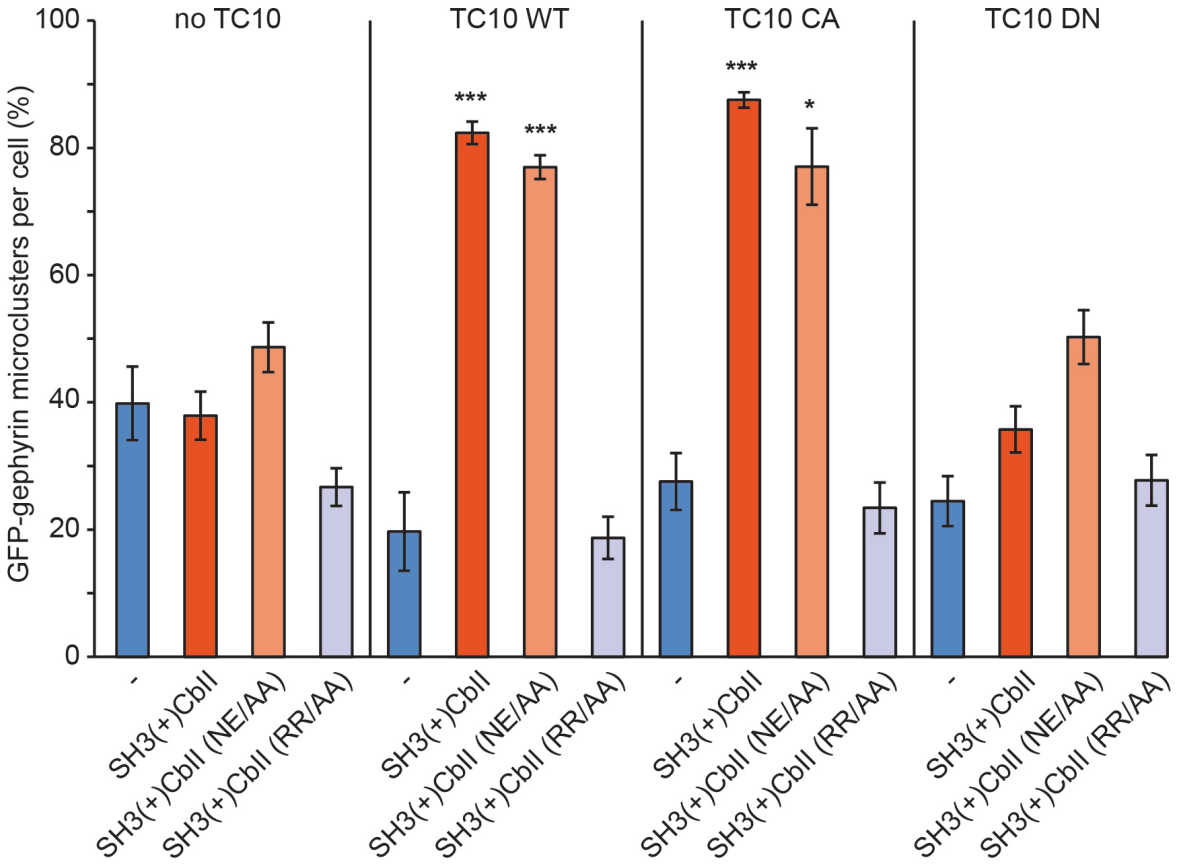

Figure 14: GFP-gephyrin microcluster formation occurs in the presence of GTP-TC10 when SH3(+)CbII binds PI(3)P. Percentages of microclusters (0.04-0.4 μm^2) per cell from images of transfected COS-7 cells, as indicated (n=6-34 cells). Statistical significance was tested between the conditions without coexpression of HA-TC10 (first four columns) and those in the presence of TC10 (WT, CA or DN), results are means ± SEM. Experiments were performed by myself, images were prepared by Theofilos Papadopoulos, image analysis was performed conjointly with Theofilos Papadopoulos.

3.1.4 Cdc42 triggers Cb-dependent gephyrin microcluster formation

While the involvement of Cdc42 in Cb-mediated gephyrin clustering at inhibitory postsynaptic sites is not essential (Reddy-Alla et al., 2010), the ability of Cdc42 to trigger Cb-dependent microcluster formation in non-neuronal cells has not been tested so far. Therefore, we performed the same experiments as with TC10, and cotransfected GFP-gephyrin, Myc-SH3(+)CbII and HA-Cdc42 (brain isoform) in COS7 cells. Coexpression of HA-Cdc42 and GFP-gephyrin led to the formation of intracellular gephyrin aggregates (Figure 15 A), but when Myc-SH3(+)CbII was coexpressed, microclusters formed (Figure 15 B). The percentage of cells classified as having SH3(+)CbII-induced GFP-gephyrin microclusters was significantly higher in the presence of Cdc42 (55.2 $\pm$ 4.5 %) than in its absence (28.2 $\pm$ 4.8 %, Figure 15 E). As with TC10, microcluster formation occurred when GFP-gephyrin was coexpressed with HA-Cdc42 and Myc-SH3(+)CbII NE/AA, the GEF-deficient Cb mutant (Figure 15 C), but not when Myc-SH3(+)CbII RR/AA, the lipid-binding deficient mutant, was coexpressed (Figures 15 D and 8).

Performing the same automated analysis of GFP-gephyrin cluster numbers and sizes, we found that the total number of GFP-gephyrin puncta per cell was significantly increased in the presence of HA-Cdc42 and Myc-SH3(+)CbII compared to coexpression of GFP-gephyrin and Myc-SH3(+)CbII only (Figure 16 A). A significant increase in the percentage of microclusters (0.04-0.4 μm^2) per cell was observed when GFP-gephyrin was coexpressed with HA-Cdc42 and Myc-SH3(+)CbII NE/AA (Figure 16 B and C).

In conclusion, we found that Cdc42 and TC10 share the ability to enhance microcluster formation in the presence if SH3(+)CbII WT and SH3(+)CbII NE/AA, but not together with SH3(+)CbII RR/AA, possibly by relieving the autoinibition of the SH3 domain. These results are in agreement with previous studies in neurons, where it has been shown that overexpression of Cdc42 influences gephyrin cluster formation at synaptic sites (Tyagarajan et al., 2011).

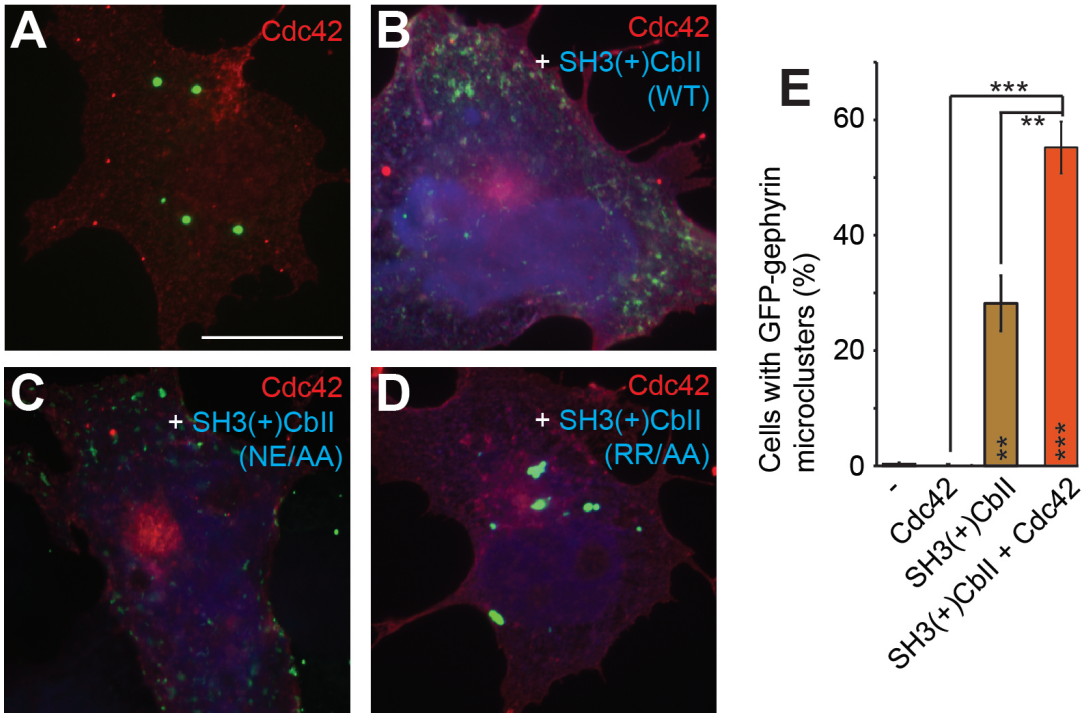

Figure 15: Cdc42 triggers Cb-mediated redistribution of GFP-gephyrin into microclusters. (A-D) Epifluorescent images of COS7 cells transfected with GFP-gephyrin and Myc-Cb and HA-Cdc42 (brain) as indicated. Scale bar: 20 μm. (E) Quantification of the percentage of GFP-gephyrin (co-) transfected cells classified as having GFP-gephyrin microclusters (>50 puncta per cell). Results are means (± SEM, N=3 independent transfections, n= 284-500 cells per transfection condition). The significance level compared to cells transfected only with GFP-gephyrin (first bar) is indicated vertically at the bottom of the bars, significance levels compared to cells transfected with GFP-gephyrin, HA-Cdc42 and Myc-SH3(+)CbII are indicated on top of the bars.

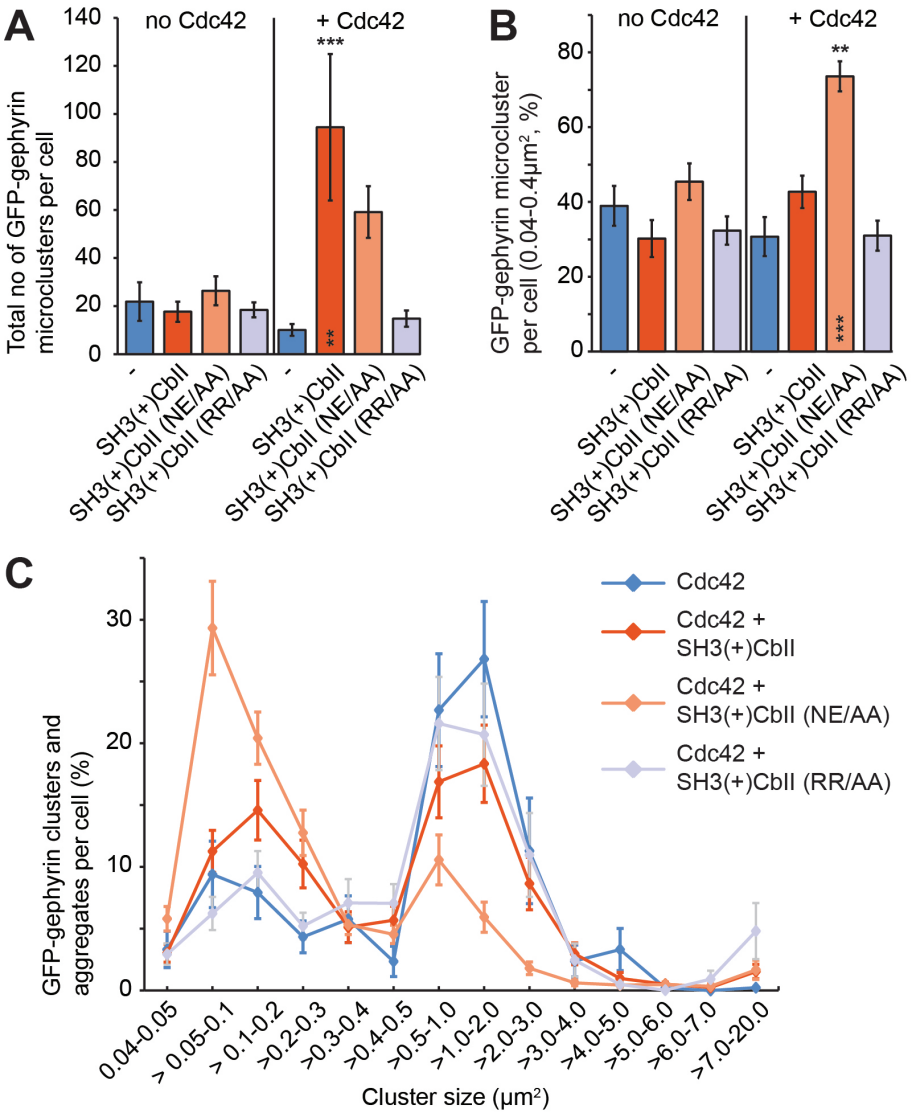

Figure 16: Quantification of Cdc42-triggered Cb-dependent GFP-gephyrin microclusters. (A) Quantification of the total number of GFP-gephyrin clusters and aggregates from epifluorescent images of COS7 cells transfected with Myc-Cb and HA-Cdc42. (B) Percentages of microclusters (0.04-0.4 μm^2) per cell as counted in (A). (A-B) The significance level compared to cells transfected only with GFP-gephyrin (first bar) is indicated vertically at the bottom of the bars. Significance compared to control conditions without Cdc42 on top of the bars. (C) Gephyrin puncta counted in (A) were binned according to their size. Results are means (± SEM, n=23-37 cells per condition).

3.2 The GEF activity of Cb may contribute to gephyrin clustering

When overexpressing gephyrin, SH3(+)CbII and either TC10 or Cdc42 in non-neuronal cells, the GEF activity of Cb is not required for gephyrin microcluster formation (3.1). However, only GTP-loaded TC10 can trigger Cb-dependent gephyrin microcluster formation (3.1.3). Hence, the question arises whether Cb can act as a GEF for TC10 and thus contribute to the accumulation of GTP-TC10. We therefore aimed at investigating, whether Cb, in addition to its reported GEF activity toward Cdc42 (1.2.4.3) (Reid et al., 1999; Tyagarajan et al., 2011), could have catalytic activity towards TC10.

3.2.1 Cb does not activate TC10 *in vitro*

We first tested the nucleotide exchange of Cdc42 and TC10 by SH3(+)CbII and ΔSH3CbII *in vitro* using fluorescence spectroscopy (Jaiswal et al., 2013). All proteins, C-terminally truncated TC10 and Cdc42, SH3(+)CbII and ΔSH3CbII, were bacterially expressed and purified using the GST tag. Subsequently, the tag was removed and small GTPases were labelled with mant-GDP. The exchange rate of Cdc42 and TC10 in the presence or absence of 20-fold higher concentrations of SH3(+)CbII or ΔSH3CbII was then measured by the decrease in fluorescence as mant-GDP was exchanged for GDP, which was provided in excess. The data was fitted to a single exponential function to obtain rate constants. In agreement with results from hPEM-2 (Jaiswal et al., 2013), Cb only accelerated the nucleotide exchange rate of Cdc42, but not TC10 (Figure 17 B). Interestingly, SH3(+)CbII or ΔSH3CbII were similarly capable of catalyzing the nucleotide exchange on Cdc42, with 4.8- and 7.5-fold rate accelerations, respectively (Figure 17 A and B). Samples taken at the end of the *in vitro* exchange reaction and analysed by SDS-PAGE and Coommassie staining, show that this cannot be explained by proteolytic cleavage of the SH3 domain during the incubation (Figure 17 C). This indicates that the catalytic site for Cdc42 is not inhibited in the closed conformation of Cb, when SH3 domain is present. This finding is in agreement with a recently published study, where it was shown that the GEF activity of Cb towards Cdc42 is independent of the presence of the SH3 domain (Tyagarajan et al., 2011). However, previous *in vitro* data using CbI and CbII showed differences in the GEF activity of these two isoforms towards Cdc42 (Xiang et al., 2006), indicating that the C-terminus, rather than the presence or absence of the SH3 domain, may account for the differences in nucleotide exchange activity observed.

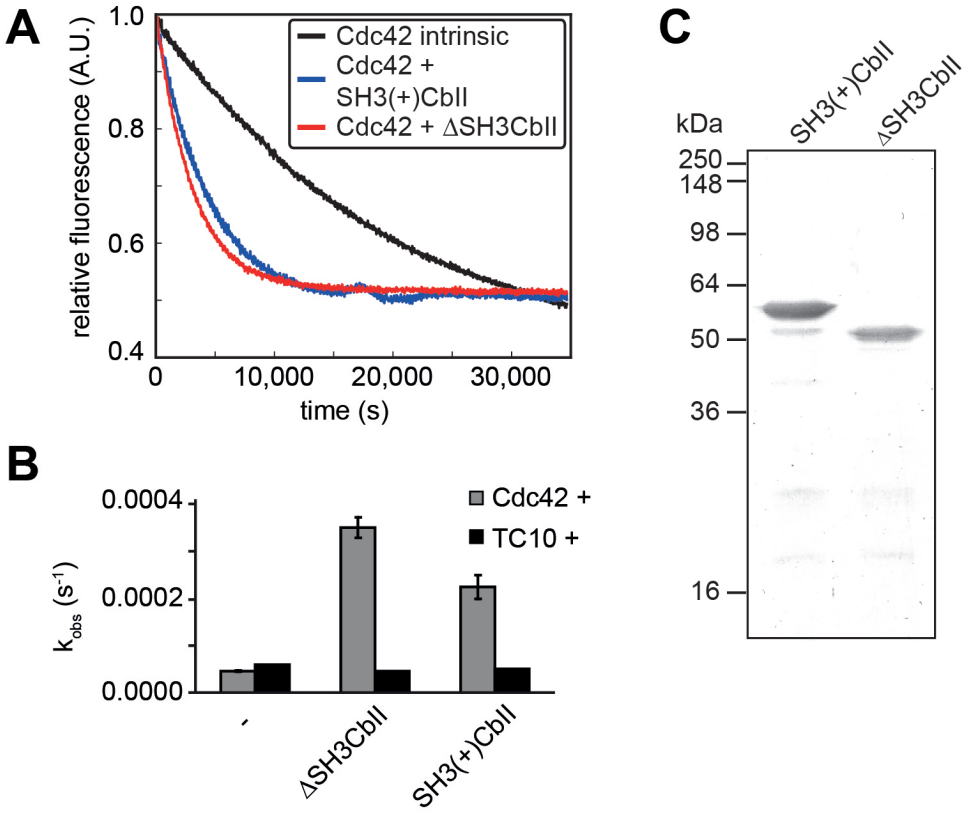

Figure 17: SH3(+)CbII and ΔSH3CbII activate Cdc42, but not TC10 *in vitro*. Spectroscopic measurements of nucleotide exchange from methylanthraniloyl-GDP (mant-GDP) to GDP on C-terminally truncated Cdc42 and TC10 in the presence or absence of ΔSH3CbII or SH3(+)CbII. All proteins were bacterially expressed and purified. (A) Exemplary traces of spectroscopic measurements of mant-GDP dissociation from Cdc42, without the addition of a GEF (black), or in the presence of SH3(+)CbII (blue) or ΔSH3CbII (red), A.U.: arbitrary units. (B) The rate constants (k_{obs}) of intrinsic, ΔSH3CbII-catalyzed or SH3(+)CbII-catalyzed reactions of either Cdc42 or TC10 were obtained by single exponential fitting of the data. For Cdc42, the mean values (± SEM) of three to four independent experiments are shown. (C) Analysis of samples taken at the end of the *in vitro* exchange assay by SDS-PAGE and Coomassie staining showing that no degradation of proteins occurred. TC10 and Cdc42 proteins for these assays were prepared by Mamta Jaiswal, experiments were carried out jointly by myself and Mamta Jaiswal (Institute for Biochemistry and Molecular Biology II, Medical Faculty, Heinrich-Heine-University, Düsseldorf, Germany).

3.2.2 Cb activates TC10 in cells

Since the enzymatic activity of GEFs may be influenced by the cellular context, we next tested the activation of TC10 by Cb in cells, using an established assay, in which GTP-bound GTPases specifically bind to an immobilised effector domain (Reddy-Alla et al., 2010). We expressed TC10 in the presence and absence of SH3(+)CbII and ΔSH3CbII in HEK293 cells, lysed the cells and analyzed the co-sedimentation with immobilized GST-PAK1-PBD. We found that ΔSH3CbII WT coexpression significantly

increased the GTP-TC10 content in cells, due to its GEF activity, since the GEF-deficient ΔSH3CbII NE/AA mutant did not show the same effect (Appendix Figures 27 A1, B and 8). The PI(3)P-binding ability was, however, not required for the accumulation of GTP-TC10 in cells, since the or ΔSH3CbII RR/AA (Figure 8) was as efficient as ΔSH3CbII WT in enhancing GTP-TC10 formation (Appendix Figure 27 A2, B).

In contrast, SH3(+)CbII coexpression with TC10 did not lead to an increase in GTP-TC10 (Appendix Figure 27 A3, B). In order to fully mimic the conditions used in the COS7 cell microcluster formation assay, we coexpressed GFP-gephyrin along with Cb isoforms and TC10. Surprisingly, we found an accumulation of GTP-TC10 in the presence of GFP-gephyrin and ΔSH3CbII or SH3(+)CbII WT or RR/AA, but not with SH3(+)CbII NE/AA (Appendix Figure 27 A3, B).

In summary, these results show that the GEF activity of Cb towards TC10 is regulated in a more complex manner than the GEF activity of Cb towards Cdc42. While TC10 nucleotide exchange is not catalyzed by Cb *in vitro*, in cells, catalysis is possible when Cb is in its open conformation. Gephyrin enhances the GEF activity of SH3(+)CbII towards TC10, possibly by changing Cb structure upon binding in such a way that TC10 binding to the DH domain is promoted and hence catalysis can occur.

3.3 Binding between Cb and TC10

While we could show that GTP-TC10 accumulation is enhanced through the GEF activity of Cb (3.2.2), this is not essential for Cb-dependent gephyrin microcluster formation in COS7 cells (3.1.2). Instead, we addressed the question whether a binding interaction between TC10 and Cb could mediate gephyrin clustering in a manner that depends on the nucleotide-loaded state of TC10, since only GTP-TC10 could trigger Cb-dependent gephyrin clustering in COS7 cells (3.1.3).

We perfomed *in vitro* binding assays using bacterially expressed and purified proteins. GST-tagged Cb protein isoforms coupled to agarose beads were incubated with His-tagged TC10 preloaded with GDP, GTPγS or trapped in its nucleotide-free state. Co-sedimentation of His-TC10 with immobilized GST-Cb was assayed by Western Blotting. As a positive control for the nucleotide binding reactions, we used immobilized GST-PAK1-PBD, a known effector of TC10, which interacts specifically with GTP-TC10 (3.2.2), as a negative control immobilized GST was used.

3.3.1 Cb has two binding sites for TC10

First, we used the constitutively active Cb isoform lacking the SH3 domain in the binding assays (Figure 8). We found that TC10 bound weakly to ΔSH3CbII in the GDP-bound form and when nucleotide-free, whereas binding was strong in the GTP-bound form (Figure 18 A). Since we found that Cb-dependent gephyrin microcluster formation is not possible in the presence of the PI(3)P-binding deficient mutant of Cb (Figure 8), we also tested whether in addition to being important for lipid binding, these residues would also be involved in the binding to TC10. However, ΔSH3CbII RR/AA bound to TC10 in the same way as ΔSH3CbII WT, with the strongest binding in the GTP-bound state (Figure 18 A). These findings show that the Arg residues in the PH domain necessary for PI(3)P binding are not required for the interaction with TC10. Therefore the inhibitory effects of the RR/AA mutation in the Cb PH domain on gephyrin microcluster formation may not be explained by a loss of binding to TC10 and potentially other small GTPases, but are likely crucial for membrane attachment, as suggested previously (Reddy-Alla et al., 2010).

We next sought to determine the binding site for TC10 on Cb. Therefore, we used SH3(+)CbII and truncated Cb proteins in binding assays with His-TC10. TC10 bound GST-SH3(+)CbII weakly and relatively independently of the nucleotide-bound state of TC10 (Figure 18 B). A mutant lacking the PH domain (GST-SH3(+)CbIIΔPH, Figure 8) bound TC10 predominantly in its GDP-bound form, while the PH domain alone (GST-PH, Figure 8) showed a very strong interaction specifically with GTPγS-TC10. This interaction was comparable to that of GST-ΔSH3CbII (Figure 18 A) and GST-PAK1-PBD (Figure 18 A and B). This indicates that the major binding site for TC10 on Cb is in the PH domain and is specific for the GTP-bound state of TC10, resembling the interaction between GTPases and their effectors (1.3.1).

Taken together, these results indicate that there are two binding sites for TC10 on Cb: One GDP-specific in the DH domain, one GTP-specific in the PH domain.

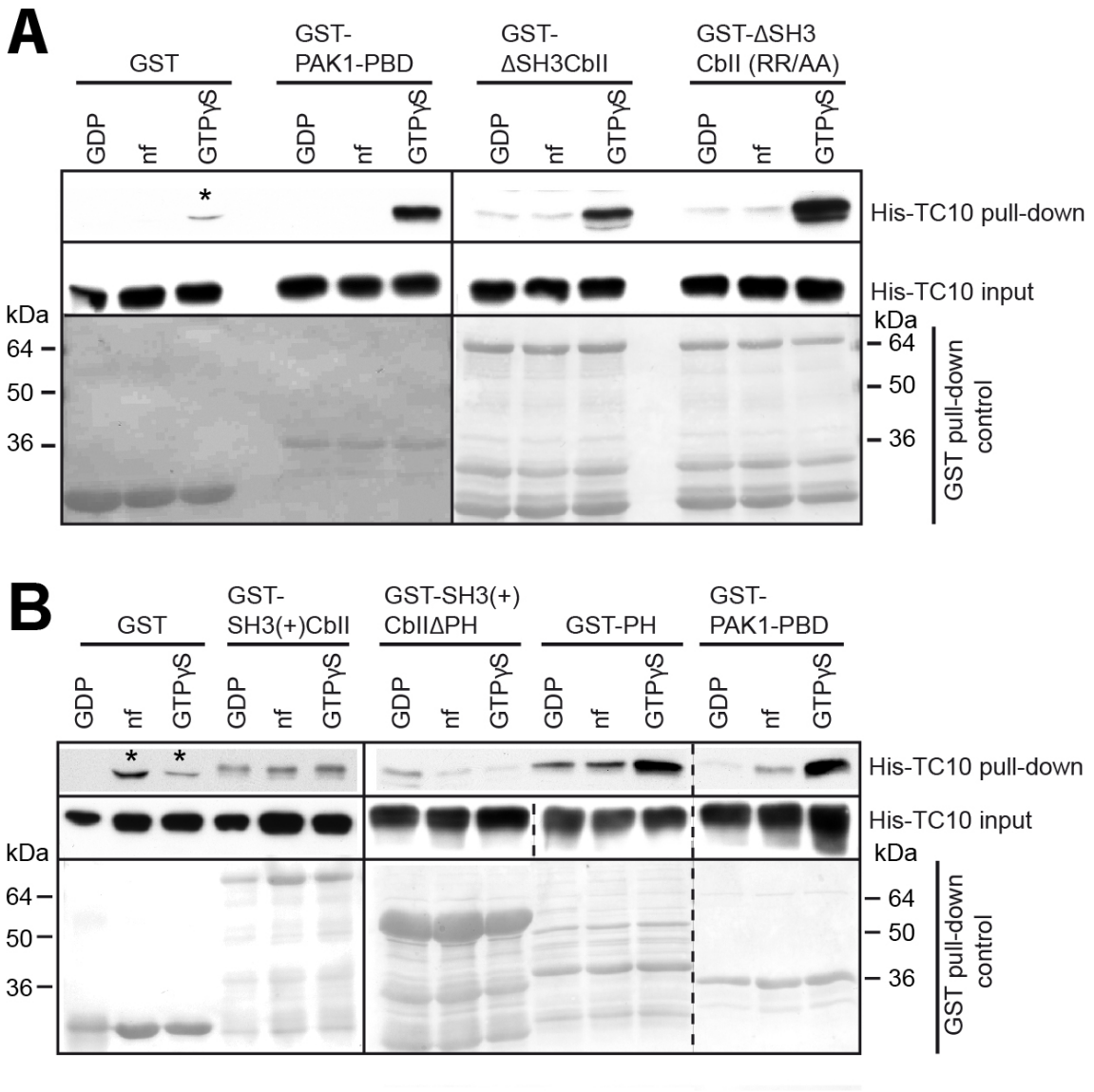

Figure 18: GTP-TC10 binds to the PH domain of Cb. Incubation of purified His-TC10, either nucleotide-free (nf) or preloaded with GDP or GTPγS, with the indicated immobilized GST-tagged proteins (shown as a MemCode staining of the pulldown in the bottom panel). Bound and input His-TC10 was detected by Western blotting (top and middle panel, respectively). (A) Binding assays using GST-ΔSH3CbII WT and RR/AA, and GST and GST-PAK1-PBD as controls. (B) Binding assays using GST-SH3(+)CbII, GST-SH3(+)CbIIΔPH and GST-PH, and GST and GST-PAK1-PBD as controls. Asterisks in A and B indicate non-specifically stained bands migrating at a lower molecular weight than His-TC10.

3.3.2 Binding between endogenous TC10 and Cb

Having shown that Cb can bind TC10 *in vitro* (3.3.1), we aimed at showing an interaction between endogenous TC10 and Cb in the brain. In order to achieve this, we used rat brain extracts and established a protocol to immunoprecipitate Cb using a polyclonal rabbit anti Cb antibody. We then tested whether TC10 co-immunoprecipitates with Cb by Western Blotting using an anti-TC10 antibody. Due to the strong binding of GTP-TC10 with Cb in the *in vitro* binding assays, we hypothesized that this might also occur in the brain and hence preincubated brain lysates with GTPγS or GDP prior to the immunoprecipitation (IP). However, only in one out of three experiments, we could observe a faint TC10-specific band in the IP samples that were GTPγS-loaded (Figure 19). Since we could not reproducibly detect this weak interaction, we conclude that it is difficult to show the binding interaction between GTP-TC10 and Cb in the brain using IP, probably because of its low-affinity, transient nature.

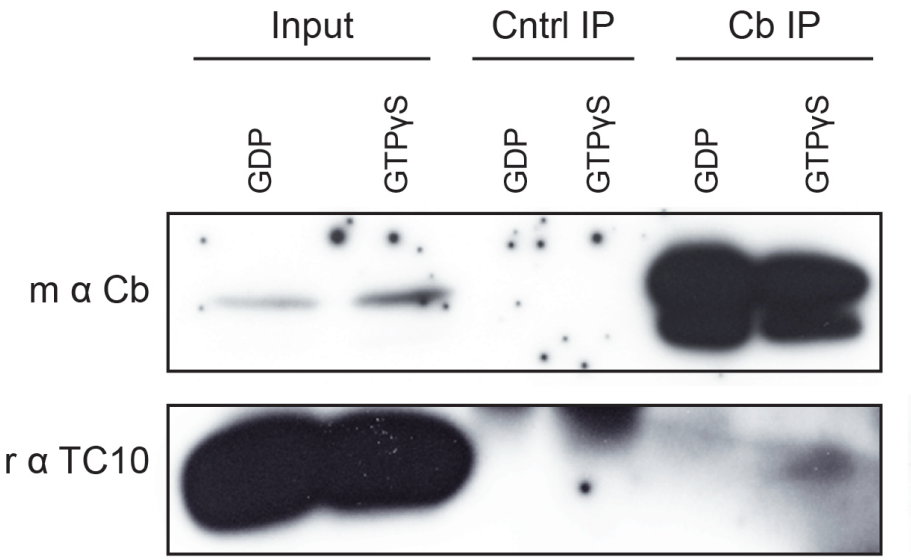

Figure 19: Potential co-immunoprecipitation of TC10 and Cb. Extracts from adult rat brain preincubated with GDP or GTPγS in conditions that allow nucleotide-loading onto GTPases. Immunoprecipitation was performed using a rabbit antibody against Cb (Cb IP) or ChromoPure rabbit IgG (Cntrl IP) followed by Western blotting using a mouse anti-Cb monoclonal antibody (top panels) or a rabbit anti-TC10 polyclonal antibody (bottom panels). Input samples show that equal amounts of lysate, containing similar amounts of Cb and TC10, were used in both conditions.

3.4 TC10 overexpression affects inhibitory postsynapses

Having shown that TC10 may be activated by Cb in cells (3.2.2) and in its GTP-bound form can bind to the Cb PH domain (3.3.1) and hence trigger gephyrin clustering in non-neuronal cells (3.1.3), we aimed at determining how TC10 overexpression affects inhibitory synapses. We therefore overexpressed TC10 CA and TC10 DN along with SH3(+)CbII in dissociated rat hippocampal neurons and analyzed the effect on synaptic gephyrin clustering (3.4.1) and inhibitory synapse function (3.4.2).

3.4.1 TC10 overexpression alters synaptic gephyrin clustering

To determine the effect of TC10 on the synaptic clustering of endogenous gephyrin, we transfected dissociated rat hippocampal neurons at days *in vitro* (DIV) 4 with Myc-ΔSH3CbII, and Myc-SH3(+)CbII in the presence or absence of HA-TC10 CA or HA-TC10 DN. Cells were fixed after ten days and immunocytochemistry for gephyrin, vesicular inhibitory amino acid transporter (VIAAT) and Myc- and HA-tags was performed. TC10 CA overexpression resulted in an increase in perisomatic gephyrin cluster density and size as well as in an increase in dendritic gephyrin cluster size (Appendix Figure 28). Conversely, TC10 DN overexpression induced a decrease in perisomatic and dendritic gephyrin cluster density (Appendix Figure 28). These postsynaptic changes were not accompanied by changes in the inhibitory presynapse marker VIAAT (Appendix Figure 29), indicating that TC10 only regulates inhibitory postsynapses.

In conclusion, in neurons, GTP-TC10 enhances the gephyrin clustering like in non-neuronal cells, while GDP-TC10 has a dominant negative effect and diminishes synaptic gephyrin clustering.

3.4.2 TC10 overexpression regulates inhibitory postsynaptic strength

In addition to examining the consequences of TC10 overexpression on gephyrin recruitment to inhibitory postsynaptic sites, we also analyzed the impact of TC10 overexpression on inhibitory neurotransmission by recording mIPSCs in rat hippocampal neurons transfected with GFP and Myc-SH3(+)CbII in the absence or presence of HA-TC10 CA or DN. We found a significant increase in mIPSC amplitudes when TC10 CA was overexpressed and a significant decrease in mIPSC frequency when TC10 DN was overexpressed (Appendix Figure 30 A1-A3). These observations may be explained by an increase in the number of functional postsynaptic $GABA_A$Rs upon TC10 CA and

Myc-SH3(+)CbII overexpression and a loss of $GABA_ARs$ from some postsynapses upon TC10 DN and Myc-SH3(+)CbII overexpression. This interpretation is further corroborated by the finding that amplitudes of IPSCs evoked by a local rapid increase in the extracellular K^+ concentration were increased significantly in TC10 CA-coexpressing neurons compared to controls (Appendix Figure 30 B1-B2).

3.5 Molecular mechanism of TC10-mediated gephyrin clustering

GTP-TC10 binding to the PH domain of Cb can enhance gephyrin clustering at synaptic sites and hence inhibitory neurotransmission. The molecular mechanism through which TC10 achieves this and in which subcellular compartment this interaction takes place is, however, unclear.

The N- and C-termini of small GTPases are highly variable and especially the C-terminus plays an important role in regulating their biological function by influencing the subcellular localisation (1.3.1).

Previously, the mechanism through which TC10 achieves GLUT4 exocytosis in adipocytes has been studied in detail and various mutations in the N- and C-termini that affect a specific interaction of TC10 with other proteins or lipids have been characterized (1.3.2.2).

We therefore sought to analyze which function of TC10 is important for mediating Cb-dependent gephyrin clustering by using the previously described mutations of HA-TC10 in the Myc-SH3(+)CbII-dependent GFP-gephyrin microcluster formation assay in COS7 cells (3.1).

3.5.1 The TC10 C-terminus is required for gephyrin clustering

A first indication that the TC10 C-terminus plays an important role in Cb-dependent gephyrin clustering was obtained by analyzing the ability of chimerical constructs, in which the C-terminus of TC10 was exchanged by the one from H-Ras or K-Ras, as was initially described by Watson et al. (2001). The C-termini of H-Ras and K-Ras are characterized by a CaaX box (Figure 20, highlighted in green and grey); additionally, H-Ras has a dual palmitoylation site in the C-terminus (Figure 20, highlighted in blue), while K-Ras has a polybasic stretch in the C-terminus (Figure 20, highlighted in red). The C-terminus of TC10 contains these three functional elements (Figure 20). The localisation of TC10 WT and TC10-H-Ras was reported to be identical, with a

subcellular accumulation perinuclearly and at the plasma membrane, while TC10-K-Ras was localised only at the plasma membrane (Watson et al., 2001).

In the COS7 cell assay, only 34.9 ± 5.8 % of cells coexpressing HA-TC10-H-Ras, GFP-gephyrin and Myc-SH3(+)CbII and 21.6 ± 4.3 % of cells coexpressing HA-TC10-K-Ras, GFP-gephyrin and Myc-SH3(+)CbII showed GFP-gephyrin microclusters compared to 100% of cells expressing HA-TC10 WT together with Myc-SH3(+)CbII and GFP-gephyrin (Figure 21). This indicates that the C-terminus of TC10 is essential for mediating Cb-dependent gephyrin clustering. Neither a similar pattern of palmitoylation, as found in H-Ras, nor a long polybasic stretch, as in K-Ras, was sufficient to induce Cb-dependent gephyrin microcluster formation.

Figure 20: Sequence alignment of the C-termini of small GTPases. Sequence alignment of the last 22 amino acids of the Rho family GTPases TC10 and Cdc42 (placental isoform) and the Ras family GTPases H-Ras and K-Ras (4B). Subcellular localisations as listed in Roberts et al. (2008). PM, plasma membrane; ER, endoplasmic reticulum; Golgi, Golgi apparatus; NE, nuclear envelope. Basic residues are highlighted in red, palmitoylated Cys are highlighted in blue, prenylated Cys are highlighted in green, the proteolytically cleaved amino acids are highlighted in grey. The phosphorylation site on TC10 is highlighted in purple.

We therefore performed a more detailed analysis of the different functional elements of the TC10 C-terminus to unravel the contributions of prenylation, palmitoylation and the polybasic residues to Cb-dependent gephyrin microcluster formation.

3.5.1.1 TC10 prenylation is essential for Cb-dependent gephyrin microcluster formation

In order to test the importance of membrane anchoring of TC10 by prenylation, we mutated the Cys residue of the CaaX box (C210S, highlighted in green in Figure 20). This mutation leads to a cytosolic distribution of the protein (Watson et al., 2003). As expected based on the observation that the TC10-H-Ras and TC10-K-Ras chimeras could not mediate gephyrin clustering, TC10 C210S also led to the accumulation of gephyrin in cytoplasmic aggregates in the presence of SH3(+)CbII in the majority of

cells (Figure 21). This result is in line with the notion that the prenylation of small GTPases is essential for their function (Winter-Vann and Casey, 2005).

The nature of the CaaX box may be important in directing posttranslational modifications of small GTPases (Roberts et al., 2008). To test whether the differences in their ability to induce gephyrin microclustering between TC10 WT and TC10-H-Ras might be due to such a regulatory effect, we exchanged the three terminal amino acids of TC10 with those of H-Ras. We found that this chimera (TC10 LIT211-3VLS) was able to trigger Cb-dependent gephyrin microcluster formation in 91.1 ± 11.8 % of cells compared to 100 % of cells in the presence of TC10 WT (Figure 21).

3.5.1.2 TC10 palmitoylation is not required for Cb-dependent gephyrin microcluster formation

A second, reversible mode of membrane attachment is the palmitoylation of TC10 at two Cys residues (C206 and C209, highlighted in red in Figure 20, Michaelson et al. (2001); Roberts et al. (2008)). Based on previous studies, mutating only C206 has little effect on the subcellular localisation of TC10, while mutating C209 or both C206 and C209 leads to the exclusion from lipid raft compartments with a predominant localisation of the protein at the plasma membrane (Watson et al., 2003). However, treatment with a palmitoylation inhibitor does not affect TC10 plasma membrane and endomembrane localisation (Roberts et al., 2008; Valero et al., 2010).

Mutating all three Cys residues thereby abolishing lipid anchoring at membranes led to a loss of gephyrin microcluster formation, with 38.0 ± 1.9 % of cells showing SH3(+)CbII induced GFP-gephyrin microclusters compared to 100% in the presence of HA-TC10 WT (Figure 21). However, mutating one or both of the palmitoylated Cys residues did not affect TC10-triggered Cb-dependent gephyrin clustering negatively (Figure 21).

The lipid raft localisation of TC10 has been described to be regulated by phoshorylation of T197 by Cdk5 (highlighted in purple in Figure 20, Okada et al. (2008)). In line with the observation that lipid raft localisation of TC10 is not required for gephyrin clustering in COS7 cells, phosphomimetic or phosphodeficient mutations of this residue did not affect gephyrin microcluster formation either (Figure 21).

3.5.1.3 Basic residues are important for TC10-mediated Cb-dependent gephyrin microcluster formation

As described for K-Ras, basic amino acid residues can act as a second membrane attachment motif in addition to prenylation. Since the palmitoylation of TC10 is not required for plasma membrane and endomembrane localisation, this may indicate an

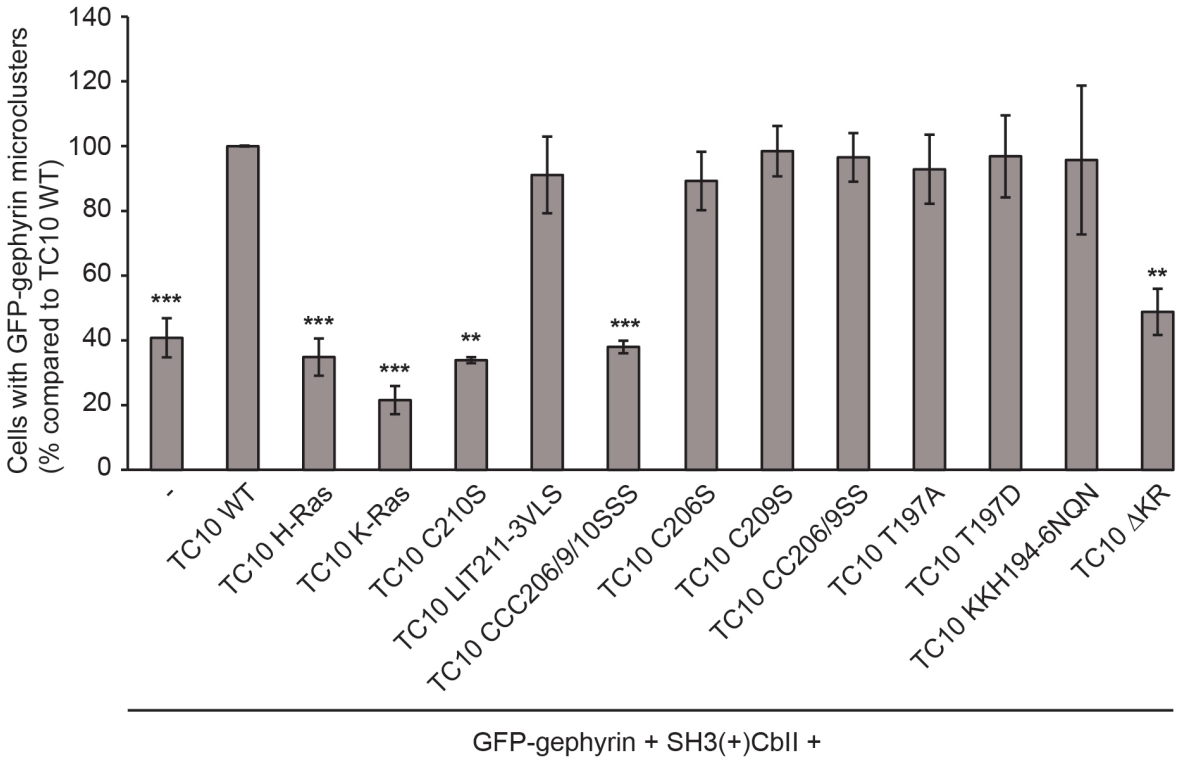

Figure 21: TC10-triggered Cb-dependent microcluster formation depends on the C-terminus of TC10. Quantification of the percentage of cells cotransfected with GFP-gephyrin Myc-SH3(+)CbII in the presence or absence of HA-TC10 mutants classified as having GFP-gephyrin microclusters (>50 puncta per cell). Values were normalized to the level of GFP-gephyrin microclusters in cells transfected with GFP-gephyrin, Myc-SH3(+)CbII and HA-TC10 WT. Results are means (± SEM, N=3-4 independent transfections, n= 348-630 cells per transfection condition). The significance level compared to cells transfected only with GFP-gephyrin, Myc-SH3(+)CbII and HA-TC10 WT (second bar) is indicated.

important role of the basic residues of TC10 in subcellular targeting (Valero et al., 2010). The TC10 C-terminus contains six basic amino acid residues (highlighted in red in Figure 20). Two of these residues in conjunction with the adjacent His (KKH) have been described to be important to prevent sorting of TC10 to the lysosomal compartment (Valero et al., 2010).

Coexpression of HA-TC10 KKH194-6NQN with Myc-SH3(+)CbII and GFP-gephyrin, resulted in the formation of GFP-gephyrin microclusters in the majority of cells, with no statistically significant difference compared to coexpression of TC10 WT (Figure 21).

Mutating the four Lys residues in the TC10 C-terminus does not affect the subcellular localisation of TC10, according to a previous study (Watson et al., 2003). In order to determine the contribution of the basic residues to TC10 function in gephyrin clustering, we therefore replaced all Lys and Arg residues with Ala residues (highlighted

in red in Figure 20, denoted as TC10 ΔKR). This mutant could not trigger the formation of Cb-dependent gephyrin microclusters in COS7 cells: only 48.8 ± 7.1 % of cells displayed GFP-gephyrin microclusters compared to 100% of those expressing TC10 WT (Figure 21). This indicates that the anchoring of TC10 to membrane through the interaction of its polybasic stretch with negatively charged membrane lipids may be important for triggering Cb-dependent gephyrin microcluster formation.

3.5.2 The TC10 N-terminus is required for gephyrin clustering

The N-terminus of TC10 is extended compared to other Rho GTPases such as Cdc42 (Figure 7). TC10 exerts its cellular function not only by binding to effectors in its GTP-bound state, but also through essential interactions with the cortical actin cytoskeleton though its N-terminal 16 amino acids in adipocytes (Chunqiu Hou and Pessin, 2003). This is mediated through the GPG or GAG sequence motifs in the N-terminus (Figure 22, Chunqiu Hou and Pessin (2003)). We therefore tested the involvement of the TC10 N-terminus in Cb-dependent GFP-gephyrin microcluster formation by transfecting COS7 cells with different N-terminal TC10 mutants along with GFP-gephyrin and Myc-SH3(+)CbII. Deletion of the 16 N-terminal amino acids (TC10 ΔN), led to a significant reduction of GFP-gephyrin microclusters (45.7 ± 8.6 % of TC10 WT-induced microclusters) compared to TC10 WT (Figure 23). However, mutating either one or both the two sequences motifs suspected to be involved in cortical actin disruption, GAG3-5 and GPG12-14, did not significantly decrease TC10- and Cb-induced gephyrin microcluster formation (Figure 23). Therefore, either further residues in the N-terminus than those six are involved in inducing cortical actin disruption, or the N-terminus has a different, novel function that is essential for Cb-dependent gephyrin clustering.

Figure 22: Sequence alignment of the N-termini of TC10 variants. Comparison of the first 16 amino acids of the human TC10 (hTC10), and mouse TC10 (mTC10) α and β. GAG / GPG motifs mediate cortical actin disruption and are highlighted in red.

Finally, there are two TC10 isoforms in the mouse, mTC10α and β, which are transcribed from two different genes and differ mostly in their N- and C-termini (Figure

7, Chiang et al. (2002)). While their subcellular localisation was reported to be similar, likely due to the similarity of their C-termini, only TC10α is capable of disrupting cortical actin in adipocytes (Chiang et al., 2002).

We therefore tested the ability of these two isoforms to trigger Cb-dependent gephyrin microcluster formation. While mTC10α potently triggered the formation of GFP-gephyrin microclusters in the presence of Myc-SH3(+)CbII with 85.3 ± 18.6 % of cells showing microclusters compared to TC10 WT, mTC10β induced GFP-gephyrin microclusters only in 47.7 ± 7.9% of cells compared to TC10 WT. This difference was statistically not significantly different from TC10 WT. However, mTC10β induces microclusters in significantly fewer cells than mTC10α, showing that the N- and C-termini may be functionally important in the mechanism underlying TC10-triggered Cb-dependent gephyrin microcluster formation.

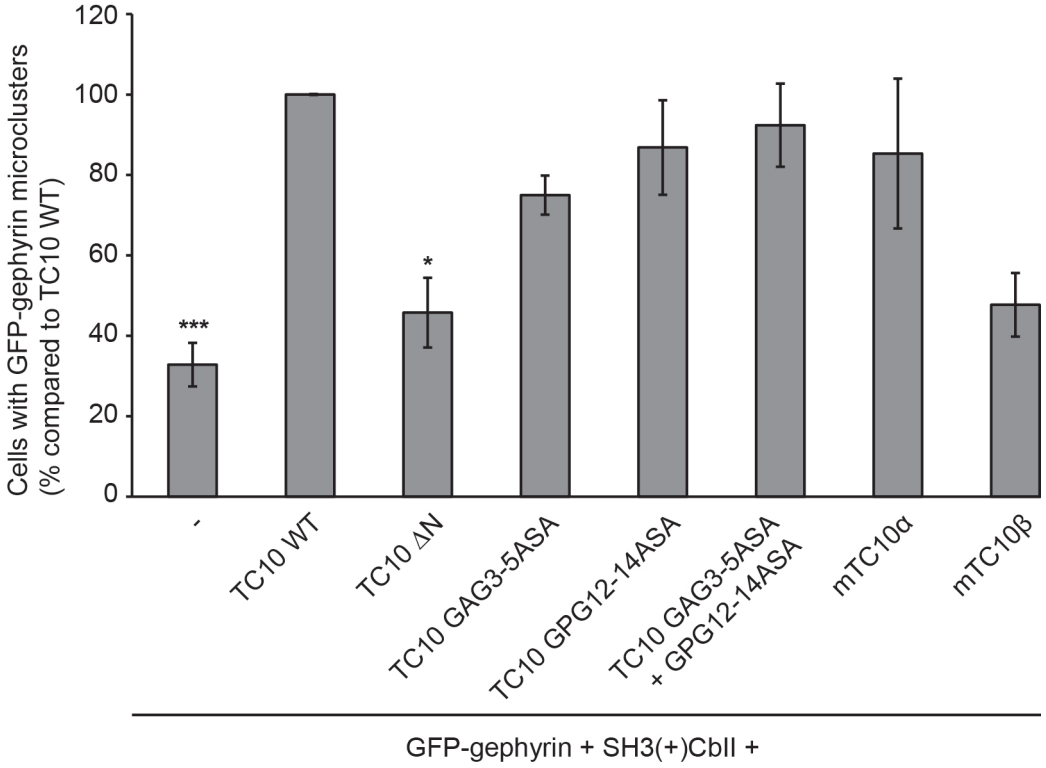

Figure 23: TC10-triggered Cb-dependent microcluster formation depends on the N-terminus of TC10. Quantification of the percentage of cells cotransfected with GFP-gephyrin Myc-SH3(+)CbII in the presence or absence of HA-TC10 mutants classified as having GFP-gephyrin microclusters (>50 puncta per cell). Values were normalized to the level of GFP-gephyrin microclusters in cells transfected with GFP-gephyrin, Myc-SH3(+)CbII and HA-TC10 WT. Results are means (± SEM, N=3-4 independent transfections, n= 389-532 cells per transfection condition). The significance level compared to cells transfected only with GFP-gephyrin, Myc-SH3(+)CbII and HA-TC10 WT (second bar) is indicated.

3.6 Loss of function of endogenous TC10

So far, our experiments have focused on overexpressing TC10 together with Cb and analysing the effects on gephyrin clustering in cell culture and in neurons. However, it is unclear, whether TC10 is required for gephyrin clustering. Due to the observation that Cdc42 can also activate SH3(+)CbII in COS7 cells and hence trigger gephyrin clustering (3.1.4) and Cdc42 deletion does not affect gephyrin clustering (Reddy-Alla et al., 2010), one possibility is that TC10 and Cdc42 act in a redundant manner.

Since no KO mouse of TC10 is available so far (Figure 6), we aimed at generating one using embryonic stem cells containing the appropriate targeting vector provided by the European Conditional Mouse Mutagenesis Program (EUCOMM). The quality control by EUCOMM failed, however, so that we could not start generating the mice within the framework of this dissertation.

Instead, we sought to identify a suitable TC10 knockdown strategy. We tested three different miRNA constructs (provided by Invitrogen) and four different shRNA constructs (provided by Genecopoeia). We tested three miRNAs and two shRNAs with target sequences in the coding sequence of TC10 further, by measuring the knockdown efficiency of human HA-TC10, which had been mutagenized to obtain rat-specific sequences at the miRNA or shRNA binding sites. After cotransfection of these HA-TC10 constructs with the knockdown vectors, we obtained the highest HA-TC10 knockdown efficiency (approximately 60 % compared to cells transfected with the negative control vector) with miRNA 4D. We then chained two miRNA4D in the same vector, however, this decreased the knockdown efficiency.

We then tested the knockdown efficiency of endogenous TC10 by generating clonal lines of Rat2 cells expressing this miRNA through selection in blasticidine-containing medium. Additionally, all four shRNA constucts were tested with this approach. Lysates of these semistably transfected cells were analyzed by Western Blotting with a TC10-specific antibody and a tubulin-specific antibody as a loading control (Figure 24 A). None of the shRNA-transfected cells showed a significant decrease in TC10 protein levels compared to the negative control. However, a reduction in TC10 protein levels was observed in preliminary experiments, therefore we analyzed the knockdown efficiency more quantitatively using the LiCor Odyssey Infrared Imager (Figure 24 A). If considering the negative control clone with the highest TC10 level (negative control clone 2) as a reference for calculating the knockdown efficiency of the TC10 knockdown clone with the lowest relative TC10 level (TC10 miRNA 4D clone 1), the knockdown efficiency is only 51 % (Figure 24 B). Even smaller knockdown efficiencies would result from comparing the other clonal lines. Surprisingly, the expression level of the reporter emGFP was only detected in a small proportion of cells in these semistable cell lines,

but this does not necessarily correlate with a loss of miRNA expression according to the manufacturer. Since we could not obtain high enough knockdown efficiencies for TC10 so far, other miRNAs or shRNAs need to be tested or different constructs need to be combined to achieve higher knockdown efficiencies, required to assess the contribution of inhibitory synapse function.

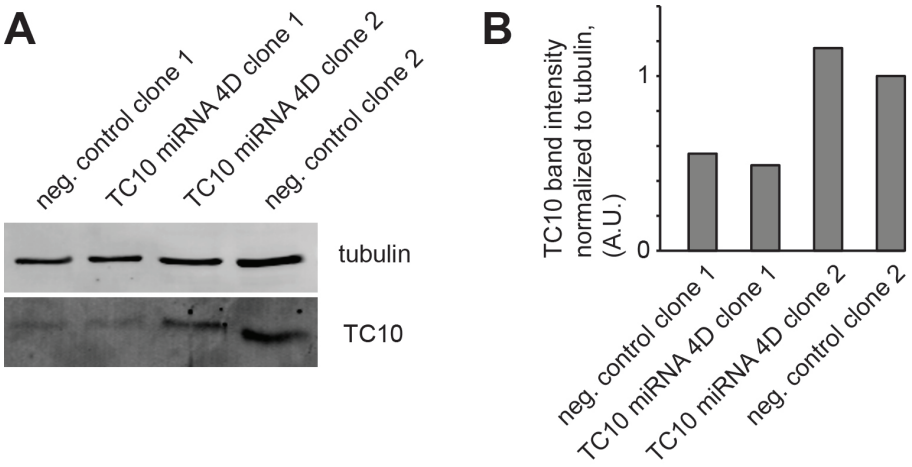

Figure 24: Knockdown efficiency of TC10-specific miRNA. Rat2 cells were transfected with miRNA-constructs at a low cell density. Clones were picked and expanded in blasticidine-containting medium. Cell lysates were analysed by Western Blotting with a TC10-specific antibody and a tubulin-specific antibody as a loading control. (A) Two clones of a negative (neg.) control miRNA and two clones of a TC10-specific miRNA (miRNA 4D) were analyzed by Western Blotting with the Odyssey scanner. (B) TC10-expression in each of the clones in shown in (A), normalized to the tubulin. A.U., Arbitrary Unit.

4

Discussion

Inhibitory synapses are crucial in neuronal networks and their dysfunction is implicated in a host of psychiatric and neurodevelopmental disorders. Their functionality depends on the precise alignment of pre- and postsynaptic specialisations as well as the regulated recruitment of proteins involved in signal transduction to synaptic sites. The GEF Cb plays a key role in the formation of inhibitory postsynapses since it links transsynaptic signalling through NLs with the stabilisation of inhibitory postsynapses by recruiting the scaffold protein gephyrin (Papadopoulos and Soykan, 2011). Therefore, the loss of Cb is detrimental for the formation and maintenance of a subset of inhibitory postsynapses in the mammalian brain (Papadopoulos et al., 2007, 2008).

Most Cb isoforms possess an autoinhibitory SH3 domain, so activation is required prior to their function in synapse formation (Kins et al., 2000; Harvey et al., 2004). Several synaptic proteins have been described to interact with the SH3 domain and thereby relieve this autoinhibition (Poulopoulos et al., 2009; Hoon et al., 2011; Saiepour et al., 2010), however, they cannot explain the activation of Cb at all types of synapses, so other activating proteins must exist (Brose, 2013).

Furthermore, Cb is characterized by a tandem DH-PH domain, the hallmark of GEFs for small Rho-like GTPases, which are involved in the regulation of the actin cytoskeleton. Cdc42 is the only Rho-like GTPase activated by Cb (Jaiswal et al., 2013) and controls the actin cytoskeleton, a component of inhibitory postsynapses. Therefore it has been suspected that activation of Cdc42 by Cb might be involved in inhibitory synapse formation. In support of this hypothesis, Cdc42 has been shown to bind to both Cb and gephyrin, and overexpression of Cdc42 in neurons leads to enhanced gephyrin clustering (Tyagarajan et al., 2011). However, conditional deletion of Cdc42 in the forebrain did not impact on gephyrin or $GABA_AR$ clustering (Reddy-Alla et al., 2010). Therefore, it is an open question which role small GTPases play in inhibitory synapse assembly.

In this study, we have investigated a potential involvement of the small Rho family GTPase TC10, the closest homolog of Cdc42 (Figure 6), in Cb-dependent gephyrin clustering. We show that TC10 can enhance gephyrin clustering and thereby increase inhibitory neurotransmission by binding to the PH domain in its GTP-bound state and providing a second membrane anchor for Cb. Thus GTP-TC10 and GTP-Cdc42 can relief the autoinhibition of Cb exerted by the SH3 domain through a novel mechanism.

4.1 Small GTPases can trigger Cb-dependent gephyrin clustering

The potential of proteins to relieve the autoinhibition of Cb can be tested by heterologous expression in non-neuronal cells. While GFP-gephyrin coexpression with SH3(+)CbII leads to the accumulation of gephyrin in cytoplasmic aggregates, we show that if TC10 is additionally coexpressed, gephyrin is redistributed from intracellular aggregates to submembraneous microclusters (Figures 9 and 10). We have quantified this redistribution both on the level of the population of transfected cells, as well as on the level of individual cells, to show that there is a significant increase in the number of cells displaying GFP-gephyrin microclusters and that the distribution of cluster sizes is different when comparing cells coexpressing GFP-gephyrin and SH3(+)CbII with those expressing TC10 additionally (Figure 11). Interestingly, the GFP-gephyrin microclusters induced by the coexpression of TC10 and SH3(+)CbII were significantly smaller than those formed in the presence of ΔSH3CbII. This may indicate that a different molecular mechanism is involved in the formation of these two types of microclusters. Furthermore, we find that a mutant of TC10 that is constitutively GTP-bound, TC10 CA, can trigger Cb-dependent GFP-gephyrin microcluster formation in COS7 cells equally efficiently as TC10 WT, whereas TC10 DN, a constitutively GDP-bound TC10 variant, cannot (Figure 13).

In dissociated hippocampal neurons, coexpression of TC10 CA or DN with SH3(+)CbII has differential effects on synaptic gephyrin clustering and inhibitory neurotransmission. While GTP-TC10 enhances gephyrin clustering at perisomatic and dendritic synapses and hence inhibitory synaptic strength, GDP-TC10 reduces gephyrin clustering and thus inhibitory synaptic strength (Appendix Figures 28 and 30). The increase in mIPSC and evoked IPSC amplitudes upon TC10 CA coexpression is likely caused by an increase in the number of functional $GABA_A$Rs at synaptic sites, concomitant with the increase in gephyrin cluster size and density. Interestingly, overexpression of NL2 also leads to an increase in IPSC amplitudes (Chubykin et al., 2007).

The reduction in mIPSC frequency observed upon TC10 DN coexpression can be interpreted as a loss of $GABA_A$Rs at a subset of synaptic sites concomitant with a loss of gephyrin clusters in dendrites. The consequence is a decrease in the number of functional inhibitory postsynaptic sites. TC10 DN thus has a dominant negative effect on gephyrin clustering at synaptic sites and inhibitory postsynaptic currents. This can be explained by the sequestration of GEFs by DN GTPases (Heasman and Ridley, 2008) - here TC10 DN sequesters SH3(+)CbII, making it unavailable for the interaction with other binding partners, which are required to achieve gephyrin clustering at synaptic sites.

Furthermore, we have aimed to resolve the controversy on the potential involvement of Cdc42 in gephyrin clustering. We show that like TC10, Cdc42 induces the formation of Cb-dependent GFP-gephyrin microclusters in COS7 cells (Figures 15 and 16). This is in agreement with a previous study showing that Cdc42 overexpression in neurons leads to changes in synaptic gephyrin clustering (Tyagarajan et al., 2011). In contrast, overexpression of a dominant negative Cdc42 variant with the constitutively active Cb isoform ΔSH3CbII and GFP-gephyrin did not have any effect compared to controls in which only ΔSH3CbII and GFP-gephyrin were coexpressed (Reddy-Alla et al., 2010). Moreover, overexpression of the Cdc42 binding domain of WASP, an effector of Cdc42, with ΔSH3CbII and GFP-gephyrin, did not affect GFP-gephyrin microcluster formation despite preventing the formation of filopodia (Reddy-Alla et al., 2010). Taken together, this indicates that SH3 domain-containing Cb isoforms can be regulated by Cdc42, whereas constitutively active Cb isoforms are likely not affected by the presence or absence of GDP- or GTP-bound Cdc42. Additionally, microcluster formation does not seem to depend on the ability of Rho GTPases to induce filopodia.

In summary, we show that both TC10 and Cdc42 potently stimulate the formation of GFP-gephyrin microclusters in the presence of SH3(+)CbII in COS7 cells. The same was observed when the synaptic proteins NL2, NL4 or $GABA_A$R$-\alpha 2$ were coexpressed with GFP-gephyrin and SH3(+)CbII in non-neuronal cells (Poulopoulos et al., 2009; Hoon et al., 2011; Saiepour et al., 2010). Furthermore, overexpression of either TC10 CA along with SH3(+)CbII, or NL2 in neurons leads to an increase in IPSC amplitudes (Chubykin et al., 2007). We therefore propose that these Rho GTPases may relieve the autoinhibition exerted by the SH3 domain, in analogy to the mechanisms described for the synaptic activators (Papadopoulos and Soykan, 2011).

However, it is unclear whether the activation of SH3 domain-containing Cb isoforms by one or both of these small GTPases is required for synaptic gephyrin clustering. It has been shown that Cdc42 is not essential for gephyrin clustering in the forebrain indicating that if it is involved in inhibitory postsynapse formation, its loss may be

compensated by other proteins acting in a redundant manner (Reddy-Alla et al., 2010). For TC10, no KO mouse is available so far. Therefore, we aimed at identifying suitable knockdown strategies, but efficiencies were unsatisfactory (3.6). An indication that TC10 might be involved in Cb-dependent gephyrin clustering is the observation that the expression of TC10 is spatiotemporally correlated with inhibitory synaptogenesis in brain regions in which gephyrin clustering depends on Cb (Tanabe et al., 2000; Mayer et al., 2013). In the near future, it will be possible to determine whether TC10 is required for inhibitory synaptogenesis, since the International Mouse Phenotyping Consortium has recently generated conditional TC10 KO mice.

Based on the observation that both TC10 and Cdc42 can relieve the autoinhibition of SH3(+)CbII, it is likely that when either Cdc42 or TC10 is lost, the other can compensate, so no effects on gephyrin and $GABA_AR$ clustering might be observed in TC10 KO mice also. This being the case, double-KO mice that lack both Cdc42 and TC10 in the developing forebrain can be generated, to determine if at least one of the two Rho family GTPases that can trigger Cb-dependent gephyrin clustering is required for the formation of inhibitory postsynapses.

Furthermore, the relation between Cb activation through small GTPases and synaptic proteins is unclear so far. Knockout studies of NL2 and the $GABA_AR$-α2 subunit have identified synapses, at which gephyrin clustering depends on these synaptic proteins. Loss of NL2 resulted only in the loss of gephyrin and $GABA_AR$-γ2 subunit immunoreactivity from perisomatic areas (Poulopoulos et al., 2009). Additionally, loss of NL2 affected only synapses of parvalbumin-positive interneurons onto excitatory neurons in the neocortex (Gibson et al., 2009). Mice lacking the $GABA_AR$-α2 subunit show a loss of gephyrin both in dendrites and at perisomatic synapses, however, NL2 was only concomitantly lost at dendrites (Panzanelli et al., 2011). These results indicate that different mechanisms for inhibitory synapse formation exist in different subcellular compartments and may be acting in concert in some, while distinctly in others.

The relative contribution of small GTPases to inhibitory synaptogenesis remains to be determined. Several scenarios are possible. First, activation through binding on two distinct domains of Cb by synaptic proteins and small GTPases may synergistically allow relief from autoinhibition. Second, the interactions may be spatiotemporally separated, such that initially activation occurs intracellularly or at non-synaptic sites through small GTPases and is stabilized at synaptic sites by the interaction with synaptic proteins. Third, activation of Cb by these different proteins may be entirely independent and each mechanism may function at distinct subcellular localisations or in different neuron types and brain areas. In support of the latter notion, the observation

that loss of NL1-3 does not impair synaptogenesis (Varoqueaux et al., 2006) indicates that other mechanisms of Cb activation may function early in development, while NL2 may stabilize Cb at mature synaptic contacts. The interplay between Cb activation by small GTPases and synaptic proteins can be studied in the future by crossing mice with a conditional deletion of TC10 and/or Cdc42 with mice lacking genes encoding NL2, NL4 or $GABA_AR$-α2.

4.2 Cb provides GTP-TC10 through its GEF activity

According to experiments performed *in vitro* and in cells, different rat and human Cb isoforms have been described to act as a GEF specifically for Cdc42 (Reid et al., 1999; Xiang et al., 2006; Jaiswal et al., 2013). Corroborating these results, we show that neither SH3(+)CbII, nor ΔSH3CbII act as a GEF for TC10 *in vitro*, whereas both catalyze the nucleotide exchange on Cdc42 with similar (low) efficiency (Figure 17). In agreement with this finding, in cells, both SH3(+)CbII and ΔSH3CbII act as a GEF for Cdc42, showing that the intramolecular interactions between the SH3 domain and the DH-PH tandem do not influence GEF activity towards Cdc42 (Tyagarajan et al., 2011).

In a cellular context, however, SH3(+)CbII and ΔSH3CbII can both act as a GEF for TC10, even though SH3(+)CbII requires the presence of gephyrin to achieve this (Appendix Figure 27). It has been suggested previously that Rho GEF specificity is determined not only by the *in vitro* intermolecular interaction between the GEF and the GTPase, but also by the cellular context, where adaptor and scaffolding proteins as well as posttranslational modifications and interactions with membranes can affect substrate specificity (Jaiswal et al., 2013). In support of this notion, it has been previously observed that the Cb GEF activity is regulated in a complex manner, with both the C-terminus and the binding to gephyrin potentially influencing the catalytic activity of the DH domain (Xiang et al., 2006)

These results indicate that in neurons, GTP-TC10 can be generated through the GEF activity of SH3 domain-containing Cb isoforms in the presence of gephyrin. Subsequently, GTP-TC10 can bind to the PH domain and hence induce gephyrin clustering, exerting a feedforward regulation.

However, the GEF activity of Cb is not essential for triggering gephyrin microcluster formation when all proteins are overexpressed in COS7 cells, as seen by the ability of the GEF-deficient Cb mutant to trigger gephyrin microcluster formation in the presence of TC10 or Cdc42 (Figures 12,15 and 16). Similarly, overexpression of the

same GEF-deficient mutant in the context of constitutively active ΔSH3CbII did not prevent GFP-gephyrin clustering in non-neuronal cells and in neurons (Reddy-Alla et al., 2010).

In conclusion, expression of recombinant proteins in either non-neuronal cells or WT dissociated hippocampal neurons, indicates that the GEF activity of Cb towards TC10 and Cdc42 is not required for gephyrin clustering, despite possibly contributing to the production of GTP-bound GTPases. However, we cannot exclude the possibility that the GEF activity of Cb plays an important role in gephyrin clustering *in vivo*.

Interestingly, mutating a residue in the linker region between the DH and PH domains of Cb (E239 of ΔSH3CbII), which makes contact with Cdc42 as shown in the crystal structure of Cb and Cdc42 (Xiang et al., 2006), prevents the formation of gephyrin microclusters in COS7 cells according to our preliminary data. This indicates that the binding of GDP-bound or nucleotide-free GTPases to the DH domain may be essential for Cb-dependent gephyrin recruitment to synaptic sites.

4.3 Cb acts as an effector of TC10

The finding that only TC10 CA and WT, but not TC10 DN, can trigger Cb-dependent GFP-gephyrin microcluster formation in COS7 cells (Figure 14) indicates that GTP hydrolysis by TC10 is not required for triggering Cb-dependent gephyrin clustering. In contrast, GTP hydrolysis is required for the fusion of GLUT4-containing vesicles (Kawase et al., 2006).

Additionally, our binding assays show that there are two distinct binding sites for TC10 in Cb: GDP-TC10 binds Cb weakly to the DH domain, whereas GTP-TC10 binds Cb strongly to the PH domain (Figure 18). In agreement with these results, Cdc42 also binds Cb preferentially in its GTP-bound state (Tyagarajan et al., 2011). Taken together, these results indicate that the preferential binding of GTP-TC10 or GTP-Cdc42 to the Cb PH domain is likely crucial for triggering Cb-dependent gephyrin clustering.

This interaction is not typical for a GEF and a small GTPase, where binding is mediated predominantly by the catalytic DH domain and occurs preferentially in the nucleotide-free state (1.3.1). Instead, the preferential binding of TC10 to Cb in the GTP-bound state on a domain not directly involved in catalysis is reminiscent of the interaction between small GTPases and their effectors. Several GTPases bind to their GEFs in a GTP-bound state on a domain that is not directly involved in catalysis. An example is RhoA, which interacts with its GEF, PDZ-RhoGEF (PRG), both in the canonical manner at the DH domain and in an unconventional manner at

the PH domain (Chen et al., 2010). Such interactions may have different regulatory roles and affect, for instance, the catalytic activitiy of the GEF or its subcellular localisation (Margarit et al., 2003; Cohen et al., 2007). Interestingly, in a different system, hPEM-2 has been identified as a downstream effector of heterotrimeric G-protein signalling, which could also be mediated by the binding of GTP-GTPases to the PH domain (Nagae et al., 2011). In the future, point mutations in both TC10 and Cb that abolish effector-type binding may demonstrate even more clearly that this interaction is essential for triggering gephyrin microcluster formation.

Several point mutations of TC10 have been described to be essential for the binding to effectors. For example, Murphy et al. (1999) have described that mutating amino acids in the effector binding domain (T49A and Y54C) in TC10 CA leads to a reduction in the ability of TC10 to induce filopodia. Mutating D52A in the same region leads to a loss of binding of TC10 CA to its effector PIST (Neudauer et al., 2001). Preliminary experiments showed no significant reduction in the ability of TC10 WT to trigger Cb-dependent gephyrin clustering in COS7 cells when Y54 or D52 were mutated. However, the biggest effect on filopodia formation was observed when T49 was mutated (Murphy et al., 1999), so mutation of this residue may be more likely to abolish TC10-triggered Cb-dependent gephyrin cluster formation.

While PH domains are one of the classical protein domains binding phosphoinositides, only around 10 % of the PH domains in the human proteome bind phosphoinositides strongly and specifically (Lemmon, 2008). Interactions with other binding partners are therefore likely to be mediated by PH domains. Mutation of residues in the Cb PH domain critical for GTP-TC10 binding should prevent TC10-triggered gephyrin clustering according to the proposed mechanism. Such residues could be identified by comparison with GTP-GTPase binding sites in other PH domains, such as PRG (Chen et al., 2010), or four-phosphate-adaptor protein 1 (FAPP1) (He et al., 2011). If such residues were identified, it would also be very interesting to examine whether they only prevent gephyrin clustering by TC10, and possibly also Cdc42, in SH3 domain-containing isoforms, or whether they also prevent gephyrin microcluster formation in the constitutively active Cb isoform ΔSH3CbII. This would indicate whether the binding of GTP-bound GTPases to the PH domain is only involved in inducing the relief from autoinhibition, or whether additionally, even active Cb isoforms use the binding of small GTPases to the PH domain as a means of membrane attachment in addition to binding to PI(3)P. The observation that coexpression of the Cdc42-binding domain of WASP (which would bind to GTP-Cdc42 and GTP-TC10, albeit with lower affinity Hemsath et al. (2005), and hence make these proteins unavailable for Cb) with ΔSH3CbII and GFP-gephyrin does not lead to a reduction in gephyrin microcluster

formation might indicate that ΔSH3CbII does not require binding to GTP-bound small GTPases at its PH domain to induce gephyrin clustering (Reddy-Alla et al., 2010). Interestingly, a point mutation in the PH domain of Cb was recently described in a patient diagnosed with mental retardation (E400K, de Ligt et al. (2012)), so it is tempting to speculate that this residue might be involved in binding to GTP-bound GTPases. In the light of these new findings, the loss of function of hPEM-2 due to the loss of a functional PH domain in a patient with various neurological symptoms may be a compound effect of the loss of PI(3)P and small GTPase binding (Kalscheuer et al., 2009).

We postulate that the effector-type interaction between Cb and GTP-TC10 leads to the activation of SH3(+)CbII, releasing it from its compact, inactive state (4.1). Indeed, several Rho GTPases induce a conformational change in their effectors that leads to the relief of autoinhibition and thereby control the function of effector proteins (Wu et al., 2008). A prominent example is N-WASP, a protein that controls the formation of actin filaments and is autoinhibited due to intramolecular interactions. N-WASP is activated cooperatively by binding of GTP-Cdc42 and PI(4,5)P_2 (Prehoda et al., 2000; Kim et al., 2000). Similarly, we show that the PH domain of Cb interacts with phosphoinositides and GTP-TC10 at different sites (Figure 18 A) and that binding to phosphoinositides through two Arg residues is essential, even in the presence of GTP-TC10 (Figure 12).

At this point, it is uncertain whether the interactions of the Cb PH domain with GTP-TC10 and PI(3)P occur simultaneously or subsequently and if the former is true, whether they act synergistically. However, the electrostatic interactions between proteins and phosphoinositides through specific protein domains, such as PH domains, or through unstructured domains, as in the case of the C-termini of some small GTPases, are of low affinity (Di Paolo and De Camilli, 2006). In order to allow a reliable recruitment of cytosolic proteins to specific membrane compartments, phosphoinositides and small GTPases therefore frequently act as co-receptors and hence conjointly achieve a high affinity binding with effector proteins (Figure 25, Di Paolo and De Camilli (2006)). Our preliminary experiments show that coexpression of either SH3(+)CbII or ΔSH3CbII with TC10 WT or CA, but not TC10 DN, results in a change in the localisation of Cb from the cytosol to vesicular structures and the plasma membrane, with a colocalisation of TC10 and Cb at these sites. This would indicate that indeed the interaction between Cb and PI(3)P is too weak to allow membrane recruitment, so a second membrane-targeting signal, here provided by binding to GTP-TC10 at the PH domain, is required for membrane localisation. This would imply a model of coincidence detection, where coincident signals are amplified by a cooperative action

of two different ligands at two different binding sites (Prehoda et al., 2000). Consequently, this would also mean that even Cb in its open conformation requires a second membrane attachment motif through the binding of small GTPases.

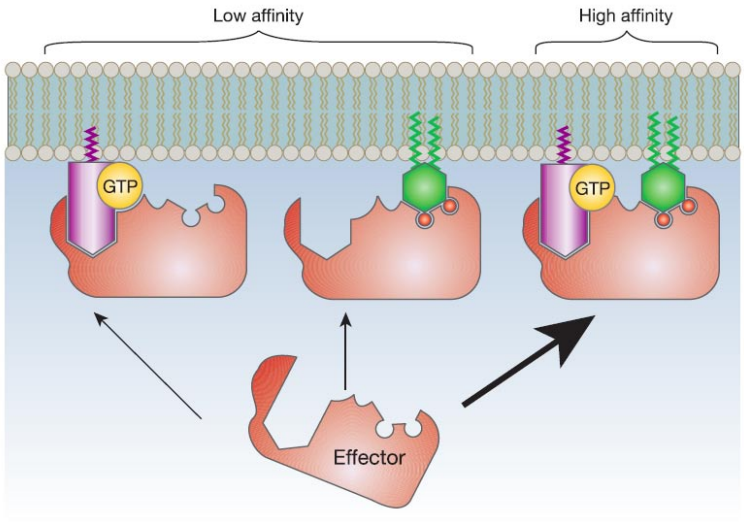

Figure 25: Phosphoinositides and small GTPases function as co-receptors for cytosolic proteins. GTP-bound GTPases (purple) and phosphoinositides (green) act cooperatively to recruit effectors to specific membrane compartments. Source: Di Paolo and De Camilli (2006).

4.4 Membrane targeting of Cb and TC10 is essential for gephyrin clustering

The PH domain of Cb plays an essential function in the control of synaptic gephyrin clustering since it interacts both with PI(3)P and with GTP-bound GTPases. However, the localisation of these interactions in unclear.

In order to approach the question where the interaction between Cb and GTP-TC10 takes place, different mutations in the C-terminus of TC10, the major determinant of TC10 localisation, were made. Interestingly, the membrane attachment of TC10 by prenylation is essential for Cb-dependent gephyrin microcluster formation (Figure 21). This finding indicates that TC10 does not merely function as an adaptor between Cb and another protein, but rather provides a means of membrane attachment for Cb, supporting the proposed model in which TC10 and PI(3)P function as coreceptors at the membrane (Figure 25).

In order to further investigate the contribution of TC10 localisation to Cb-dependent gephyrin clustering, different functional elements of the C-terminus were

studied. The first interesting observation was that the replacement of the TC10 C-terminus with that of H-Ras or K-Ras prevented TC10-triggered Cb-dependent gephyrin microcluster formation (Figure 21) indicating that neither dual palmitoylation, nor interactions with phospholipids through basic residues by themselves are sufficient. In contrast, TC10-H-Ras and TC10 WT, which show a similar subcellular distribution, were reported to potently inhibit insulin-stimulated GLUT4 translocation (Watson et al., 2001).

Palmitoylation is known to enhance the recruitment of proteins to lipid rafts (Levental et al., 2010), and inhibitory postsynapses may have raft-like properties based on different lines of evidence such as the observation that gephyrin may be palmitoylated (Kang et al., 2008; Renner et al., 2009). Therefore, one hypothesis would be that TC10 recruits the Cb-gephyrin complex to lipid rafts at inhibitory synapses. A recent study indicates that gephyrin is phosphorylated by Cdk5 and thereby recruited to synaptic sites in a Cb-dependent manner (Kuhse et al., 2012). Cdk5 also phosphorylates TC10 in the C-terminus leading to the recruitment of TC10 to lipid raft domains (Okada et al., 2008). An interesting possibility would therefore be that TC10 is the missing link between Cb and Cdk5-dependent gephyrin recruitment to inhibitory synapses. However, mutating the palmitoylation sites or the Cdk5 phosphorylation site in the TC10 C-terminus did not affect GFP-gephyrin microcluster formation in the presence of SH3(+)CbII (Figure 21). In contrast, lipid raft localisation of TC10 through palmitoylation is required for the inhibition of GLUT4 trafficking in adipocytes (Watson et al., 2003).

A second membrane attachment motif in TC10 is the cluster of basic amino acid residues, which interact with negatively charged lipid head groups (Valero et al., 2010). Mutating these abolishes the ability of TC10 to trigger Cb-dependent gephyrin clustering (Figure 21). K-Ras also has a polybasic motif, but it is considerably different from the one of TC10 (Figure 20), with eleven Lys residues compared to four Lys and two Arg residues in TC10. Additionally, the amino acid residues surrounding the basic residues are also important in determining the interaction with membrane lipids (Di Paolo and De Camilli, 2006). In the TC10 C-terminus the KKR motif is flanked by hydrophobic residues on both sides (Val and Ile, Figure 20) and these residues can strengthen attachment by partially penetrating the membrane (Di Paolo and De Camilli, 2006). Furthermore, the interaction with the membrane through basic residues also prevents solubilisation of GTPases by GDI (Das et al., 2012). The polybasic stretch may therefore play an important role in stably localising TC10 at the appropriate membrane compartments, in addition to the prenylation. With regard to Cb-dependent gephyrin clustering this result supports the idea that TC10 provides a

second membrane anchor for Cb, in addition to the weak binding of the PH domain to PI(3)P. Furthermore, the stable attachment of TC10 to a specific membrane through prenylation and basic residues, seems to be required. In agreement with this interpretation, preliminary results indicate that mutating the basic residues in the C-terminus of Cdc42, which have been implicated in COPI, PI(4,5)P_2 and PI(3,5)P_2 binding (1.3.2.1, Johnson et al. (2012); Wu et al. (2000)), also leads to a pronounced reduction in the ability of Cdc42 to trigger Cb-dependent gephyrin microcluster formation in non-neuronal cells.

The only phosphoinositide known to interact with Cb is PI(3)P (1.2.4.3). However, this interaction has only been demonstrated in very unphysiological conditions in lipid overlay assays, so in cells the lipid binding specificity of Cb may be different (Kalscheuer et al., 2009; Reddy-Alla et al., 2010). PI(3)P is the defining phosphoinositide at early endosomes (Di Paolo and De Camilli, 2006) and can only be locally produced at the plasma membrane upon stimulation (Falasca and Maffucci, 2009). Similarly, TC10 is predominantly localised at the plasma membrane and in the endosomal system (1.3.2.2). It would therefore be interesting to investigate whether TC10 binds a specific phosphoinositide through its C-terminal polybasic residues. If it shares a preferential binding to PI(3)P with Cb, this could reinforce such an interaction, be it at endosomes or at the plasma membrane. Alternatively, it is possible that TC10 binds to a negatively charged membrane lipid constitutively localised at the plasma membrane. In such a scenario, the binding of Cb to PI(3)P would be important during the trafficking through the secretory system, which may involve endosomal compartments, while TC10 would bind to plasma membrane-enriched negatively charged lipids. Alternatively, binding of GTP-TC10 to the PH domain might also modify the lipid binding specificity of Cb so that binding to plasma membrane-enriched negatively charged lipids is favoured.

Most of the mutations in the TC10 C-terminus used here were initially described in the regulation of GLUT4 exocytosis by TC10. While initially it was tempting to speculate that TC10 might act through the same mechanisms in neuronal Cb-dependent gephyrin clustering, the analysis has revealed that there are considerable mechanistic differences. In the future, it will be interesting to study whether some of the effectors of TC10 in GLUT4 trafficking also play a role in Cb-dependent gephyrin clustering. For example, the exocyst complex, which is involved in vesicle fusion, interacts with both TC10 and Cdc42 (Wu et al., 2010; Pommereit and Wouters, 2007) and may therefore be a good candidate for further investigation.

4.5 Potential regulation of the actin cytoskeleton at inhibitory synapses by small Rho GTPases

As discussed (1.2.6), the actin cytoskeleton is essential for the formation of inhibitory synapses (Bausen et al., 2006) and may be regulated by the binding of profilin and Mena/Vasp to gephyrin (Mammoto et al., 1998; Giesemann et al., 2003). Additionally, small GTPases function as classical switches in the control of the actin cytoskeleton and are also involved in regulating the actin cytoskeleton at inhibitory synapses. For example, Cdc42 also binds to Cb directly, independently of its nucleotide-bound state (Tyagarajan et al., 2011). This implies that this interaction is not regulating the actin cytoskeleton directly, but rather enhances the recruitment of Cdc42 to Cb-gephyrin complexes. Subsequently, Cb is able to catalyze the nucleotide exchange on GDP-Cdc42 producing GTP-Cdc42 (1.2.4.3). GTP-Cdc42 can bind to the Cb (Tyagarajan et al., 2011) and thereby likely cause a conformational change in Cb (Figures 15 and 16).

Interestingly, actin depolymerising drugs induce the formation of smaller gephyrin clusters in spinal cord neurons (Kirsch and Betz, 1995). TC10 has been described to disrupt cortical actin as well (Chunqiu Hou and Pessin, 2003). Hence the formation of smaller GFP-gephyrin microclusters in COS7 cells in the presence of TC10 and SH3(+)CbII compared to ΔSH3CbII may be due to the ability of TC10 to disrupt cortical actin. In line with this interpretation, deleting the first 16 amino acids of TC10 abolishes the actin disrupting abilities of TC10 at the same time as preventing TC10-triggered Cb-dependent gephyrin clustering (Chunqiu Hou and Pessin, 2003). Corroborating this interpretation, treatment of HEK293 cells that express gephyrin with actin filament depolymerizing drugs leads to the formation of actin patches and the redistribution of gephyrin to submembraneous microclusters (Bausen et al., 2006).

4.6 An updated mechanistic model of inhibitory postsynapse assembly

In this study, we have shown that both Cdc42, the Rho-like GTPase known to be activated by the GEF Cb, and its closest homolog TC10 are able to trigger Cb-dependent gephyrin clustering at synapses, likely by binding to the PH domain of Cb in a GTP-dependent manner. This has two consequences for Cb function. First, a second mode of membrane attachment of Cb through a membrane-bound small GTPase is provided in addition to its interaction with PI(3)P (4.4). Second, the activation of SH3 domain-

containing Cb isoforms occurs through the PH domain rather than the SH3 domain, as is the case with synaptic proteins (Poulopoulos et al., 2009; Hoon et al., 2011; Saiepour et al., 2010).

We therefore suggest an updated mechanistic model of inhibitory postsynapse assembly (Figure 26). Cb and gephyrin form a complex in the cytoplasm (Figure 26, step 1), likely in cytoplasmic aggregates which are observed in early stages of development (Colin et al., 1996, 1998; Sassoe-Pognetto and Wässle, 1997; Papadopoulos et al., 2008; Patrizi et al., 2012). Most Cb isoforms contain an autoinhibitory SH3 domain, which makes extensive contact with the DH and PH domains (Papadopoulos and Soykan, 2011). While this does not prevent nucleotide exchange (Figure 17, Tyagarajan et al. (2011)), in this conformation, the PH domain cannot bind to PI(3)P so that Cb cannot mediate the transport of gephyrin-Cb complexes to the plasma membrane (Papadopoulos and Soykan, 2011). In this state, Cdc42, and possibly also TC10, can bind to gephyrin in a nucleotide-independent manner (Tyagarajan et al., 2011). Both Cdc42 and TC10 can be activated through the GEF activity of the DH domain of Cb in cells (Figure 26, step 2). However, it is unclear whether Cb acts as a GEF for these small GTPases in neurons to induce gephyrin clustering, since overexpression of a GEF-deficient mutant in neurons did not prevent gephyrin microcluster formation (Reddy-Alla et al., 2010). In the GTP-bound form, these GTPases bind to the PH domain of Cb (Figure 26, step 3). This interaction results in a conformational change in Cb, to a more extended conformation, in which the PH domain can interact with PI(3)P at the membrane (Figure 26, step 4). The Cb-gephyrin complex is now stably associated with the membrane through the dual binding of the PH domain to PI(3)P and membrane-anchored GTPases. While all these interactions occur either at intracellular membranes, such as on endosomes, or at the plasma membrane, the subsequent recruitment of the complex to synaptic sites on the plasma membrane, in apposition to presynaptic specialisations, is achieved by the interaction with synaptic proteins (Figure 26, step 5). The maintenance of the open conformation of Cb could also be enhanced by the interaction of NL2, NL4 or the α2-subunit of $GABA_A$Rs with the SH3 domain. Finally, gephyrin binds to both $GABA_A$Rs and microtubules, and actin filaments through different actin binding proteins, to ensure the stabilisation of neurotransmitter receptors at synaptic sites (Figure 26, step 6). Furthermore, small Rho GTPases may also be involved in regulating actin dynamics at synapses.

While the timing of events and the relative contributions of synaptic Cb activators and small GTPases are unclear as discussed (4.1), this is one potential scenario, in agreement with the finding that NL2 is only required for inhibitory synapse stabilisation, but not synaptogenesis *per se* (Varoqueaux et al., 2006).

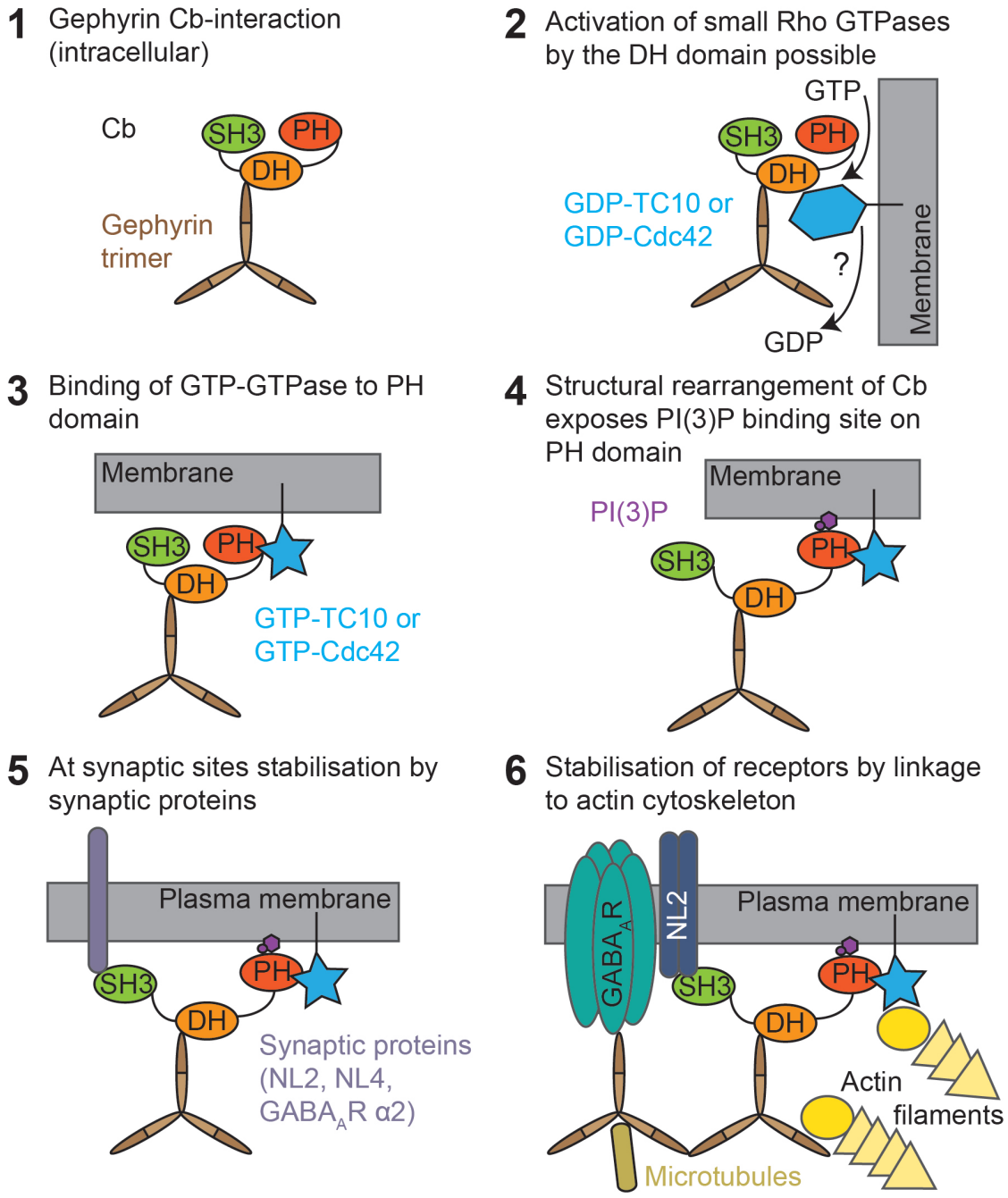

Figure 26: Mechanistic model of Cb function in inhibitory postsynapse formation. (1) Intracellularly, gephyrin trimers bind to Cb in its closed conformation. (2) Cb can catalyze the nucleotide exchange on Cdc42 and TC10 in the DH domain, whether this happens *in vivo* in uncertain. (3) GTP-TC10 or GTP-Cdc42 interact with the PH domain and thereby anchor it at the membrane. (4) The ensuing structural rearrangement exposes the PI(3)P binding site on the PH domain. (5) At synaptic sites on the plasma membrane, the open conformation of Cb is maintained by the interaction of NL2, NL4 or the α2-subunit of GABA$_A$Rs with the SH3 domain. (6) Gephyrin interacts with and thus limits the diffusion of GABA$_A$Rs. Additionally, both gephyrin and TC10/Cdc42 interact with actin binding proteins such as profilin (yellow circle), in order to induce actin filament assembly (yellow). Gephyrin also interacts with microtubules.

5

Summary and Conclusions

In this study, we have identified a novel mechanism that can trigger gephyrin recruitment to synaptic sites through the GEF Cb. We therefore present a new mechanistic model for Cb-dependent gephyrin clustering at synaptic sites (Figure 26). The findings presented here reconcile controversies in the field regarding the contribution of Cdc42 to Cb-dependent gephyrin clustering by showing that while Cdc42 can relieve the autoinhibition of Cb, at least one other small GTPase acts in the same manner. The possible redundancy may thus explain the lack of effects on gepyhrin and $GABA_AR$ clustering of Cdc42 KO mice.

However, it is important to note that further work is required to understand the contribution of small GTPases to Cb-dependent gephyrin clustering in more detail. Biochemically, it would be interesting to study, which cellular factors allow Cb to act as a GEF for TC10 only in cells and why gephyrin is required for SH3(+)CbII to act as a GEF for TC10. On a cell biological level, it would be interesting to investigate the contribution of the actin cytoskeleton rearrangements triggered by the Rho GTPases to Cb-dependent gephyrin clustering in more detail. For example, it would be important to understand whether regulation of the actin cytoskeleton by small GTPases is required for the transport from intracellular aggregates to submembraneous microclusters or whether actin rearrangements triggered by Cdc42 or TC10 are mostly important to achieve clustering once the complex is at the plasma membrane. In order to fully understand the regulation of Cb activation through the SH3 and the PH domain, the relative contribution of each mechanism for gephyrin clustering needs to be determined. Several novel cell adhesion proteins regulating inhibitory synapses have been characterized recently, and it will be crucial to determine their influence on the regulation of inhibitory synapse formation by Cb. Furthermore, several other means of Cb regulation have been identified in the past years. For example, Cb may be involved in regulating both local protein translation by recruiting translation factors

and protein degradation through its ubiquitination by Smurf1.

Furthermore, the function of gephyrin, which has been used as a main readout for Cb function in this study, at synapses also requires further clarification. Open questions concern the ability of gephyrin to form a hexagonal scaffold underneath the synaptic plasma membrane, and the contribution of posttranslational modifications to gephyrin function. The interdependence between gephyrin and $GABA_AR$ clustering is not fully understood either. Indeed, gephyrin-independent mechanisms of inhibitory synapse formation and stabilisation exist and may be regulated by Cb. An indication for this is the observation that Cb has also been shown to colocalise with α-dystroglycan-positive synapses in the cerebellum (Patrizi et al., 2012).

In conclusion, this work has enhanced our mechanistic understanding of Cb-dependent gephyrin clustering and inhibitory synapse formation. In networks, these synapses play a crucial role and are required for diverse functions such as learning and memory. Inhibitory synapses are very diverse, with a plethora of mechanisms regulating their formation and dynamic remodeling in different subcellular localisations, cell types and brain regions of the mammalian brain. Mutations in the gene encoding Cb in patients with diverse neurological and psychiatric disorders show that understanding Cb-dependent mechanisms of inhibitory postsynapse regulation is crucial to gain a deeper understanding of these diseases.

6

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Appendix

The following experiments were performed by Theofilos Papadopoulos and Rohit Kumar and figures were taken from our recent publication (Mayer et al., 2013). To allow a coherent discussion, these results are presented in an abbreviated manner in the results section (3).

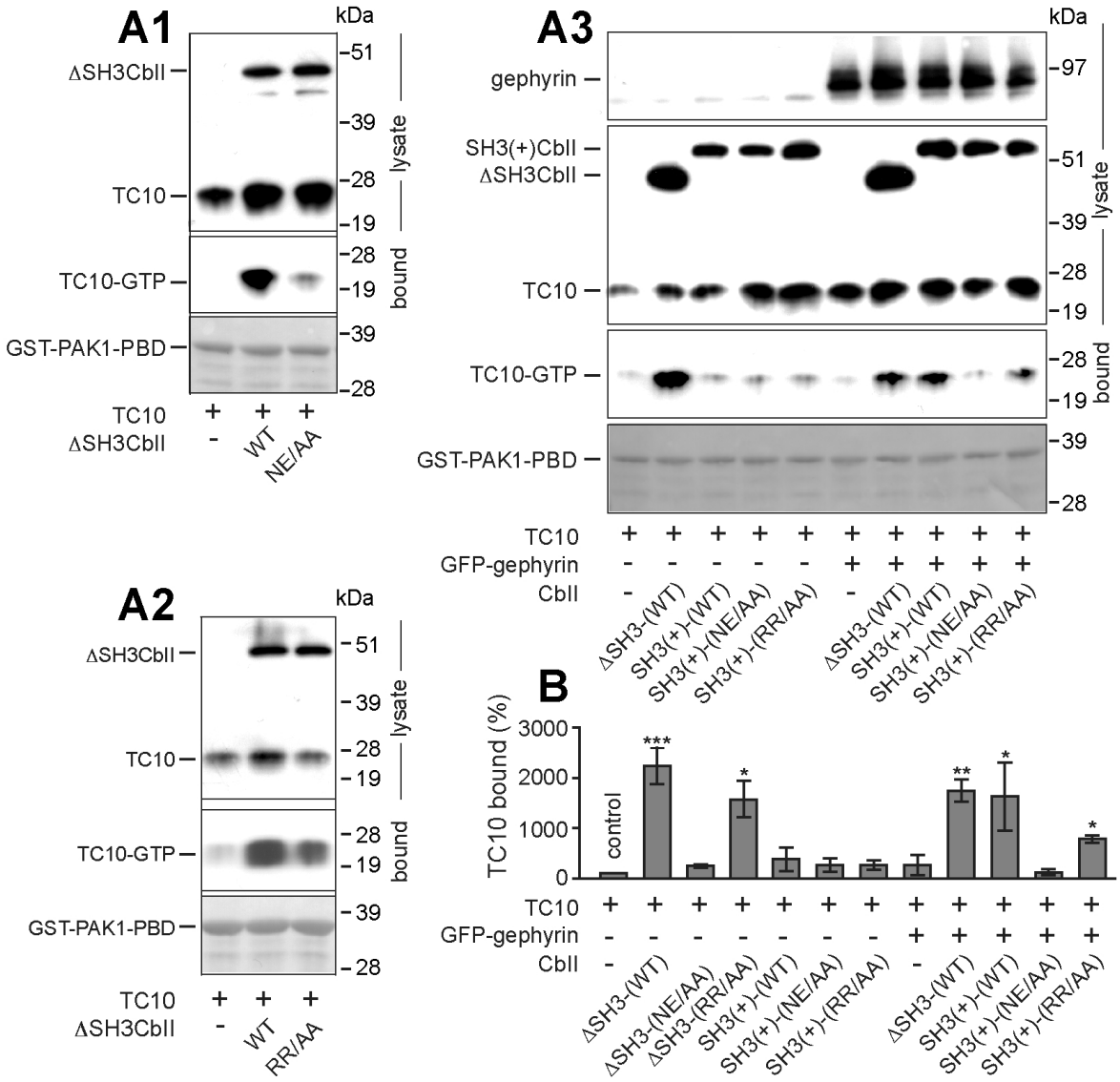

Figure 27: ΔSH3CbII and SH3(+)CbII differentially activate TC10 in non-neuronal cells. (A1-A3) Myc-TC10 was transfected either alone (-) or together with the indicated HA- (A1) or Myc-tagged (A2-A3) Cb constructs in the absence (A1-A2) or presence (A3; top panel, last 5 lanes) of GFP-gephyrin into HEK293 cells. Cell lysates were used for cosedimentation with immobilized GST-PAK1-PBD. GTP-bound TC10 was detected by Western blotting with an anti-Myc antibody. MemCode staining (bottom panels) was used to confirm that equal amounts of GST-PAK1-PBD had been added to each lysate. (B) Relative band intensities of TC10 bound to GST-PAK1-PBD (N=3-4 independent experiments). Statistical significance was compared to Myc-TC10 expressed alone (first bar).

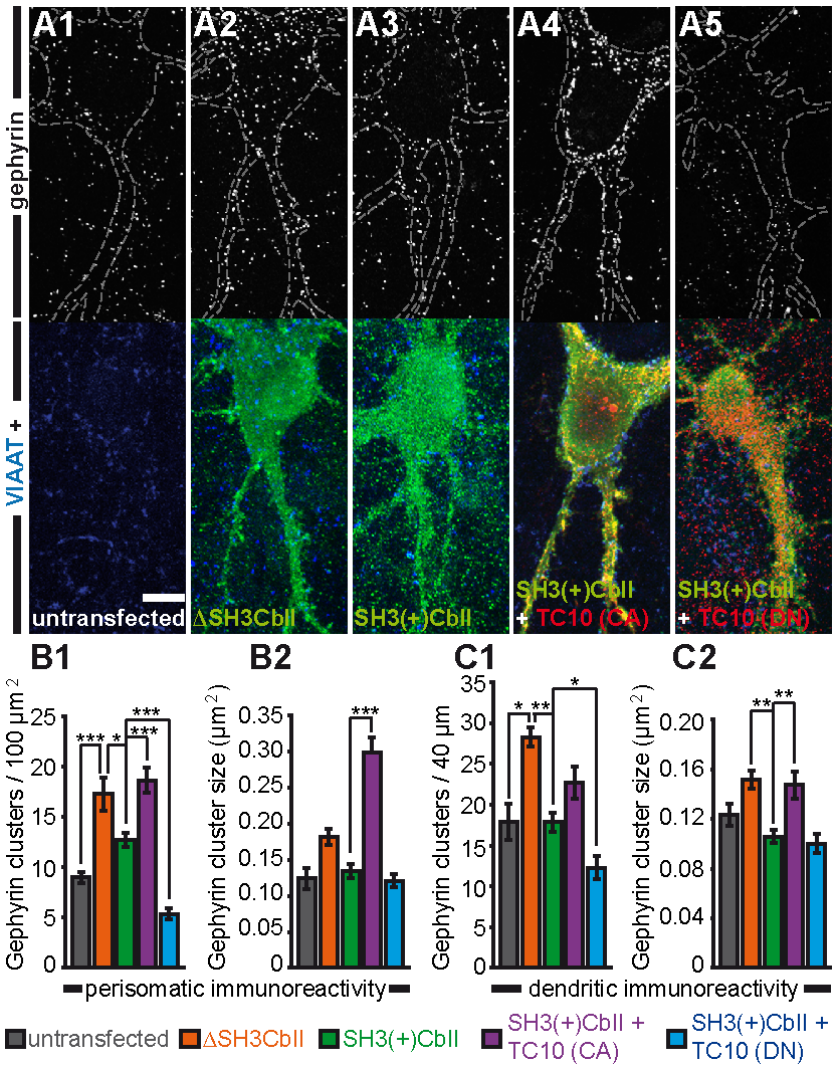

Figure 28: TC10 activity enhances SH3(+)CbII-mediated clustering of gephyrin in cultured hippocampal neurons. (A1-A5) Cultured rat hippocampal neurons were cotransfected at DIV 4 with the empty pcDNA3 vector and either the Myc- SH3CbII (A2) or Myc-SH3(+)CbII (A3) cDNAs, or cotransfected with Myc-SH3(+)CbII and HA-TC10 CA (A4) or HA-TC10 DN (A5), respectively; untransfected cultures (A1) served as control. At DIV 14, the cells were fixed and immunostained for gephyrin, VIAAT, HA and Myc. For clarity, only endogenous gephyrin immunoreactitivy is shown in the upper panels and the corresponding costainings in the bottom panels. Scale bar, 10 μm (A1-A5). Dotted lines in A1-A5 indicate the borders of transfected cells. (B1-C2) Bar diagrams of (B1) perisomatic gephyrin cluster densities per 100 μm^2 area and (B2) average sizes of perisomatic gephyrin clusters (n=258-1344 clusters analyzed), (C1) gephyrin immunoreactive clusters per 40 μm dendrite length and (C2) average sizes of dendritic gephyrin clusters (n=179-590 clusters analyzed). Bars correspond to results obtained from the somata and randomly selected second-order dendrites of N=10-28 individual neurons (N=3 independent cultures).

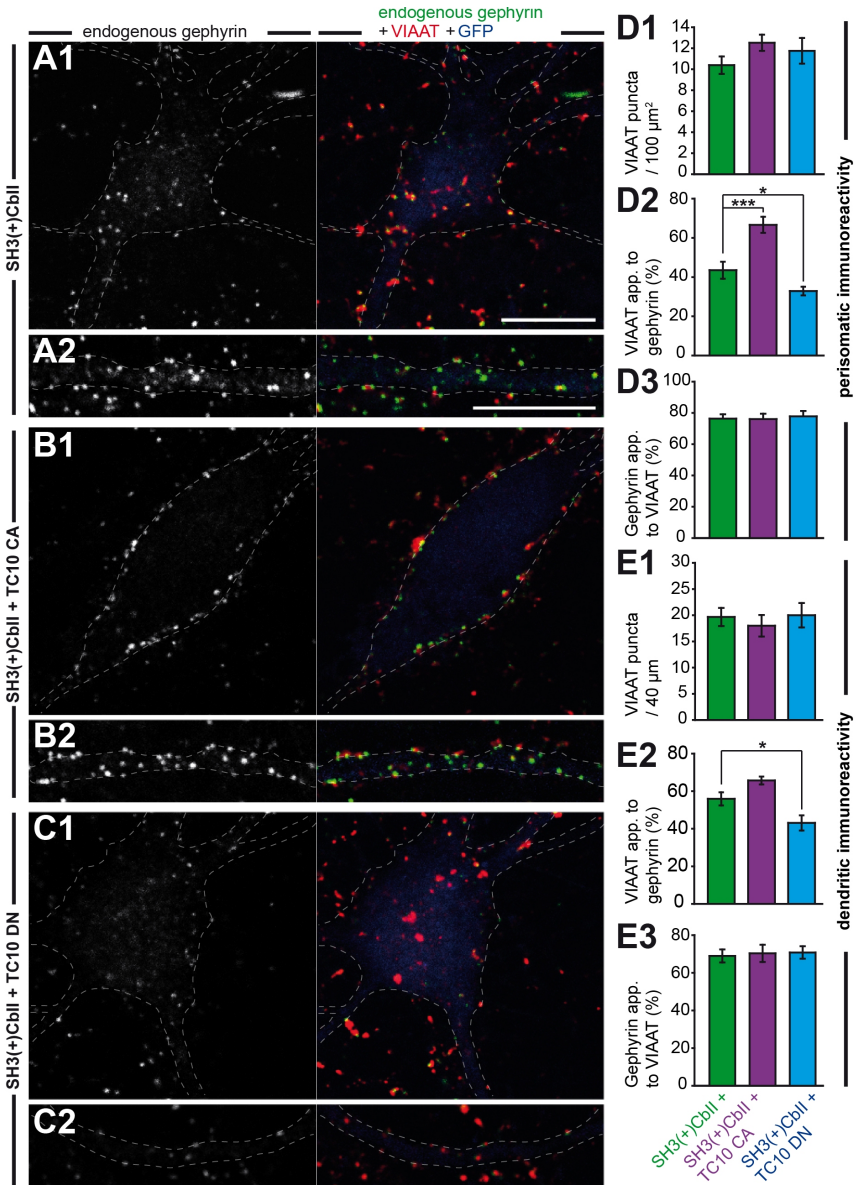

Figure 29: TC10 activity regulates SH3(+)CbII-mediated gephyrin clustering without affecting presynaptic VIAAT immunoreactivity. (A-C) Rat hippocampal neurons were transfected with plasmids encoding GFP and Myc-SH3(+)CbII (A1-A2) or GFP, Myc-SH3(+)CbII and either HA-TC10 CA (B1-B2) or DN (C1-C2), fixed and stained for gephyrin, VIAAT, Myc and HA. Only endogenous gephyrin (green) and VIAAT (red) immunoreactivities are shown in confocal images from representative somatic (A1-C1) or dendritic (A2-C2) areas of transfected neurons. GFP (pseudocolored blue) was used to identify transfected neurons. Scale bar: 10 μm (A1-C2). Dotted lines in A1-C2 indicate the borders of the transfected neurons. (D-E) Quantifications of perisomatic (D1-D3) or dendritic (E1-E3) VIAAT immunoreactivities and percentages of gephyrin clusters apposed to VIAAT or VIAAT puncta apposed to gephyrin in perisomatic (D2-D3; n=423-562 analyzed clusters) or dendritic (E2-E3; n=260-315 analyzed clusters) areas of neurons transfected as described above. Each bar in D1-E3 corresponds to counts performed on the somata and randomly selected second-order dendrites of N=13-15 individual neurons. Data represent means (± SEM) from 2 independent experiments.

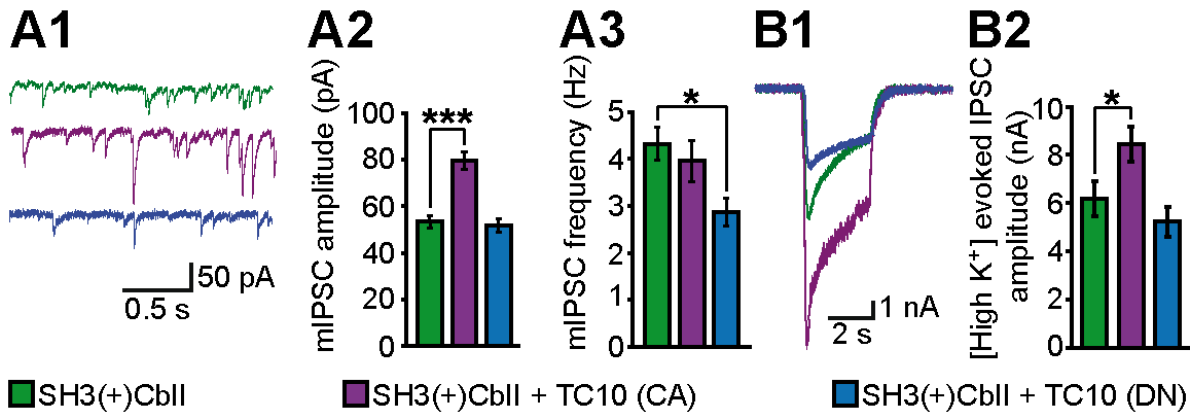

Figure 30: TC10 activity enhances GABAergic mIPSCs in cultured hippocampal neurons. (A1) Representative traces of mIPSCs recorded from neurons coexpressing GFP and Myc-SH3(+)CbII without (green) or together with HA-TC10 CA (purple) or HA-TC10 DN (blue), respectively. (A2-A3) mIPSC mean amplitudes (A1) and frequencies (A2) of transfected neurons as described in A1. (B1-B2) Representative recordings (B1) and mean amplitutes (B2) of high potassium evoked IPSCs from neurons transfected as described in A1. Data in A2-A3 were obtained from n=96-135 neurons and in B2 from n=41-55 neurons. N=4 independent experiments.

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Publications

2013	Collybistin activation by GTP-TC10 enhances postsynaptic gephyrin clustering and hippocampal GABAergic neurotransmission. Mayer S, Kumar R, Jaiswal M, Soykan T, Ahmadian MR, Brose N, Betz H, Rhee JS, Papadopoulos T. Proc Natl Acad Sci U S A, 2013 Dec 17;110(51):20795-8002013
2011	Spatiotemporal transcriptome of the human brain. Kang HJ, Kawasawa YI, Cheng F, Zhu Y, Xu X, Li M, Sousa AM, Pletikos M, Meyer KA, Sedmak G, Guennel T, Shin Y, Johnson MB, Krsnik Z, Mayer S, Fertuzinhos S, Umlauf S, Lisgo SN, Vortmeyer A, Weinberger DR, Mane S, Hyde TM, Huttner A, Reimers M, Kleinman JE, Sestan N. Nature, 2011 Oct 26;478(7370):483-9

Awards and Honours

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