UNIVERSITÄT ULM, Department of Neurology

Workgroup "Molecular Analysis of Synaptopathies"

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"Loss of COMMD1 and copper overload disrupt

zinc homeostasis and influence an autism-associated

pathway at glutamatergic synapses"

Dissertation zur Erlangung des Doktorgrades

der Medizin der Medizinischen Fakultät

der Universität Ulm

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Geislingen/Steige

2017

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Tag der Promotion: 25.10.2018

Please note that parts of this thesis were already published in the peer-reviewed journal *Biometals* by Springer:

Baecker T, Mangus K, Pfaender S, Chhabra R, Boeckers TM and Grabrucker AM: Loss of COMMD1 and copper overload disrupt zinc homeostasis and influence an autism-associated pathway at glutamatergic synapses. *Biometals* 27: 715-730 (2014)

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LIST OF ABBREVIATIONS

°C	centigrade
AAS	atomic absorption spectrometry
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APS	ammonium persulfate
Arg	arginine
ASD	autism spectrum disorders
ATP7A /ATP7B	copper-transporting p-type ATPases
ATPase	adenosin triphosphatase
CA	cornu ammonis region
CER	cerebellum
CNS	central nervous system
CO_2	carbon dioxide
COMMD	Copper- metabolism gene MURR1-domain
CortBP1	cortactin-binding protein 1
CTR1	copper transporter 1
ctrl	control
CTX	cortex
Cu	copper
$CuCl_2$	copper chloride

LIST OF ABBREVIATIONS

d	days
DAPI	4,6-di-amidino-2-phenylindol
DG	dentate gyrus
DIV	days *in vitro*
DlgA	discs large A
DMEM	Dulbecco's modified eagle medium
DMT1	divalent metal transporter 1
DNA	desoxy-ribonucleic-acid
DNase	deoxyribonuclease
dNTP	nucleoside triphosphates containing deoxyribose
E 18	embryonic day 18
ECL	enhanced chemiluminiscent reagent
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EtOH	ethanol
FBS	fetal bovine serum
FCS	fetal calf serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein

LIST OF ABBREVIATIONS

GKAP	guanylate kinase-associated protein
Glu	glutamine
GluA2	AMPA receptor subunit 2
GluN1	NMDA receptor subunit 1
GluR	glutamate receptor
GRIP	glutamate receptor-interacting protein
H_2O	aqua bidest
H_2S	hydrogen sulfide
HBSS	Hank's balanced salt solution
HCl	hydrochloric acid
HEK 293	cell line derived from human embryonic kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HiK^+	potassium chloride
HIP	hippocampus
His	histidine
HMBS	hydroxymethylbilane synthase gene
HRP	horseradish peroxidase
ICP-MS	inductively coupled plasma mass spectrometry
IgG	immunoglobulin G
KCl	potassium chloride

LB	lysogeny broth
LDL	low density lipoprotein
LTP	long term potentiation
max	maximum
mGluR	metabotropic glutamate receptor
MAGUK	membrane-associated guanylate kinases
min	minimum
mo	months
mRNA	messenger RNA
MT	metallothionein
MTF-1	metal-regulatory transcription factor 1
MURR1	mouse U2af1-rs1 region 1
n	number
Na_2S	sodium sulfide
NaCl	sodium chloride
NB	neuro basal
NIH 3T3	cell line derived from primary mouse embryonic fibroblast cells
Nlgn	neuroligin
NMDA	N-Methyl-D-aspartate
NO	nitric oxid

Nrxn	neurexin
p	significance level
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDD-NOS	pervasive developmental disorder-not otherwise specified
PDZ	PSD-95, DlgA and ZO-1
PFA	paraformaldehyde
ProSAP	proline-rich synapse-associated protein
PVDF	polyvinylidene fluoride
PSD	postsynaptic density
PSD-95	postsynaptic density protein-95
RDA	recommended dietary allowance
RNA	ribonucleic acid
RT	room temperature
SAM	sterile α-motif
scr	scrambled
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SH3	Src homology 3

LIST OF ABBREVIATIONS

Shank	Src homology 3 domain and ankyrin repeat-containing
shRNA	short hairpin RNA
S.O.C.	Super Optimal broth with Catabolite repression
SPN	Shank/ProSAP N-terminal
SSTRIP	somatostatin receptor-interacting protein
STR	striatum
suppl	supplemented
TAE	tris-acetate-EDTA
TBS	tris-buffered saline
TBS-T	tris-buffered saline with tween 20
TEMED	N-tetramethylethylenediamine
TPEN	N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine
Tris	tris-hydroxymethyl-aminomethane
WB	Western Blot
ZO-1	zona occludens-1
Zn	zinc
$ZnCl_2$	zinc chloride

1 INTRODUCTION

1.1 The central nervous system and the synapse

The central nervous system of humans and other higher vertebrates consists out of 10^{11} neurons and at least the same number of glial cells whose task is, among others, to protect and support the neurons (Kandel, 1991). Neurons are able to receive, process and then transmit information via synapses. Each neuron, even though very different in size and shape, consists of dendrites, a soma, an axon and a multitude of synapses. The dendrites are the afferent part of the neuron and receive incoming information from other neurons. The axon as the efferent part, which can be over 1 m long, transmits the information after it was processed. At the end of each axon there are many synaptic terminals, which consist of pre- and postsynaptic membranes separated by the synaptic cleft. Synapses are used to transfer information from one neuron to another. This kind of communication between neurons is the basis for proper brain functioning and for memory formation (Kandel, 1991; Suedhof and Malenka, 2008). There exist chemical and electrical synapses. Chemical synapses use chemical substances, so-called neurotransmitters, to transmit information. These neurotransmitters are released into the synaptic cleft from vesicles where they are stored upon a neuronal impulse (a so-called action potential) reaching the presynaptic terminal of an axon. The release occurs by fusion of the neurotransmitter vesicle membrane with the presynaptic membrane initiated by Ca^{2+} influx (Ziv and Garner, 2004). After diffusion through the synaptic cleft, the neurotransmitters can bind to receptors or ion channels located on the postsynaptic membrane. This leads to opening or closing of postsynaptic ion channels, which in turn leads to the generation of postsynaptic potentials to complete the transmission of information. The postsynaptic membrane localized on the opposite side of the synaptic cleft is characterized by the so-called postsynaptic density (PSD) (Kandel, 1991; Garner et al., 2002; Scannevin and Huganir, 2000).
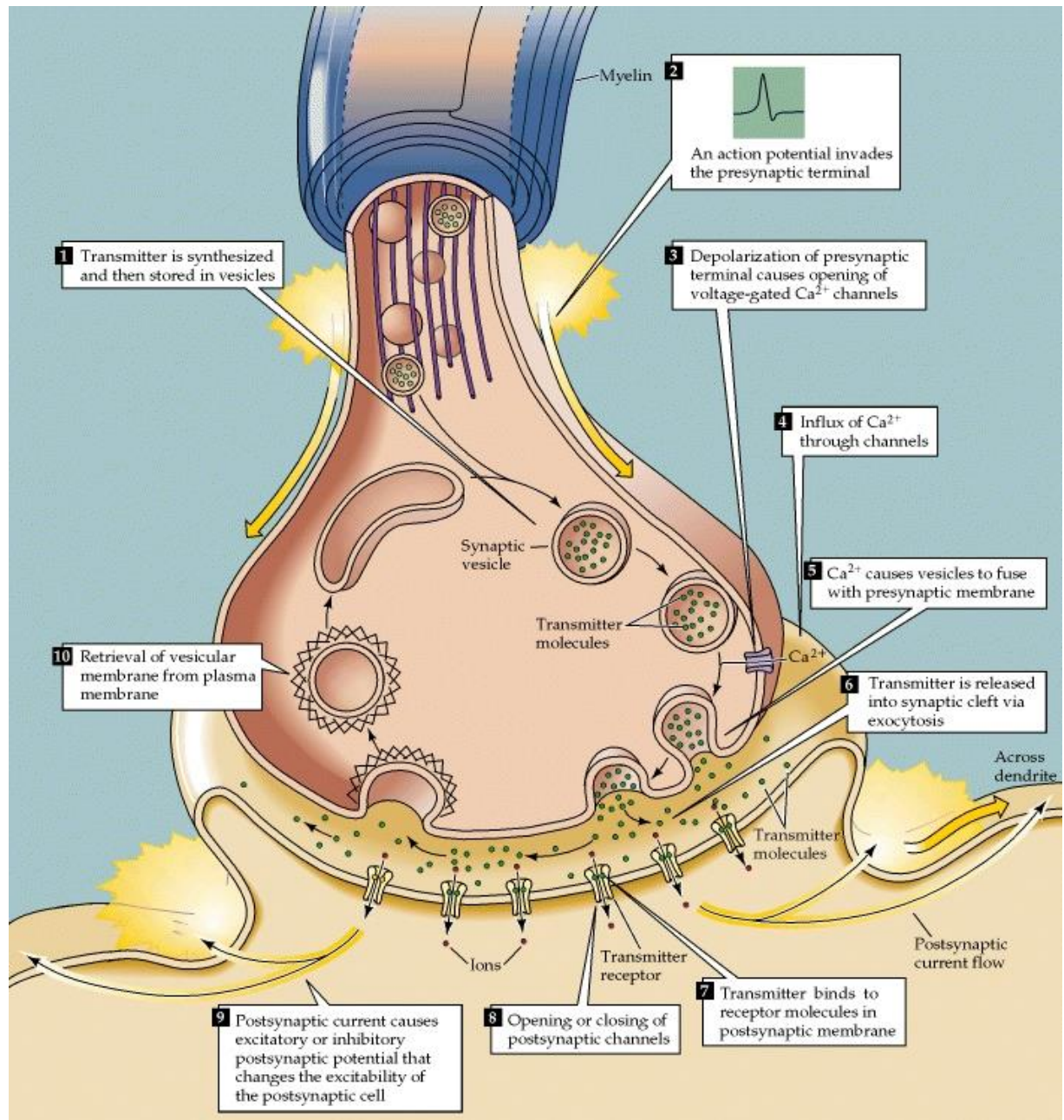
Figure 1: Schematic illustration of a chemical synapse: A neuronal impulse (action potential) reaches the presynaptic terminal of an axon. This depolarization of the presynaptic membrane is followed by influx of Ca^{2+} which causes neurotransmitter release into the synaptic cleft from vesicles by fusion of the vesicle membrane with the presynaptic membrane. The transmitters bind to receptors of the postsynaptic membrane causing opening or closing of ion channels there. Postsynaptic potentials are created which completes the transmission of information from one neuron to the other (Purves et al., 2004; reprinted with permission from Oxford University Press).

1.2   The postsynaptic density

The postsynaptic density (PSD) is an electron-dense protein network that is 40 nm thick and a few hundred nanometers wide (Harris and Stevens, 1989) (figure 2).
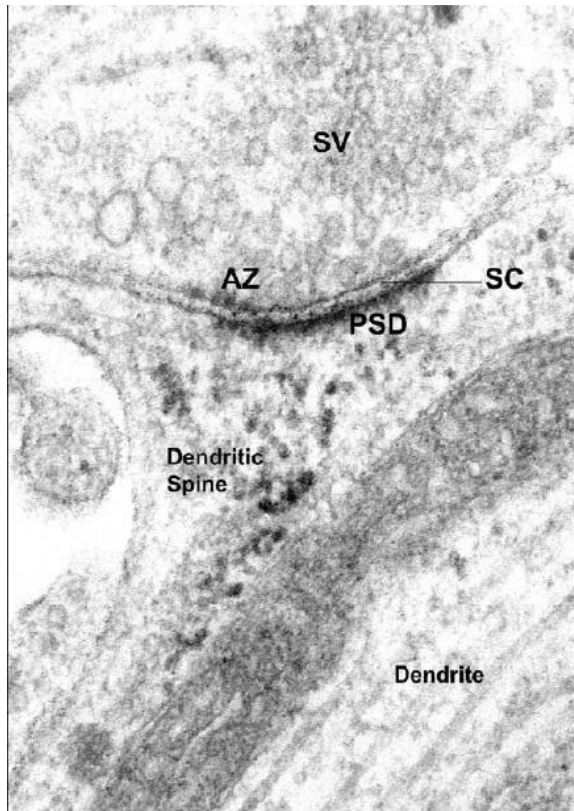
Figure 2: High magnification of a synaptic ultractructure: A presynaptic axon terminal with vesicles with a diameter of 50nm (SV) can be seen in the active zone region (AZ). Between the pre- and postsynaptic membrane the synaptic cleft (SC) is visible. The electron-dense band of the postsynaptic membrane is the postsynaptic density (PSD) which is located at the top of a dendritic spine, a small subcompartment of a dendrite of the neuron (Boeckers, 2006; reprinted with permission from Springer).

It is composed of a specialized network underneath the postsynaptic membrane of excitatory synapses consisting of cell-adhesion molecules, cytoskeletal and scaffold proteins as well as membrane-bound receptors and channels, G-proteins and signaling molecules like kinases/phosphatases (Klauck and Scott, 1995; Boeckers et al., 2002). Typical PSDs can be found at type 1 glutamatergic excitatory synapses which use glutamate as neurotransmitter (Boeckers, 2006; Ziff, 1997). The mentioned proteins are involved in clustering synaptic membrane proteins and anchor the postsynaptic structure to the actin cytoskeleton. The PSD organizes postsynaptic signal transduction and coordinates changes in postsynaptic structures dependent of development and activity. It

is also important for so-called synaptic plasticity (Ziff, 1997). Within the postsynaptic membrane, various types of glutamate receptors (GluRs) are clustered such as AMPA receptors (AMPARs), NMDA receptors (NMDARs) and group I metabotropic GluRs (mGluRs) (Sheng, 2001). These glutamate receptors interact either directly or indirectly (for example via the scaffold protein Homer) with scaffold proteins of the ProSAP/Shank family, which organize the PSD (Boeckers et al., 2002). The major components of the PSD and a model of the molecular structure can be seen in figure 3.
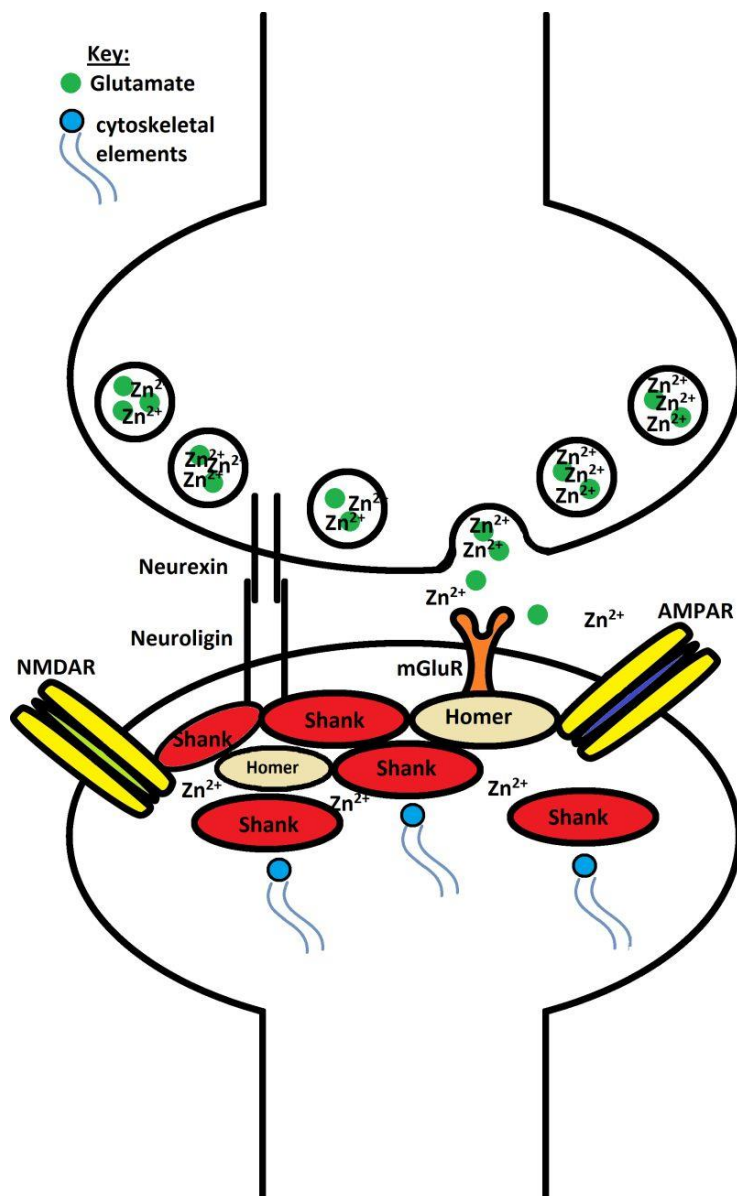

Figure 3: Model of the molecular structure of the postsynaptic density at excitatory synapses: The ProSAP/Shank proteins build platforms with the help of zinc at the PSD by interacting with NMDAR/AMPAR complexes, Homer-mGluR complexes and the cytoskeleton.

1.3   The ProSAP/Shank protein family

The ProSAP/Shank protein family was identified in 1998/1999 and it consists of three known members: Shank1, ProSAP1/Shank2 and ProSAP2/Shank3 (Du et al., 1998; Boeckers et al. 1999a; Naisbitt et al., 1999). There exist several synonymous names for this protein family such as Spank proteins (Boeckers et al., 2002). The Shank (Src homology 3 domain and ankyrin repeat-containing) proteins are important for the regulation of formation and maturation of excitatory synapses as well as for synaptic stability and plasticity (Boeckers et al., 2001; Lim et al., 1999; Grabrucker et al., 2011b). As Shank proteins contain conserved proline-rich domains they were also named proline-rich synapse-associated proteins (ProSAPs). There exist at least three isoforms of ProSAP1/Shank2, a big variety of isoforms of ProSAP2/Shank3 and also several splice variants of Shank1 (Boeckers et al., 1999a; Lim et al., 1999; Naisbitt et al., 1999; Wang et al., 2014). The ProSAP/Shank proteins can be found in large quantities at PSDs of excitatory synapses in the CNS (Boeckers et al., 1999a). They can cluster neurotransmitter receptors as group I mGluRs and bind trans-synaptic complexes of Neuroligins (Nlgn) and Neurexins (Nrxn) (Verpelli et al., 2011; Meyer et al., 2004). Thus, presynaptic molecules like Piccolo can be regulated Nrxn/Nlgn dependent via trans-synaptic signaling by ProSAP2/Shank3 (Arons et al., 2012). ProSAP/Shank proteins also interact with other scaffold proteins like GKAP, Homer or GRIP and together they anchor the receptor complexes in the postsynapse (Boeckers et al., 1999b; Tu et al., 1999). Therefore, correct organization of the PSD can be ensured. Based on this function, ProSAP/Shank proteins are also termed "master" scaffold proteins (Sheng and Kim, 2000; Boeckers et al., 2001). Actin-binding proteins such as cortactin and α-fodrin form a link to the cytoskeleton (Naisbitt et al., 1999; Boeckers et al., 2001; Boeckers et al., 2002). Because of their key function in organizing the PSD, the ProSAP/Shank proteins can regulate synaptic assembly, stability and plasticity, which are necessary for learning and memory formation (Boeckers et al., 2006; Grabrucker et al., 2011a).

The ProSAP/Shank proteins are relatively large proteins (>180 kDa). They contain five domains that are presumably important for protein interactions. The N-terminus contains 5-6 ankyrin repeats followed by the Src homology3 (SH3) domain, the PSD-95/Discs large/zona occludens-1 (PDZ) domain, several proline-rich regions, a cortactin-binding

domain (ppI) and the C-terminal sterile α-motif (SAM) domain (Boeckers et al., 1999a; Boeckers et al., 1999b).

The newly found Shank/ProSAP N-terminal (SPN) domain consists of about 90 amino acids and is located N-terminal to the ankyrin repeats. It seems to be important for the regulation of ligand binding to the anykrin repeats (Mameza et al., 2013). In Figure 4 the three family members of the ProSAP/Shank scaffold proteins in their major isoforms with their domains are illustrated.
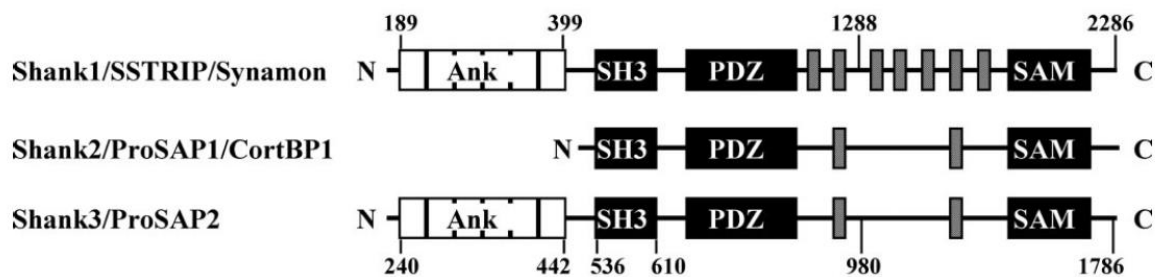
Figure 4: The three members of the ProSAP/Shank protein family with its respective protein interaction domains: SSTRIP: somatostatin receptor-interacting protein; ProSAP: proline-rich synapse-associated protein; CortBP1: cortactin-binding protein 1; Ank: ankyrin repeat; SH3: Scr homology3; PDZ: PSD-95/DLG/ZO-1; SAM: sterile α-motif (Boeckers et al., 2001; reprinted with permission from the American Society for Biochemistry and Molecular Biology).

The C-terminus with the SAM domain is needed for correct targeting of ProSAP1/Shank2 and ProSAP2/Shank3 to the synapse (Boeckers et al., 2005). The SAM domain is able to bind zinc (the expression "zinc" or "zinc ions" is used instead of Zn^{2+} to simplify reading) which leads to stabilization of antiparallel assemblies. Thereby, large sheets of helical fibers can be formed between several ProSAP2/Shank 3 proteins (Baron et al., 2006). Zinc may have the ability to enhance assembly and packaging of these assemblies and are also able to re-cluster dissolved PSDs (Gundelfinger et al., 2006). Thereby, zinc directly modulates the structure and also the function of the PSD. Additionally, a depletion of zinc leads to a loss of postsynaptic localization of ProSAP/Shank proteins and therefore to a disintegration of immature PSDs (Grabrucker et al., 2009). The PDZ domain mediates indirect binding to NDMAR/AMPAR via GKAP, direct binding to mGluRs and it is important for synaptic localization of Shank1 (Kim et al., 1997; Tu et al., 1999; Sala et al., 2001). The ankyrin repeats bind to spectrin and α-fodrin (Boeckers et al., 2001). There are binding

sites for cortactin at the C-terminus (Naisbitt et al., 1999). Both proteins are F-actin associated.
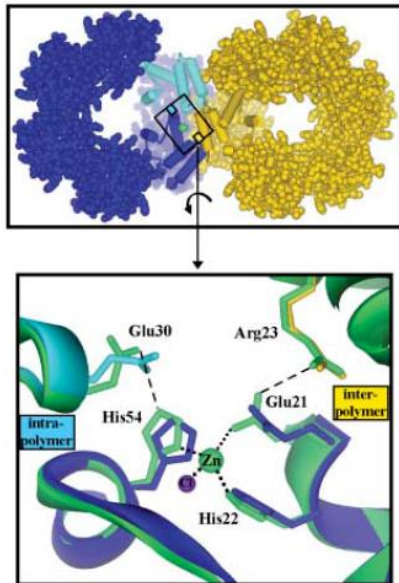
Figure 5: Crystral structure of the SAM domain of ProSAP/Shank proteins: The crystal structure of the SAM domain of ProSAP/Shank proteins shows that it is bound to zinc and that a tetrahedron is formed zinc being bound to Glu21,His22, His54 and a chloride ion. The zinc-binding site is located where intra- and interpolymer protein parts meet and seems to stabilize two salt bridges across the two parts: His54 with Glu30 and Glu21 with Arg23, respectively (Baron et al., 2006; reprinted with permission from The American Association for the Advancement of Science).

At different developmental stages all three ProSAP/Shank family members can be detected in various tissues. While ProSAP1/Shank 2 and ProSAP2/Shank3 appear early at the synapse and therefore are important for synapse formation, the zinc-insensitive form Shank1 appears there last and seems to be more important for maturation (Grabrucker et al., 2011a). Only Shank1 seems to be brain specific (Lim et al., 1999). However, all ProSAP/Shank proteins are expressed in the brain and can be found at the PSD of excitatory synapses (Boeckers et al., 1999b; Naisbitt et al, 1999). Interestingly, Shank1, ProSAP1/Shank2 and ProSAP2/Shank3 are highly expressed in the hippocampus and cortex, which are regions with higher cognitive function. In the cortex and hippocampus ProSAP1/Shank2 and ProSAP2/Shank3 proteins are co-expressed in neurons. In the cerebellum there is a complementary distribution: While ProSAP1/Shank2 is found in Purkinje cells, ProSAP2/Shank3 is expressed in the granular cell layer (Boeckers et al., 2004).

It is also interesting to see that mouse models for autism spectrum disorders with mutations/deletions of ProSAP1/Shank2, ProSAP2/Shank3 or Shank1 revealed deficits in social interactions along with reduced synaptic functioning and plasticity. For example, these mice show autistic-like behavior injuring themselves by repetitive grooming and displaying impaired learning and memory abilities (Bozdagi et al., 2010; Wang et al., 2011). ProSAP/Shank knockout mice also show abnormal social behavior and less complex vocal communication, which is also a behavior that can be seen in autistic children (Wang et al., 2011; Peça et al., 2011; Schmeisser et al., 2012).

1.4   Zinc

Zinc is an essential trace-mineral that can be found in all parts of the human body such as liver, kidneys, blood and brain (Hambidge et al., 1986). It is important for the functioning of more than 300 enzymes, hormone receptors or transcription factors as zinc ions are able to bind to proteins as ligands (Williams, 1989; Prasad, 2012; Oteiza and Mackenzie, 2005). Additionally, zinc is a component of certain metalloproteins (MacDonald, 2000; Williams, 1989). Zinc is needed for the correct functioning of the immune system (Prasad, 1995). It also plays an important role in the proliferation of cells, DNA and RNA metabolism, differentiation and growth (Chesters, 1978). Furthermore, zinc ions are also involved in gene expression and apoptosis (Chesters, 1978; MacDonald, 2000). Normal growth and development during pregnancy, childhood and adolescence is supported by zinc (Simmer and Thompson, 1985). In the United States, the Recommended Dietary Allowance (RDA) of zinc is 11 mg/day for men and 8 mg/day for women (Food and Nutrition Board, Institute of Medicine, 2000). Zinc cannot be stored in the body and zinc uptake has to occur regularly by food consumption (Golub et al., 1995). Good food sources for zinc are eggs, meat, fish and milk products. Non-animal sources for zinc are vegetables, nuts and whole grains (Hambidge et al., 1986).

Most zinc ions in the body are bound to proteins. However, free zinc can be found in the brain. It is highly concentrated in synaptic vesicles of special glutamatergic neurons in the mammalian forebrain. All zinc-containing neurons are glutamatergic and it can be

released together with glutamate. These neurons can be found in the neocortex, striatum, amygdala and hippocampus - above all in the CA3 region (Frederickson, 2000). Zinc ions that are released from synaptic terminals of mossy fibers are important for the induction of LTP (long-term potentiation) in the CA3 region of the hippocampus (Li et al., 2001). The distribution suggests that a normal zinc homeostasis in the brain is necessary for development, synaptic plasticity, neurotransmission and proper cognitive function (Li et al., 2001; Sensi et al., 2009). Therefore zinc deficiency, especially during early development, may lead to brain dysfunction and can impair thinking, attention, learning and memory (Takeda, 2000; Bhatnagar and Taneja, 2001; Grabrucker et al., 2011a).

Furthermore, zinc ions can be found in huge amounts in the postsynaptic density (PSD) at glutamatergic synapses where they play an important role in the formation and maintenance of the stability of the PSD structure (Jan et al., 2002; Baron et al., 2006). There, zinc ions help forming two-dimensional sheets of helical fibers by binding to the SAM domain of ProSAP2/Shank3 proteins (Gundelfinger et al., 2006). They also influence the recruitment of ProSAP1/Shank2 and ProSAP2/Shank3 scaffold proteins to the PSD, which are both zinc-binding proteins. These two zinc-sensitive proteins are necessary for the assembly and stability of immature synapses. Zinc ions are consequently able to modulate synapse formation and maturation.

Zinc deficiency is in the majority of cases a consequence of low bioavailability of zinc, but can also be a consequence of malabsorption diseases, which can lead to biochemical dysfunctions (Prasad, 1983). Zinc deficiency in periods of rapid growth such as pregnancy and infancy can lead to disturbances of growth (Sandstead et al., 1967). Therefore, an increased amount of zinc is needed during pregnancy, infancy and adolescence (Favier, 1992). Clinical signs of severe acute zinc deficiency in humans are cognitive impairment, increased susceptibility to infections and impaired wound-healing, hypogonadism, skin disorders and decreased appetite (Prasad, 1991).

Zinc deficiency can be detected from various sources like plasma, hair, body liquids or tissue samples. Methods for the detection of zinc deficiency are atomic absorption spectrometry (AAS), inductively coupled plasma mass spectrometry (ICP-MS), analysis of

metallothioneins by ELISA and zinc-dependent enzymes in the plasma and zinc staining (Meadows et al., 1981; Grider et al., 1990; Ruz et al., 1992; Townsend et al., 1998). Free zinc ions are histochemically reactive and can be visualized by Timm's sulfide-silver staining or with fluorescent probes as Zinquin ethyl ester or Zinpyr-1 (Jaarsma and Korf, 1990; Frederickson, 2000; Coyle et al., 1994; Colvin et al., 2006; Walkup et al., 2000).

Zinc intoxication is rare as it requires high doses. It mainly occurs as a consequence of copper deficiency (Plum et al., 2010). Clinical signs are gastrointestinal symptoms, lethargy, staggering of gait and difficulties in writing (Fosmire, 1990).

1.5 Copper and COMMD1

Copper (the expression "copper" or "copper ions" is used instead of Cu^{2+} to simplify reading) is an essential trace element that can donate and accept electrons. Therefore, it plays an important role in oxidation-reduction reactions and takes part in many enzymatic reactions as a cofactor (Harris, 1992; Kosman et al., 1974). The daily requirement of copper is 1-2 mg (Buchet et al., 1983; Das and Ray, 2006). Its uptake occurs in the gut mediated through the copper transporter CTR1 and the divalent metal transporter DMT1 (Lutsenko, 2010; Arredondo et al., 2003). Copper can be found in various food sources such as vegetables, liver, nuts or chocolate (Linder and Hazegh-Azam, 1996). Copper is stored bound to metallothioneins and delivered into the blood via ATP7A, which is a P-type ATPase (Vulpe et al., 1993). It is then bound to the serum protein albumin and transported to the liver. From there it can be delivered to other organs being bound to the glycoprotein ceruloplasmin or excreted into the bile via the copper transporting ATPase ATP7B (Loudianos and Gitlin, 2000; Wijmenga and Klomp, 2004; Das and Ray, 2006). 75-95% of copper in the blood is bound to ceruloplasmin, which can bind six copper ions (Hellman and Gitlin, 2002; Das and Ray, 2006).

The entry of copper into the brain is still poorly understood. CTR1, ATP7A and ATP7B seem to play a role at the entry of copper into the brain through the blood-brain-barrier (Choi and Zheng, 2009). Brain regions with high copper levels are the hypothalamus in rats and human hippocampus (Rajan et al., 1976; Dobrowolska et al., 2008). Almost all

copper in the brain is bound to proteins and its distribution is tightly regulated (Que et al., 2008; Lutsenko et al., 2010). High levels of copper in the brain can inhibit AMPA and NMDA receptors and also AMPA receptor-mediated synaptic transmission (Weiser and Wienreich, 1996; Peters et al., 2011). At NMDA receptors, copper leads to an inhibition of hippocampal LTP formation which is important for learning and memory (Doreulee et al., 1997; Lynch, 2004).

Usually copper deficiency occurs because of too little copper uptake and can also be a consequence of malabsorption (Danks, 1988). Copper deficiency manifests in anemia and impaired mobilization of iron, leuko- and neutropenia, decreased levels of superoxide dismutase, ceruloplasmin and cytochrome c oxidase as copper is a cofactor for these enzymes and copper-binding proteins (Williams, 1983; Plum et al., 2010). It can lead to cardiomyopathy, muscle weakness and increased plasma levels of LDL (low density lipoprotein) cholesterol as well (Danks, 1988).

Severe copper intoxication is rare in humans as long as a normal capacity of copper excretion through the bile is available. Main causes are infections, inflammation, excessive uptake, systemic lupus erythematosus, trauma or Wilson's disease (Russo and DeVito, 2011). Copper toxicity mainly occurs as a consequence of low levels of zinc (Blaurock-Busch et al., 2012). It affects first the liver, but also the kidneys and brain function. It can progress to severe hemolytic anemia, hepatic necrosis, vascular collapse, coma and death (Gaetke and Chow, 2003). Tissue damage mainly occurs because of increased free radical concentrations and oxidative damage to nucleic acids, proteins and lipids (Bjørklund, 2013; Rosenzweig, 2000).

ATP7B is non-functional in patients with Wilson's disease, which manifests in copper overload in the liver and the brain, which in turn can lead to neurodegeneration and cognitive impairment (Bost et al., 2012; Huster, 2010). ATP7B transfers intracellular copper ions to apoceruloplasmin followed by conversion to ceruloplasmin, which is further released into the blood stream in order to deliver copper to the brain (Das and Ray, 2006; de Bie et al., 2005). The expression level and stability of ATP7B and its trafficking within cells is regulated by COMMD1 as it can bind to the N-terminus of ATP7B

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(Tao et al., 2003; de Bie et al., 2005; Miyayama et al., 2010; Wang et al., 2011). COMMD1 (Copper- metabolism gene MURR1-domain 1) also mediates degradation of misfolded, mutant ATP7B proteins (Materia et al., 2012).

The COMMD1 gene is located on the human chromosome 2p15. The COMMD proteins 1-10 contain a MURR1 (MURR = mouse U2af1-rs1 region) domain that is located near the C-terminus and is also called COMM domain. The conserved unique motif is leucine-rich, consists of 70 to 85 amino acids and is important for protein interactions (Burstein et al.2005). The copper binding site is located in the exon 2 product which can bind one copper ion. (Narindrasorasak et al., 2007). COMMD1 is ubiquitously expressed (Klomp et al., 2003).
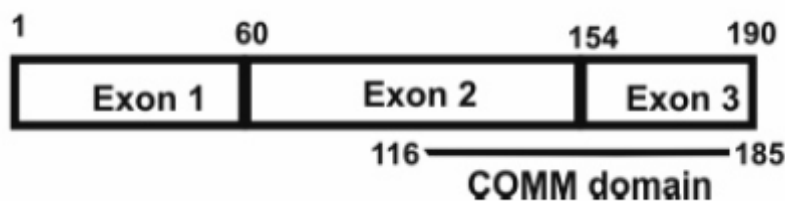
Figure 6: Schematic representation of COMMD1: All three exons of COMMD1 are shown with the amino acid residues that are the boundaries of each exon. The COMM domain consists of 70 to 85 amino acids (Narindrasorasek et al., 2007; reprinted with permission from the American Chemical Society).

Knockdown of COMMD1 *in vitro* and *in vivo* leads to increased intracellular copper levels (Burstein et al. 2004; Vonk et al., 2011). There exists an inverse correlation between the COMMD1 protein and the intracellular level of copper (Burstein et al., 2005). Therefore COMMD1 can be seen as a regulator of copper metabolism. It has been identified as the mutated gene that causes copper toxicosis in Bedlington terriers by complete loss of functional COMMD1 (van de Sluis et al., 2002). COMMD1 plays an important role in controlling copper homeostasis in hepatocytes and together with ATP7B in copper excretion (deBie et al., 2007; Vonk et al., 2011).

Within the body, copper and zinc have antagonistic roles which means that an overload of copper leads to zinc deficiency and vice versa (Hill and Matrone, 1970; Mills, 1985; Hall et al., 1979). Usually the ratio of copper to zinc in the blood is close to 1:1. (Faber et al, 2009; Van Weyenbergh et al., 2004).

1.6 Metallothioneins

Most intracellular zinc ions are bound to metal-binding proteins, above all to proteins of the metallothionein family (MTs). Metallothioneins are small proteins that consist of 61- 68 amino acids. 20 of these amino acids are highly conserved cysteines that bind seven zinc ions or other metals including copper with high affinity. MTs form zinc clusters thus shielding the zinc from the environment (Maret, 2000).

Four different isoforms of MTs are known in mammals. The MT gene family is located on the human chromosome 16. Three out of four known MT isoforms are found in the nervous system. Both MT-1 and MT-2 are expressed in several organs, however the MT-3 isoform can only be found in the brain (Palmiter et al., 1992). While MT-1 and MT-2 are expressed mainly in glial cells, MT-3 is mostly localized in zinc-containing neurons (Aschner et al., 1997; Masters et al., 1994). Therefore, MT-3 is preferentially present in regions with a high density of zinc-containing neurons, such as the hippocampus (Masters et al., 1994). MT-1 and MT-2 expression levels in the cerebellum are higher than MT-3 (Aschner et al., 1997). MT-4 expression is restricted to skin epithelium and the upper part of the gastrointestinal tract (Quaife et al. 1994; Aschner et al., 1997).

Metallothioneins can provide, recycle or buffer zinc. They are important for the detoxification of metal ions (Erickson et al., 1997). From MTs zinc ions can be released into the cytoplasm with the help of nitric oxide (NO) by oxidizing sulfhydryl groups in MTs or by destroying zinc-sulfur clusters (Kröncke et al., 1994; Aravindakumar et al., 1999). Both zinc and copper ions can induce the transcription of metallothioneins via MTF-1, a metal-regulatory transcription factor that enhances the transcription of MT genes (Brugnera et al., 1994; Heuchel et al., 1994). Levels of metallothioneins rise when copper levels increase (Faber et al., 2009). Copper is bound more firmly to MTs than zinc (Sakulsak, 2012). Disturbed functioning of MTs may lead to zinc deficiency and may be combined with copper excess (Bjørklund, 2013).

1.7   Autism spectrum disorders

Autism spectrum disorders (ASD) belong to a group of neurodevelopmental disorders and define a very heterogeneous group including disorders such as autism, Asperger Syndrome and PDD-NOS (pervasive developmental disorder-not otherwise specified; Diagnostic and Statistical Manual of Mental Disorders, V. by the American Psychiatric Association). The main clinical characteristics are stereotypic behavior, impairments in communication and language, social skills and cognition (Rapin, 1997; Lord et al., 2000). Clinical signs are present at the age of 3 at the latest. ASD patients show an altered neurodevelopment, aberrations in brain growth, cortical connectivity and changed structure of synapses and dendrites (Pardo and Eberhart, 2007; Zoghbi, 2003).

The genetics of ASD is very complex as the cause is polygenic. A lot of associated genes are involved in different developmental processes like neuronal migration, cortical organization, synaptic and dendritic conformation (Pardo and Eberhart, 2007).

1.8   Proteins involved in ASD pathogenesis

Among others, genes that have been found to be mutated/deleted in ASD patients are coding for synaptic scaffold proteins of the ProSAP/Shank family (Grabrucker et al., 2011c). Autism-associated mutations have been reported in all three ProSAP/Shank family members (Durand et al., 2007; Moessner et al., 2007; Gauthier et al., 2009; Berkel et al., 2010; Pinto et al., 2010; Sato et al., 2012). The most important ProSAP/Shank isoform associated with neurodevelopmental deficits or neurobehavioral disorders is ProSAP2/Shank3 (Durand et al., 2007; Moessner et al., 2007; Gauthier et al., 2009). In ASD patients with a de novo mutation in the ProSAP2/Shank3 protein anomalies like reduced neuronal size and truncated dendrites were observed. Disturbances there may lead to learning disabilities (Durand et al., 2012). The ProSAP2/Shank3 gene is located on chromosome 22q13.3. Microdeletions of the long arm of chromosome 22, mostly de novo, which affect ProSAP2/Shank3 expression or loss of one copy of ProSAP2/Shank3 lead to the so-called Phelan McDermid Syndrome which is a neurodevelopmental disorder (Nesslinger et al., 1994; Phelan and McDermid, 2011; Manning et al., 2004). These patients also show autistic behavior among other deficits (Durand et al., 2007).

Additionally, the disruption of the PSD platforms generated by ProSAP/Shank proteins only leads to an impairment of the function of excitatory synapses as inhibitory synapses do not contain ProSAP/Shank proteins (Naisbitt et al., 1999). This may lead to an imbalance of excitation and inhibition, which might be an important factor in neurological diseases such as ASD (Rubenstein and Merzenich, 2003). De novo mutations in the ProSAP1/Shank2 gene have also been associated to ASD patients (Berkel et al., 2010; Pinto et al., 2010).

Some other defect genes found in ASD patients are coding for other PSD proteins at glutamatergic synapses such as neurexins or neuroligins or proteins involved in the homeostasis of metal ions like metallothioneins, the transcription factor MTF-1 and copper transport proteins like ceruloplasmin or COMMD1 (Serajee et la., 2004; Tórsdóttir et al., 2005; Koumura et al., 2009; Betancur et al., 2009; Levy et al., 2011).

In some cases of ASD the COMMD1 gene was found to be homozygous deleted which led to a complete loss of function and thereby to an intracellular overload of copper (Levy et al., 2011). Ceruloplasmin, which transports copper to the brain, was also already associated with ASD. The level of ceruloplasmin reduction correlates with the loss of previously acquired language skills in children with autism (Chauhan et al., 2004).

1.9 Zinc, copper and ASD

Interestingly, a disrupted biometal-homeostasis has been found in hair and nail samples of many ASD patients which lead to elevated intracellular levels of copper (Lakshmi Priya and Geetha, 2011). Further studies indicate that the copper/zinc ratio is increased in the blood serum of patients with autism. Therefore, it was suggested to use this ratio as biomarker for children with ASD (Faber et al., 2009). The mentioned patients either showed a zinc deficiency or a copper toxicity. Especially children with ASD suffer from marginal to severe zinc deficiency. There's a significant correlation of zinc concentration with age in the autistic patients. Lowest zinc levels in hair samples have been found in the youngest children aged 0-3 years (Yasuda et al., 2011; Yasuda et al., 2013; Lakshmi Priya and Geetha, 2011). The copper level in the examined autistic children could also be correlated with the severity of their symptoms, which means that the highest copper

levels were found in children with the most severe symptoms (Lakshmi Priya and Geetha, 2011). These results suggest that infantile zinc deficiency or copper overload might contribute to the pathogenesis of autism (Yasuda et al., 2011).

A one case study by Walker also showed that copper overload during pregnancy may lead to autism and that high copper concentrations can be treated with zinc also as prevention (Walker et al., 2011 and personal correspondence of Andreas M. Grabrucker with the author).

ASD patients often show changes in the intestinal function and flora, which can lead to malabsorption of zinc, maybe also due to copper overload (de Theije et al., 2011; Finegold et al., 2012). This can be a cause for zinc deficiency as well.

Lots of ASD candidate genes can be placed into a common hypothetical pathway at glutamatergic synapses (Bourgeron, 2009; Grabrucker et al., 2011b). Therefore, the deregulation of important members of this pathway may represent major genetic risk factors for ASD. The interplay of zinc and copper may also be a core regulatory component of this disrupted pathway in ASD patients (Grabrucker, 2014).

1.10 Aim of the thesis

Autism is a neurodevelopmental disorder and alteration of specific proteins located at glutamatergic synapses was implied as causing factor. For example, mutations in all three synaptic proteins of the ProSAP/Shank family are associated with ASD (Durand et al., 2007; Moessner et al., 2007; Gauthier et al., 2009; Berkel et al., 2010; Pinto et al., 2010; Sato et al., 2012). Other changed proteins found in ASD patients are metallothioneins or COMMD1, a protein that regulates copper excretion (Koumura et al., 2009; Levy et al., 2011). It was discovered that children with ASD often suffer from marginal to severe zinc deficiency and copper overload leading to abnormally high copper/zinc ratios (Faber et al., 2009; Lakshmi Priya and Geetha, 2011). Therefore, this changed copper/zinc ratio was suggested as biomarker for children with ASD (Faber et al., 2009). However, recent data also implies a link between trace metal levels and functionality of synaptic proteins and thus, alterations of copper/zinc ratio may also be a causative factor. In particular, in this thesis, I follow the hypothesis that increased copper levels can lead to decreased zinc

levels *in vitro* and *in vivo* as within the body, copper and zinc have antagonistic roles. This means that copper overload can lead to zinc deficiency and vice versa.

Furthermore, ProSAP1/Shank2 and ProSAP2/Shank3 are zinc-sensitive via their SAM domains and are stabilized at the PSD of glutamatergic synapses in a zinc-dependent manner (Baron et al., 2006). A depletion of zinc leads to a disintegration of immature synapses and therefore zinc, among others, is key for formation, maturation and plasticity of excitatory glutamatergic synapses (Grabrucker et al., 2011a). Given that zinc deficiency leads to decreased levels of ProSAP1/Shank2 and ProSAP2/Shank3 (Grabrucker et al., 2014), we therefore hypothesize that a changed zinc homeostasis by copper overload or changed levels of the copper excretion regulator protein COMMD1 affect the autism-associated pathway at glutamatergic synapses in which ProSAP/Shank proteins are involved.

The aim of this thesis is to confirm the previously mentioned hypotheses that increased copper levels can lead to decreased zinc levels *in vitro* and *in vivo* and that a changed zinc homeostasis by copper overload or changed levels of COMMD1 affect the ProSAP/Shank dependent autism-associated pathway at glutamatergic synapses.

As autism is a neurodevelopmental disorder it should also be analyzed whether there exists a critical developmental time window for the effects of copper on the ASD associated pathway by the conduction of *in vivo* experiments on adult and prenatal mice.

To examine the effects of an altered copper concentration *in vivo* and to evaluate its influence on zinc homeostasis, copper overload in mice is induced by feeding mice with a copper enriched diet. Some of the mice are mated to investigate copper supplementation and its consequences on prenatal zinc homeostasis and the autism-associated pathway in the offspring. Zinc and copper levels in body and brain – primary in cortex and hippocampus - are measured biochemically and immunohistochemically in order to see whether copper supplementation can change these levels *in vivo*. The influence of copper supplementation on PSD proteins *in vivo* is also analyzed immunohistochemically and with molecularbiological methods on protein level.

We will also investigate immunohistochemically how changed zinc and/or copper levels influence zinc homeostasis *in vitro*. Additionally, ProSAP/Shank and other protein levels of

the PSD of glutamatergic synapses are measured immunohistochemically after alteration of zinc and copper levels *in vitro*. A knockdown of COMMD1 is performed to investigate, using immunohistochemistry, the influence of altered COMMD1 levels on zinc and copper homeostasis as well as ProSAP/Shank levels at synapses.

2   MATERIALS AND METHODS

2.1   Materials

2.1.1   Labware and machines

Blot Transfer System 11-14	Life Technologies, Gaithersburg, MD, USA
Cell culture flasks 25 cm^2	Sarstedt, Nümbrecht
Cell culture plates 24 well	Sarstedt, Nümbrecht
Cell culture dishes TC 100	Sarstedt, Nümbrecht
Centrifuge 5424	Eppendorf, Hamburg
Centrifuge Hettich Mikro 200R	Sigma-Aldrich, Steinheim
Centrifuge Multifuge 3SR Plus	Heraeus-Kendro, Hanau
Chamber Slides	Nunc, Wiesbaden
Chemiluminescence System MicroChemi 4.2	Biostep, Jahnsdorf
Cover glasses 13 mm Ø	Menzel, Braunschweig
Cryostat Leica CM3050S	Leica, Wetzlar
Dark Hood DH-40	Biostep, Jahnsdorf
Horizontal electrophoresis system GH220	Biostep, Jahnsdorf
Electrophoresis power supply	Biostep, Jahnsdorf
Eppendorf tubes 0.2/0.5/1.5/2 ml	Eppendorf, Hamburg
Falcon Tubes 15 ml, 50 ml	Sarstedt, Nümbrecht

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Filtropur S 0.2 µm	Sarstedt, Nümbrecht
Flasks 100/250/500/1000 ml	Duran, Mainz
Freezer -20°C	Liebherr, Biberach
Freezer -80°C	ThermoFisher Scientific, Waltham, MA, USA
Fridge 4°C	Liebherr, Biberach
Gel documentation system Gerix 1000	Biostep, Jahnsdorf
Homogenizer Potter S	Sartorius, Göttingen
Ice Machine	Scotsman, USA
Incubator shaker Series 25	New Brunswick, New Jersey
Incubator Heracell 240i (37°C)	ThermoFisher Scientific, Waltham, MA, USA
Incubator BBD 6220	ThermoFisher Scientific, Waltham, MA, USA
Incubator INB 400 (37°C)	Memmert, Schwabach
Integra Vacusafe extraction system	GENEO BioTechProducts GmbH, Lübeck
Magnetic stirring hot plate MR Hei-Standard	Heidolph, Schwabach
Measuring cylinder 1000 ml	SciLabware Ltd, Staffordshire, UK
Microcentrifuge Heraeus pico	ThermoFisher Scientific, Waltham, MA, USA

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Microscope Axioskop 2 mot plus	Zeiss, Oberkochen
Microscope Axiovert 40 CFL	Zeiss, Oberkochen
Microscope slides	VWR, Darmstadt
Microscope Stemi 2000-CS	Zeiss, Oberkochen
Microwave	Sharp, Hamburg
Mini Centrifuge Sprout	Heathrow Scientific, Vernon Hills, IL, USA
Nanodrop 2000	ThermoFisher Scientific, Waltham, MA, USA
Nitrocellulose Blotting Membrane Amersham Protran 0.45 µm	GE Healthcare Life Sciences, Freiburg
Neubauer counting chamber	Brand, Wertheim
Nunc Cell Scrapers	ThermoFisher Scientific, Waltham, MA, USA
Nylon cell sieve 100 µm	ThermoFisher Scientific, Waltham, MA, USA
pH meter 766	Knick, Berlin
Pipetboy	Hirschmann, Eberstadt
Pipette tips 10 µl, 200 µl, 1000 µl	Sarstedt, Nümbrecht
Pipettes 2.5 µl	Eppendorf, Hamburg
Pipettes 10 µl, 100 µl, 1000 µl	Ahn Biotechnologie, Nordhausen

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Pipettes 10 µl, 100 µl, 1000 µl	Brand, Wertheim
Pipettes (glass) 5 ml, 10 ml, 20 ml	Brand, Wertheim
Pipettes 5 ml, 10 ml, 20 ml	Greiner Bio-One, Frickenhausen
QIAshredder	QIAGEN, Hilden
SDS-PAGE gel chamber	BioRad, München
SDS-PAGE gel tray	BioRad, München
SDS-PAGE glass plates 7.3 cm x 10.1 cm	BioRad, München
SDS-PAGE mini-protean comb 10 well, 15 well	BioRad, München
SDS-PAGE PowerPac HC	BioRad, München
Shaker Standard 1000	VWR, Darmstadt
Sterile bench Laminar flow	Nunc, Wiesbaden
Sterile bench HeraSafe	Heraeus, Hanau
Sterile bench MSC Advantage	ThermoFisher Scientific, Waltham, MA, USA
Tabletop shaker KS 260 basic	IKA, Freiburg
Tank Blot machinery	Biorad, München
Test tube rotator 34528	Snijders Labs, Tilburg, Niederlande
Thermo Cycler T100	Biorad, München
Thermomixer comfort 1.5 ml	Eppendorf, Hamburg

Thermomixer 5436	Eppendorf, Hamburg
UV transilluminator	Biostep, Jahnsdorf
Vortex-Genie 2	Scientific Industries, Inc., New York, USA
Water bath	Memmert, Schwabach
Weighing system, CPA225D	Sartorius, Göttingen
Weighing system, CPA42025	Sartorius, Göttingen
Weighing system BP 2100 S	Sartorius, Göttingen
Whatman filter papers 3MM	Roth, Karlsruhe
X-ray cassette	Dr. Goos Suprema, Heidelberg
X-ray Amersham Hyperfilm ECL	GE Healthcare Life Sciences, Freiburg

2.1.2 Kits

JETQUICK Plasmid Maxi Kit 20	Genomed, Löhne
JETQUICK Plasmid Mini Prep Kit	Genomed, Löhne
JETQUICK Gel Extraction Spin Kit	Genomed, Löhne
GoTaq Green Master Mix	Promega, Mannheim
µMACS Magnetic Beads Kit	Miltenyi Biotex, Bergisch Gladbach

2.1.3 Chemical substances

Chemical substances used in this thesis were purchased from Sigma-Aldrich, Steinheim if not indicated otherwise.

2.1.4 Solutions for molecular biology

LB-medium	20.0 g LB-medium Lennox	(Roth, Karlsruhe)
	ad 1 l deionized water	
	→ autoclaved at 121°C for 20 min	
LB-agar	35.0 g LB-agar Lennox	(Roth, Karlsruhe)
	ad 1 l deionized water	
	→ autoclaved at 121°C for 20 min	
Antibiotics (stock solution)	50 mg/ml ampicillin	
	dissolved in 50% ethanol	
	→Aliquots were stored at -20°C	
	25 mg/ml kanamycin	
	dissolved in 50% ethanol	
	→Aliquots were stored at -20°C	

1x TAE buffer	100 ml 10x TAE buffer
	ad 1 l with Aqua bidest

2.1.5 Solutions for cell culture

Poly-L-lysin	5 mg poly-L-lysin	
	ad 50 ml HBSS	(PAA, Cölbe)
	sterile filtration through Filtropur S 0.2 µm	
	mix half of the solution with 25 ml fresh HBSS	

DMEM++	DMEM (4.5 g/L D-glucose, L-glutamine, without pyruvate)	(GIBCO, Karlsruhe)
	5 ml FCS	(Hyclone, Logan, USA)
	500 µl L-glutamine	(GIBCO, Karlsruhe

DMEM+++	500 µl L-glutamine	(GIBCO, Karlsruhe)
	1 ml B27 supplement	(GIBCO, Karlsruhe)
	500 µl penicillin/streptomycin	(GIBCO, Karlsruhe)
	ad 50 ml DMEM (4.5 g/L D-glucose, L-glutamine, without pyruvate)	(GIBCO, Karlsruhe)

NB ++ medium	500 µl L-glutamine	(GIBCO, Karlsruhe)
	1 ml B27 supplement	(GIBCO, Karlsruhe)
	ad 50 ml neurobasal medium	(GIBCO, Karlsruhe)
NB +++ medium	500 µl L-glutamine	(GIBCO, Karlsruhe)
	1 ml B27 supplement	(GIBCO, Karlsruhe)
	500 µl penicillin/streptomycin	(GIBCO, Karlsruhe)
	ad 50 ml neurobasal medium	(GIBCO, Karlsruhe)

2.1.6 Solutions for immunocytochemistry and immunohistochemistry

PFA	1 l 1x PBS		(Invitrogen, Karlsruhe)
	40 g PFA		
	40 g Sucrose		
PBS 0.2% Triton	100 µl	Triton X-100	
	ad 50 ml	1x PBS	(Invitrogen, Karlsruhe)
PBS 0.05% Triton	25 µl	Triton X-100	
	ad 50 ml	1x PBS	(Invitrogen, Karlsruhe)

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Blocking Solution	5 ml	10% FBS	
	ad 50 ml	1x PBS	(Invitrogen, Karlsruhe)
PBS + DAPI	1 µl	DAPI (1mg/µl)	(AppliChem, Darmstadt)
	ad 50 ml	1x PBS	(Invitrogen, Karlsruhe)
Arabic gum solution	1 kg	Arabic gum	
	2 l	Aqua bidest	
Sodium citrate buffer	25.5 g	citric acid monohydrate	(Roth, Karlsruhe)
	23.5 g	sodium citrate dehydrate	(AppliChem, Darmstadt)
	ad 100 ml	Aqua bidest	
Hydroquinone solution	2.84 g	Hydroquinone	
	50 ml	Aqua bidest	
Timm developer	60 ml	filtered Arabic gum solution	
	10 ml	Sodium citrate buffer	
	30 ml	Hydroquinone solution	

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Primary antibodies

Anti-Bassoon mouse	(Synaptic Systems, Göttingen)	1:500
Anti-Gephyrin rabbit	(Synaptic System, Göttingen)	1:1000
Anti-mGluR5 rabbit	(Abcam, Cambridge, MA, USA)	1:200
Anti-ProSAP1 rabbit	(described in Schmeisser et al., 2012)	1:500
Anti-ProSAP2 guinea pig	(described in Schmeisser et al., 2012)	1:500
Anti-Shank1 rabbit	(Novus Biologicals, Frankfurt/Main)	1:400
Anti-Homer1 rabbit	(Synaptic Systems, Göttingen)	1:500
Anti-COMMD1 goat	(Santa Cruz Biotechnology, Inc., Dallas, Texas, USA)	1:500
Anti-myc mouse	(Sigma-Aldrich, Steinheim)	1:500

Secondary antibodies

concentration 1:500

Anti-guinea-pig-IgG, Alexa Fluor 568	(Invitrogen, Karlsruhe)
Anti-guinea-pig-IgG, Alexa Fluor 647	(Invitrogen, Karlsruhe)
Anti-mouse-IgG, Alexa Fluor 488	(Invitrogen, Karlsruhe)
Anti-mouse-IgG, Alexa Fluor 568	(Invitrogen, Karlsruhe)
Anti-mouse-IgG, Alexa Fluor 647	(Invitrogen, Karlsruhe)
Anti-rabbit-IgG, Alexa Fluor 568	(Invitrogen, Karlsruhe)
Anti-rabbit-IgG, Alexa Fluor 647	(Invitrogen, Karlsruhe)

Anti-goat-IgG, Alexa Fluor 568   (Invitrogen, Karlsruhe)

2.1.7 Solutions for protein biochemistry

Lysis buffer	150 mM	NaCl	
	1%	Triton X-100	
	50 mM	Tris/HCl	

4x SDS loading buffer	2.5 ml	1 M Tris/HCl pH 6.8	
	0.8 g	SDS	(Roth, Karlsruhe)
	6 ml	50% glycerol	
	0.46 g	Dithiothreitol	
	0.002 g	Bromophenol blue	
	ad 10 ml Aqua bidest		

Separating gel 12%	4.9 ml	Aqua bidest	
	6.0 ml	30% Acrylamide	(Serva, Heidelberg)
	3.8 ml	1.5 M Tris/HCl pH 8.8	
	0.15 ml	SDS 10% [m/v]	
	0.15 ml	APS 10% [m/v]	(Merck Succhard, Hohenbrunn)
	0.010 ml	TEMED	

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Separating gel 10%	5.9 ml	Aqua bidest
	5.0 ml	30% Acrylamide
	3.8 ml	1.5 M Tris/HCl pH 8.8
	0.15 ml	SDS 10% [m/v]
	0.15 ml	APS 10% [m/v]
	0.010 ml	TEMED

Stacking gel 5%	2.1 ml	Aqua bidest
	0.5 ml	30% Acrylamide
	0.38 ml	1.5 M Tris/HCl pH 6.8
	0.03 ml	10%SDS [m/v]
	0.03 ml	10%APS [m/v]
	0.005 ml	TEMED

10% SDS	10 g	SDS	(Roth, Karlsruhe)
	ad 100 ml Aqua bidest		

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1.5 M Tris/HCl pH 8.8	181.5 g	Tris	(Affymetrix, Santa Clara, USA)
	ad 900 ml Aqua bidest		
	pH 8.8 adjusted with HCl		
	ad 1 l Aqua bidest		

1.5 M Tris/HCl pH 6.8 1	21.14 g	Tris	(Affymetrix, Santa Clara, USA)
	ad 800 ml Aqua bidest		
	pH 6.8 adjusted with HCl		
	ad 1 l Aqua bidest		

Running buffer 10x	30.3 g	Tris	(Affymetrix, Santa Clara, USA)
	144 g	Glycin	
	10 g	SDS	(Roth, Karlsruhe)
	ad 1 l Aqua bidest		

Running buffer 1x	100 ml	Running buffer 10x	
	ad 1 l Aqua bidest		

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Blotting buffer 10x	30.3 g	Tris	(Affymetrix, Santa Clara, USA)
	144 g	Glycin	
	ad 1 l Aqua bidest		

Blotting buffer 1x	100 ml	Blotting buffer 10 x	
	200 ml	Methanol	
	ad 1 l Aqua bidest		

10x TBS	200 mM (24.22 g/l) Tris	(Affymetrix, Santa Clara, USA)
	1 M (58.44 g/l) NaCl	
	ad 500 ml Aqua bidest)	
	adjust pH to 7.6 with HCl	
	ad 1 l Aqua bidest	

TBS-T 0.2%	200 ml	10 x TBS
	4 ml	Tween 20
	1800 ml Aqua bidest	

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TBS-T 0.05%	200 ml	10 x TBS
	1 ml	Tween 20
	1800 ml	Aqua bidest

Blocking solution	5 g	Dried milk powder	(Roth, Karlsruhe)
	50 µl	Tween 20
	ad 100 ml TBS

Buffer A	0.12 g	10mM HEPES	(Roth, Karlsruhe)
	5.48 g	0.32M sucrose pH 7.4
	ad 50 ml Aqua bidest

Primary antibodies:

Anti-β-Actin mouse	(Sigma-Aldrich, Steinheim)	1:100000
Anti-MTF-1 rabbit	(Acris, Herford)	1:250
Anti-ProSAP1 rabbit	(described in Schmeisser et al., 2012)	1:1000
Anti-ProSAP2 rabbit	(described in Schmeisser et al., 2012)	1:1000
Shank 1 mouse	(Sigma-Aldrich, Steinheim)	1:500
Anti-MT3 goat	(Santa Cruz Biotechnology, Inc., Dallas, Texas, USA)	1:600
Anti-GAPDH mouse	(Novus Biologicals, Frankfurt/Main)	1:1000

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Anti-COMMD1 goat	(Santa Cruz Biotechnology, Inc., Dallas, Texas, USA)	1:200
Anti-COMMD1 rabbit	(Sigma-Aldrich, Steinheim)	1:250
Anti-GFP mouse	(Merck Millipore, Darmstadt)	1:3000
Anti-myc mouse	(Sigma-Aldrich, Steinheim)	1:200
Anti-GluN1 rabbit	(Synaptic Systems, Göttingen)	1:1000
AntiGluA2 rabbit	(Synaptic Systems, Göttingen)	1:1000

HRP-conjugated secondary antibodies:

Anti-mouse Immunoglobuline/HRP	(Dako, Glostrup, DK)	1:10000
Anti-rabbit Immunoglobuline/HRP	(Dako, Glostrup, DK)	1:10000
Anti-goat Immunoglobuline/HRP	(Dako, Glostrup, DK)	1:10000

2.2 Methods

2.2.1 Methods of microbiology

2.2.1.1 Transformation of chemically competent *E. coli*

Chemically competent *E. coli* OneShot TOP 10 (Invitrogen, Karlsruhe) were used for preparation of plasmids. An aliquot of 200 µl frozen E. coli was gently thawed on ice for about 10 min. The E. coli were mixed with 1 µl plasmid DNA and then incubated on ice for 30 min followed by heat-shock at 42°C for 45 s and immediate cooling on ice for 1 min. 300 µl S.O.C. medium (Invitrogen, Karlsruhe) were added and the cells were incubated at 37°C while shaking with 300 rpm for 1 h. Then the cells were plated on a LB-agar plate with the adequate antibiotic selection which was incubated at 37°C overnight.

2.2.1.2 Cultivation of E. coli

After bacteria have been cultivated on LB-agar plates, a colony was picked and cultivated in sterile liquid LB-medium in a shaker with 200 rpm at 37°C overnight. Specific antibiotics (ampicillin or kanamycin) with a concentration of 100 µg/ml were added according to the antibiotics specificity of the plasmid with which the E. coli had been transformed. For long term storage, bacterial culture was mixed 1:1 with 50% glycerol to create glycerol stock solutions and stored at -80°C.

For plasmid isolation, 100 µl of the glycerol stock or one single bacterial colony picked from the LB-agar plate with a sterile pipette tip were inoculated in 4 ml LB-medium with the adequate antibiotic and incubated at 37°C in a shaker with 200 rpm overnight (Mini-Prep). For large scale isolation (Maxi-Prep), 200 ml LB-medium with the adequate antibiotics were mixed with the 4 ml pre-culture and incubated at 37°C in a shaker with 200 rpm overnight.

2.2.2 Methods of molecular biology

2.2.2.1 Isolation and purification of plasmid DNA

Small scale isolation of plasmid DNA

Plasmid isolation was performed using the JETQUICK Plasmid Mini Prep Kit (Genomed, Löhne) according to the manufacturer's instructions. The plasmid DNA was eluted in 50 µl JETQUICK Plasmid TE buffer (Genomed, Löhne).

Large scale isolation of plasmid DNA

Plasmid isolation was performed using the JETQUICK Plasmid Maxi Kit 20 (Genomed, Löhne) according to the manufacturer's instructions. Dried DNA precipitate was resuspended in 100-200 µl JETQUICK Plasmid TE buffer (Genomed, Löhne) according to precipitate size. DNA was stored at -20°C.

2.2.2.2 Measurement of DNA concentration

The NanoDrop was used to determine DNA concentrations. Therefore the DNA sample was exposed to ultraviolet light with a wavelength of 260 nm and the absorbance of light was measured.

2.2.2.3 Cloning of plasmids

Used vector systems

For GFP-tagged COMMD1 expression constructs the pEGFP (C1-3) vector system (Clontech, Palo Alto, CA, USA) and for COMMD1 knockdown the pSuper neo GFP (Oligoengine, Seattle, WA, USA) vector system was used.
The used myc-tagged COMMD1 vector contructs (pCMV-MYC vector; Clontech, Palo Alto, CA, USA) and the used scrambled shRNA (pSuper neo GFP vector with the same amount of guanine and cytosine as the COMMD1-shRNA; Oligoengine, Seattle, WA, USA) were generously provided by Jun. Prof. Andreas M. Grabrucker and Stefanie Grabrucker.

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Polymerase chain reaction

PCR was used for amplification of the COMMD1 gene. The used template was the myc-tagged COMMD1 vector construct by Jun. Prof. Andreas M. Grabrucker.

The used primers were:

COMMD1 Forward:

5' – TTTAAGCTTATGGCGGGCGAGCTGGAGAG – 3'

COMMD1 Reverse:

5' – TTTAAGCTTTTAGGCTGCCTGCATCAGCC – 3'

The GoTaq Master Mix containing a Taq polymerase, buffers and dNTPs from Promega (Mannheim) were used for PCR experiments.

The PCR mix contained:

DNA template (1000 ng/µl)	1 µl
primer (sense/antisense 20 pmol/µl)	4 µl each
GoTaq Green Master Mix	25 µl
aqua bidest	ad to 50 µl

Activation of the polymerase was achieved by heating the PCR mix at 95°C for 2 min. The PCR was conducted in the following steps (Number 2 to 4 were executed in 35 cycles):

1. Denaturation of double stranded DNA for the first time to separate all strands at 95°C for 2 minutes.
2. Denaturation of double stranded DNA in order to separate the two strands at 95°C for 30 seconds.
3. Annealing of each primer at its appropriate single strand at 54°C for 30 seconds.
4. Amplification of the defined part of DNA with a polymerase at 72°C for 90 seconds.
5. Amplification of all PCR products that were not finished until now at 72°C for 5 minutes.

The mix was cooled down to 4°C afterwards.

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Linearization and annealing of oligonucleotides

Complementary oligonucleotides had to be annealed to form a double stranded DNA for insertion into the used plasmid vector constructs.

The shRNA oligonucleotides were purchased from MWG Eurofins. For COMMD1 knockdown the used target sequence was:

5' – GAAGCTGTCAGAGGTGGAA – 3'.

Sense oligonucleotides:

5' – GATCCCC**GAAGCTGTCAGAGGTGGAA**TTCAAGAGA**TTCCACCTCTGACAGCTTC**TTTTT GGAAA – 3'

Antisense oligonucleotides:

5' – AGCTTTTCCAAAAA**GAAGCTGTCAGAGGTGGAA**TCTCTTGAA**TTCCACCTCTGACAGCT TC**GGG – 3'

For separation of hairpin bonds within one oligonucleotide and annealing of complementary oligonucleotides 2 µl sense oligonucleotides and 2 µl antisense oligonucleotides were mixed with 21 µl aqua bidest in a 0.5 ml sterile tube.

Incubation protocol:	90°C	4 min
	70°C	10 min
	37°C	10 min
	room temperature	10 min
	4°C	20 min

The mixture was stored at -20°C for further use.

Restriction enzyme digestion of DNA

The ApE-plasmid Editor v2.0.30 was used to find the appropriate cutting sites of the used plasmid DNA to choose the right restriction enzymes and buffers. For cloning of myc-COMMD1, the restriction enzyme *HindIII-HF* (5' - AAGCTT – 3'; 20000 U/ml) was used. For cloning of COMMD1-shRNA, the restriction enzymes *HindIII-HF* and *BglII* (5' - AGATCT - 3'; 10000 U/ml) were used.

For digestion of DNA using restriction enzymes the following components were mixed and

incubated at 37°C for 90 min:

plasmid DNA	10 µg
restriction endonuclease buffer	10 µl
restriction enzymes	5 µl each
aqua bidest	ad to 100 µl

Digestion was controlled by agarose gel electrophoresis. All used restriction enzymes and appropriate buffers were purchased from NewEngland BioLabs, Frankfurt/Main.

Agarose gel electrophoresis

For preparation of the 1% agarose gel, 0.5 g agarose in 50 ml 1xTAE buffer was boiled and cooled down. Nucleic acids were stained using RedSafe Nucleic Acid Staining Solution (iNtRON, Gyeonggi-do, Korea) at a concentration of 1:10. The gel was poured into a casting tray and a comb was added to create sample wells. DNA samples were mixed with 6x loading buffer (ThermoFisher Scientific, Waltham, MA, USA) 1:6. After solidification, the comb was removed from the gel and it was put into the electrophoresis chamber filled with 1x TAE buffer. 10 µl of the GeneRuler 1 kb DNA ladder (Invitrogen, Karlsruhe) as size standard and the samples were pipetted into the created wells. After the gel was run at 90 V for 30 min the DNA bands were photographed under UV light using a gel documentation station (BioStep).

Elution of DNA fragments from agarose gel

For cloning, the adequate band of the DNA had to be cut out of the gel using a scalpel and was placed into a sterile microcentrifuge tube. The DNA was extracted using the JETQUICK Gel Extraction Spin Kit (Genomed, Löhne) according to the manufacturer's instructions. The DNA was dissolved in 30 µl Plasmid TE buffer from the kit and then stored at -20°C or used for ligation.

Ligation

The DNA insert was ligated into the linearized vector (using restriction enzymes) using T4 DNA ligase and the appropriate ligation buffer (NewEngland BioLabs, Frankfurt/Main).

Ligation reaction mixture	T4 DNA ligase	1 µl/ 1 U
	10x T4 ligation buffer	1 µl
	vector DNA	1 µl
	insert DNA	7 µl

The reaction mixture was incubated at RT for 2 h. After ligation the DNA was transformed into *E.coli* (see 2.2.1.1) and cultivated on LB-agar plates.

Sequencing of DNA constructs

After culture forming on the LB-agar plates single bacterial colonies were picked from them, inoculated in LB-medium (see 2.2.1.2) and the DNA was isolated as described in 2.2.2.1. Then the sequence of the constructs was checked. Therefore 1-2 µg DNA in 30 µl TE buffer (Genomed, Löhne) was sent to the company MWG Eurofins, Ebersberg, for sequencing. The construct with the right sequence was amplified using large scale isolation of plasmid DNA for use in further experiments.

2.2.3 Cell culture

2.2.3.1 NIH/HEK cell culture

NIH cells (mouse embryonic fibroblasts, DSMZ, Braunschweig) and HEK 293 (human embryonic kidney cells, DSMZ, Braunschweig) cells were stored in liquid nitrogen at -196°C. They were cultivated in 25 cm² cell culture flasks in DMEM++ at 37°C in 5% CO_2 and passaged twice per week (1:10) in order to use them for several weeks. Cell density and condition were examined by light microscopy and DMEM++ was warmed to 37°C before the old culture medium was discharged. Then trypsin (TrypLE Express; GIBCO,

Karlsruhe) was added for about 2 min at 37°C to detach the cells. To fully remove them it was necessary to tap the flask. Cells were resuspended in fresh warm DMEM++ in a 1:10 dilution and stored in the incubator. All this was done under a laminar flow hood. For immunocyctochemistry, NIH cells were plated on 24-well-plates.

2.2.3.2 Transfection of NIH/HEK cells

NIH cells were transferred into 94mm cell culture dishes (for protein biochemistry) or 24-well-plates (for immunocytochemistry) for transfection (1:10). HEK cells were transferred to chamberslides for transfection. All cells were cultured until they were approximately 80% confluent. Cells were transfected using PolyFect Transfection Reagent (Qiagen, Hilden) according to manufacturer's instructions. After 24 h cells were examined for GFP-positive signals with the microscope and then lysed in order to obtain protein lysate for Western Blotting (see 2.2.5).

2.2.3.3 Preparation of hippocampal cultures

Cell culture of primary hippocampal neurons from rat (E18) was prepared as described in Goslin and Banker (1991) with some modifications (Dresbach et al., 2003). Briefly, a pregnant rat (E18) was killed using carbon dioxide, the rat embryos were taken out and then decapitated. A petri dish was filled with ice-cold HBSS (PAA, Cölbe) and their brains were collected there. Hippocampi were prepared and then collected in a falcon tube filled with ice-cold HBSS. The next steps were performed under the laminar flow hood. After three washing steps with 5 ml HBSS without Ca^{2+} and Mg^{2+} each (PAA, Cölbe) hippocampi were transferred into a volume of 1.8 ml HBSS. 200 µl of 2.5% trypsin (Invitrogen, Karlsruhe) were added and hippocampi were incubated for 20min at 37°C followed by five careful washing steps with 5ml HBSS each to remove trypsin. A microcentrifuge tube was filled with 1.6 ml of the suspension and 400 µl DNAse I (Invitrogen, Karlsruhe) were added to achieve a final concentration of 0.01%. Hippocampi were slowly pipetted up and down ten times to disperse the cells and then transferred in drops into a 50 ml falcon tube using a nylon sieve (aperture size: 100 µm). 18 ml DMEM+++ were added. A

Neubauer counting chamber was used to determine cell density. In order to get a density of 2-3x 10^4 cells per well the suspension was diluted with DMEM+++. Neurons were seeded on sterile glass coverslips that had previously been placed into 24-well-plates, coated with poly L-lysin and incubated for 1 h at 37°C followed by three washing steps with deionized water. Cells were incubated for 6-12 h. Then DMEM+++ was removed, the cells were covered with NB+++ medium and incubated at 37°C, 5% CO_2.

2.2.4 Transfection of primary hippocampal neurons

Primary hippocampal neurons were transfected at DIV 10 by using Optifect Transfection Reagent (Invitrogen, Karlsruhe). For each well 2 µg DNA was diluted in 50 µl neurobasal medium (NB medium; GIBCO, Karlsruhe). In a separate tube 5 µl Optifect Transfection Reagent was diluted in 50 µl neurobasal medium. After an incubation time of 5 min at RT the diluted Optifect Reagent was gently mixed with the DNA and incubated for another 30 min at RT. The growth medium was collected from the wells before the neurons were transfected and replaced with freshly made NB++ medium. Then, 100 µl of the transfection solution was added in drops to the neurons in each well. After incubation of 6-8 h in the incubator (37°C, 5% CO2), the NB++ medium was removed and the earlier collected growth medium was given back to the neurons. Then, the neurons were incubated until DIV 14 when immunocytochemistry was performed.

2.2.4.1 Treatment of hippocampal neurons and immunocytochemistry

Some neurons were treated before they were stained for immunofluorescence. Supplementation of the growth medium with 50 mM KCl (HiK$^+$) for 1 min was used for stimulation. After the stimulation the cells were incubated again in their normal growth medium at 37°C, 5% CO_2 for 30 min. Then the cells were stained for immunofluorescence described below.

At DIV 14 ZnCl$_2$ (10 µM) and/or CuCl$_2$ (10 µM) was added as supplementation to the growth medium of some neurons followed by incubation at 37°C for one hour.

Supplemented culture medium was collected for measurement of extracellular zinc and copper concentrations (see 2.2.8)

For fluorescent zinc staining, the hippocampal neurons were incubated with a solution of 25 µM Zinquin ethyl ester or 5 µM Zinpyr-1 in 1x PBS for 40 min at 37°C, fixed with 4% PFA in 1x PBS with 4% sucrose at RT for 15 min, washed for 5 min with Aqua bidest and then the coverslips were mounted using Vecta Mount AQ (Vector Laboratories, Burlingame, CA, USA). Zinquin ethyl ester is a dye that shows fluorescence as soon as it binds to zinc (Colvin et al., 2006; Coyle et al., 1994). Zinpyr-1 is a fluorescent that can be used for intracellular zinc staining in hippocampal neurons even *in vivo* (Woodroofe et al., 2004).

For immunofluorescence, the neurons were fixed as described above and then permeabilized with PBS Triton 0.2% for 10 min. After a washing step with 1x PBS for 5 min, coverslips were incubated with blocking solution to prevent non-specific binding of the antibodies at RT for 1 h. Afterwards cells were incubated with the primary antibody diluted in blocking solution at RT for 2 h or at 4°C overnight. All following steps were carried out within little light to avoid fading of the fluorescence. After 3 washing steps with 1x PBS 5 min each, incubation with the secondary antibody conjugated to either Alexa488, Alexa 568 or Alexa 647 fluorescent dyes (1:500, in blocking solution) at RT for 1 h followed. Cell nuclei were counterstained with PBS + DAPI at RT for 5 min, washed with 1x PBS for 5 min and then coverslips were mounted as described before after a last washing step with Aqua bidest for 5 min.

NIH cells were treated the same way for immunocytochemistry.

2.2.5 Generation of mice with acute and prenatal copper overload

For the experiment 24 female mice (*Mus musculus*, strain C3H/HenRj) were used. They were purchased from Janvier at ten weeks of age and housed in standard plastic cages. The mice were held under standard laboratory conditions with an average temperature of 22°C. Food and water was available *ad libitum.* The lights were automatically turned on and off in a 12 h rhythm whereas the lights were turned on at 7 am. After acclimation of one week, the mice were divided into two groups with 12 female mice in each group. The

mice in the control group were fed with standard laboratory food (6 mg/kg copper) and tapped water. The other 12 mice were fed a copper supplemented diet (30 mg/kg copper, Research Diets Inc.) and tapped water. The mice were weighed every day. Food consumption was determined by weighing newly given food and food leftovers. After two weeks, three mice of each group were mated. Of each group four mice brains were used to generate protein lysate and for mRNA experiments. Five mice brains of each group were used for the generation of brain slices for immunohistochemistry and for the preparation of the four brain regions cortex, cerebellum, striatum and hippocampus.

12 pups were killed for the analysis of prenatal copper overload at day 3. The brains of three pups of each group (control and copper supplemented mothers) were used for protein analysis, mRNA experiments and the other three of each group were used for immunohistochemistry.

All animal experiments were performed in accordance with the guidelines for the welfare of experimental animals by the Federal Government of Germany and the local ethics committee (Ulm University) ID Number: 0.103 and 1132.

2.2.6   Methods of immunohistochemistry

2.2.6.1   Preparation of mice

The mice were killed with carbon dioxide and decapitated. A petri dish was filled with ice-cold HBSS (PAA, Cölbe) to collect the brains there. Then the cranium was opened by making two lateral cuts through the foramen magnum and one sagittal cut. The optical nerves were cut as well. Brains were cut in half through the corpus callosum. One half of each brain was frozen in liquid nitrogen and stored at -80°C until further use. Of the other half of each brain slices of 16 μm were cut sagital with a cryostat microtome at -20°C and mounted on glass slides. They were then stored at -80°C until further use.

Of some adult mice's brains the brain regions cortex, cerebellum, striatum and hippocampus were located with the help of a microscope and removed. They were stored at -80°C until further use.

2.2.6.2   Immunohistochemical staining of cryosections

Cryosections were thawed at RT for 20 min followed by incubation with Zinpyr-1 with a concentration of 10 µM at RT for 1h. To stain cell nuclei sections were washed with PBS DAPI at RT for 3 min. After final washing with 1x PBS the sections were mounted with Vecta Mount (Vector Laboratories, Burlingame, CA, USA).

For chelation of zinc, some sections were treated with 15 µM TPEN (solved in dimethyl sulfoxide) for 10 min at RT before Zinpyr-1 staining.

For Shank1, ProSAP1/Shank2 and ProSAP2/Shank3 stainings, cryosections were first thawed at RT for 20 min followed by fixation with PFA for 20-20 min. Sections were then permeabilized with PBS Triton 0.2% at RT for 2 h followed by 3 washing steps with PBS Triton 0.05% for 10 min each. Subsequently, sections were incubated with blocking solution to prevent non-specific binding of the antibodies at RT for 2 h. Afterwards sections were incubated with the primary antibody diluted in blocking solution at 37°C for 2 h. This incubation and all the following steps were carried out within little light to avoid fading of the fluorescence. After 3 washing steps with PBS Triton 0.05% for 10 min each, incubation with the secondary antibody conjugated to either Alexa488, Alexa 568 or Alexa 647 fluorescent dyes (1:500, in blocking solution) at 37°C for 1.5 h followed. Cell nuclei were counterstained twice with 1x PBS + DAPI at RT for 5 min, washed with Aqua bidest for 5 min and then mounted with Vecta Mount AQ (Vector Laboratories, Burlingame, CA, USA).

2.2.6.3   TIMM staining

TIMM staining was performed as described by Jaarsma and Korf (Jaarsma and Korf, 1990). Briefly, cryosections were thawed at RT for 20 min, washed with 0.1 N HCl and incubated at RT for 3 h. Then the sections were placed into a 4 l vacuum dessicator jar. 200ml 0.1% Na_2S solution was added, pH adjusted to 7.3 with 2 N HCl in order to produce H_2S gas. The sections were incubated in the H2S gas for 16 h and fixed with 96% EtOH for 15 min. After three washing steps with 70% and 50% EtOH and distilled water for 2 min each, the sections were stained in the Timm developer mixed with 0.5 ml silver nitrate (1.7 g were dissolved in 10 ml Aqua bidest) at 26°C in the dark for 2 h. Subsequently, sections were

rinsed in aqua bidest for 10 min and incubated with 5% sodium thiosulfate for 10 min. After washing with aqua bidest, the sections were dehydrated using a graded series of EtOH and xylene. After 1-2 h sections were embedded using Entellan (Merck Millipore) and covered with coverslips.

2.2.7 Protein biochemistry

2.2.7.1 Isolation of proteins from NIH/HEK cells

NIH or HEK cells were grown and transfected (see 2.2.3.2).

After the culture medium had been removed, the cells were washed with 1x PBS. 200 µl 4°C lysis buffer with EDTA-free protease inhibitor (Roche, Basel, Switzerland) was added, the cells were detached by using a plastic cell scraper and transferred into a 2 ml tube followed by incubation under constant movement at 4°C for 90 min. The cell lysate was centrifuged 1 min at 4°C at 7000 rpm to separate proteins from cell debris. The supernatant was transferred into a new tube and stored at -20°C until further use.

2.2.7.2 Protein isolation of brains and brain regions

All brains from adult mice were weighed and then collected in cold buffer A (10 ml/g brain) containing protease inhibitor. They were homogenized (Potter S, Sartorius, Göttingen) on ice and then centrifuged at 1000 rpm at 4°C for 5 min. The resulting S1 fraction (supernatant) was centrifuged at 10000 rpm at 4°C for 10 min. The pellet P2 was resuspended in 2 ml buffer A. The protein lysates were stored at -80°C until further use. The same procedure was performed with the collected brain regions.

Brains from pups were homogenized. The homogenate was centrifuged in QIAshredder columns with 13000 rpm at room temperature for 2 min. The rest of the protein isolation was performed as described for the adult brains. Bradford Assay was performed to make sure 20 µg protein of all brain regions was used in Western Blot.

2.2.7.3   SDS-PAGE

The 10% or 12% resolving gel was freshly prepared, poured between two glass plates and overlaid with 1 ml isopropanol to create a smooth surface of the gel. As soon as the gel was solid the isopropanol was removed and the stacking gel was prepared and poured on top. A comb was inserted into the liquid gel to create wells for the protein samples and removed after polymerization. Protein lysates were mixed 1:1 with SDS loading buffer and heated at 95°C for 5 min to denature the proteins. 20 µl (10 wells) or 15 µl (15 wells) of the sample and 9 µl of the PageRuler Prestained Protein Ladder (Fermentas, St. Leon-Rot) or the Spectra Multicolor High Range Protein Ladder according to protein size were loaded on the gel after it had been transferred to a vertical electrophoresis chamber. The electrophoresis was performed in 1x running buffer at 100 V for about 15 min (until the proteins had reached the separating gel) followed by about 1h at 200 V until the proteins had been separated.

2.2.7.4   Western Blot

After the proteins had been separated in gel electrophoresis, they were transferred from the gel onto a nitrocellulose membrane (Amersham, Freiburg). Thus, membrane and gel were sandwiched between two pieces of Whatman paper and two sponge pads equilibrated with 1x blotting buffer and then placed between plate electrodes for the protein transfer at 90 V for 90 min. The blotting chamber also contained 1x blotting buffer and a cooling jacket to prevent overheating. After blotting the membrane was incubated in blocking solution on a shaker for 2 h at RT to prevent unspecific binding of the antibody. Then the membrane was rinsed with TBS-T 0.05% for 5 min each and incubated with the appropriate primary antibody diluted in 10 ml TBS-T 0.05% at RT for 1 h while shaking. Subsequently, the membrane was washed three times with TBS-T 0.05% for 5 min and incubated with the appropriate secondary HRP-conjugated antibody for 1 h. The membrane was washed three times with TBS-T 0.2% for 15 min each followed by visualization of the protein bands using the ECL Western Blotting Substrate (Pierce, Upland, USA) or the SuperSignal detection system (Pierce, Upland, USA). The membrane was incubated in ECL solution according to manufacturer's instructions and it was either

exposed to an X-ray film (Hyperfilm ECL, Amersham Biosciences, Freiburg) or the immunoreactivity was visualized on an imaging system.

2.2.7.5 Coimmunoprecipitation

Coimmunoprecipitation was performed with the generated protein lysate from HEK cells transfected with either GFP-COMMD1 or MYC-COMMD1 according to manufacturer's instructions (µMACS Magnetic Beads Kit, Miltenyi Biotex, Bergisch Gladbach). µMACS GFP-beads or MYC-beads were used, respectively, to purify protein lysates which were then used in a Western Blot to show that the MYC- or GPF-tagged COMMD1 fusion proteins can be detected with any MYC-, GFP- or COMMD1-antibody. Controls were the pEGFP (C1-3) vector and pCMV-MYC vector (Clontech, Palo Alto, CA, USA).

2.2.8 Organ specific tissue lysate

Liver and kidneys of three mice of each group were collected in an empty falcon tube after they were killed (see 2.2.6) and the tubes were stored at -80°C until further use. Buffer A (10 ml/g organ) was added. The organs were homogenized on ice (Potter S, Sartorius, Göttingen) and using QIAshredder columns. They were centrifuged with 13000 rpm at room temperature for 2 min. 150 µl of the homogenate was transferred to a new tube. 0.5 mg/ml Proteinase K was added. The samples were incubated at 37°C for 1 h. After determination of the lysates' weights they were sent to the Department of Clinical Chemistry (ZE klinische Chemie) of the Ulm University Hospital for measurement of zinc and copper concentrations by atomic absorption spectrometry.

2.2.9 Measurement of zinc and copper concentrations

Urine and feces of three mice of each group were collected during the housing in plastic cages or during the preparation of the mice. Blood was collected of three mice of each group and coagulated for 20 to 60 min followed by centrifugation at 1000 rpm at RT for

15 min. Serum was pipetted carefully into a new tube. Concentrations of zinc and copper in brain tissue, serum, urine and feces were measured by inductively coupled plasma mass spectrometry (ICP-MS) at the "Spurenanalytisches Laboratorium Dr. Baumann" (Maxhütte-Haidhof). The concentration of zinc in blood, other organs and culture medium was measured by atomic absorption spectrometry (AAS) at the Department of Clinical Chemistry (ZE klinische Chemie) of the Ulm University Hospital.

2.2.10 Software used for analysis and statistics

2.2.10.1 Synapse, dendrite and nuclei measurement

For cell culture experiments, 10 cells per condition were imaged. For brain sections 3 animals of each group (control and copper-supplemented group) were used to obtain images of the 4 brain regions (cortex, cerebellum, hippocampus and striatum) by 3 optic fields each. The fluorescence images were obtained with an upright Axioscope microscope equipped with a Zeiss CCD camera (16 bits; 1280x1024 ppi) and AxioVision Rel. 4.6.3 software (Zeiss). The signal intensity of fluorescent puncta along dendrites was analysed and quantified using ImageJ v1.48r (National Institutes of Health, USA) and OpenView Noam Ziv (Israel Institute of Technology, Israel). Background fluorescence was determined by measuring four puncta on the dendritic compartment of a neuron where no synapse was located and the addition of 20% of the signal intensity. The remaining values – higher than the value of background fluorescence - were used to calculate the average and standard error.

The number of dendrites of ProSAP2/Shank3 stained hippocampal neurons and DAPI-positive nuclei per optic field were counted manually. A primary dendrite was defined as the dendrite that is directly connected to the cell body. A secondary dendrite is the dendrite that is branching from a primary dendrite and a tertiary dendrite is branching from a secondary dendrite.

Statistics were done with Microsoft Excel and Student's *t*-tests were used to test for significance (all values were normally distributed). Data are presented as mean ± SEM. Results were considered significant with a p value <0.05 (p <0.05: *; <0.01: **; <0.001: ***).

2.2.10.2 Western Blot analysis

Bands from Western Blots (WBs) were evaluated with ImageJ. Three animals of each group were used to perform WBs and the blots were scanned (600 dpi) to analyse the selected individual bands. Then the integrated density was measured using the freehand selection tool in ImageJ. All WBs were normalized to β-actin and the ratios were averaged and standard deviations were determined. Statistics were done with Microsoft Excel and Student's *t*-tests were used to test for significance (all values were normally distributed). Data are presented as mean ± SEM. Results were considered significant with a p value <0.05 (p <0.05*; <0.01**; <0.001***).

3 RESULTS

3.1 Alteration of copper concentration influences zinc homeostasis *in vitro*

The first question was whether changes of the concentration of zinc and/or copper can influence intracellular levels of copper and zinc ions *in vitro*. Therefore, primary hippocampal neurons were cultivated until DIV 14 and incubated with 10 µM ZnCl$_2$, 10 µM CuCl$_2$ or 10 µM ZnCl$_2$ and 10 µM ZnCl$_2$, and as control without reagents, at 37°C for 1 h. Immunocytochemistry was performed with Zinquin ethyl ester, a dye that shows fluorescence as soon as it binds to zinc. Supplementation of zinc significantly increases intracellular zinc concentrations. This increase is blocked when CuCl$_2$ is also added. The block can only be slightly overcome when zinc concentrations were further increased (20 µM zinc).
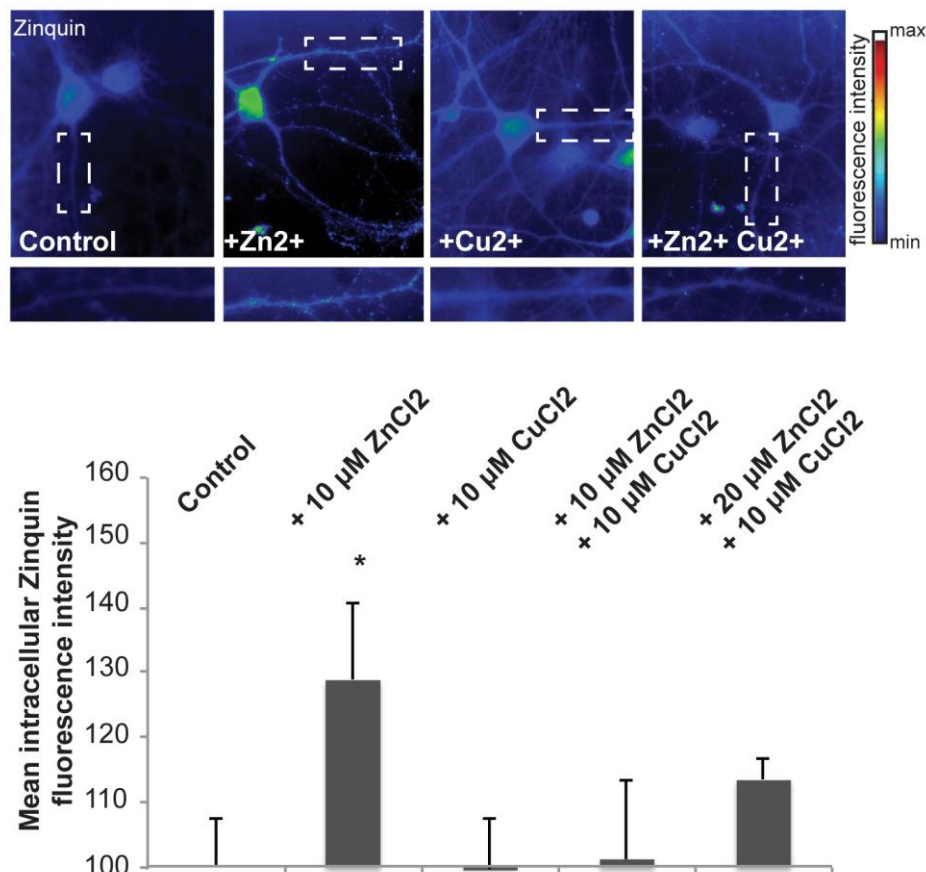
Figure 7: Alteration of zinc/copper concentration has an influence on intracellular zinc concentration in hippocampal neurons: Primary hippocampal neurons were treated at 14 DIV for 1 h with 10 µM ZnCl$_2$, 10 µM CuCl$_2$ or 10 µM ZnCl$_2$ and 10 µM CuCl$_2$. The quantification of the signal intensity values of Zinquin ethyl ester is used to detect intracellular zinc levels. Supplementation with zinc leads to a

significant increase in intracellular zinc levels; however, the application of copper together with zinc inhibits an increase of intracellular zinc. Application of 20 μM ZnCl$_2$ and 10 μM CuCl$_2$ could only slightly overcome the impaired increase by copper.

Similar effects could be observed in experiments using NIH cells.
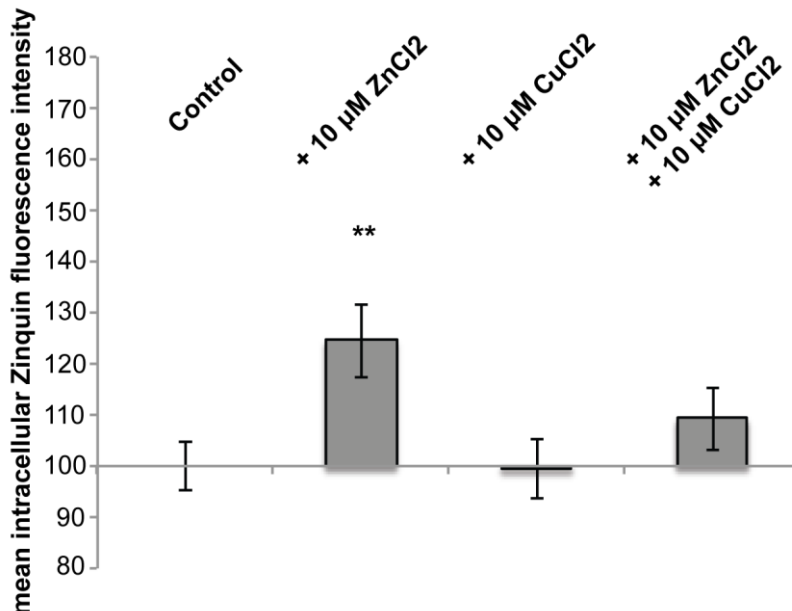
Figure 8: Alteration of zinc/copper concentration has an influence on intracellular zinc concentration in NIH cells: NIH cells were treated for 1 h with 10 μM ZnCl$_2$, 10 μM CuCl$_2$ or 10 μM ZnCl$_2$ and 10 μM CuCl$_2$. The intracellular zinc levels were detected by quantification of the signal intensity values of Zinquin ethyl ester. Similar to neurons, a significant increase in intracellular zinc levels can be observed under supplementation with zinc that is inhibited by the application of copper together with zinc.

Additionally, extracellular zinc and copper concentrations of hippocampal neurons DIV 14 supplemented with 10 μM ZnCl$_2$, 10 μM CuCl$_2$ or 10 μM ZnCl$_2$ and 10 μM ZnCl$_2$ were analyzed using atomic absorption spectrometry. After treatment with ZnCl$_2$ and CuCl$_2$ extracellular levels of zinc and copper, respectively, were significantly increased. Supplementation with ZnCl$_2$ plus CuCl$_2$ leads to even higher extracellular levels of zinc as the uptake of zinc is significantly impaired. However, application of ZnCl$_2$ together with CuCl$_2$ also impairs the uptake of copper. This impairment is underlined by the copper/zinc ratio that is significantly increased when CuCl$_2$ was applied solely, but not when CuCl$_2$ is

co-applied with ZnCl$_2$. As expected the copper/zinc ratio of the cells is significantly
decreased in cells after treatment with ZnCl$_2$.
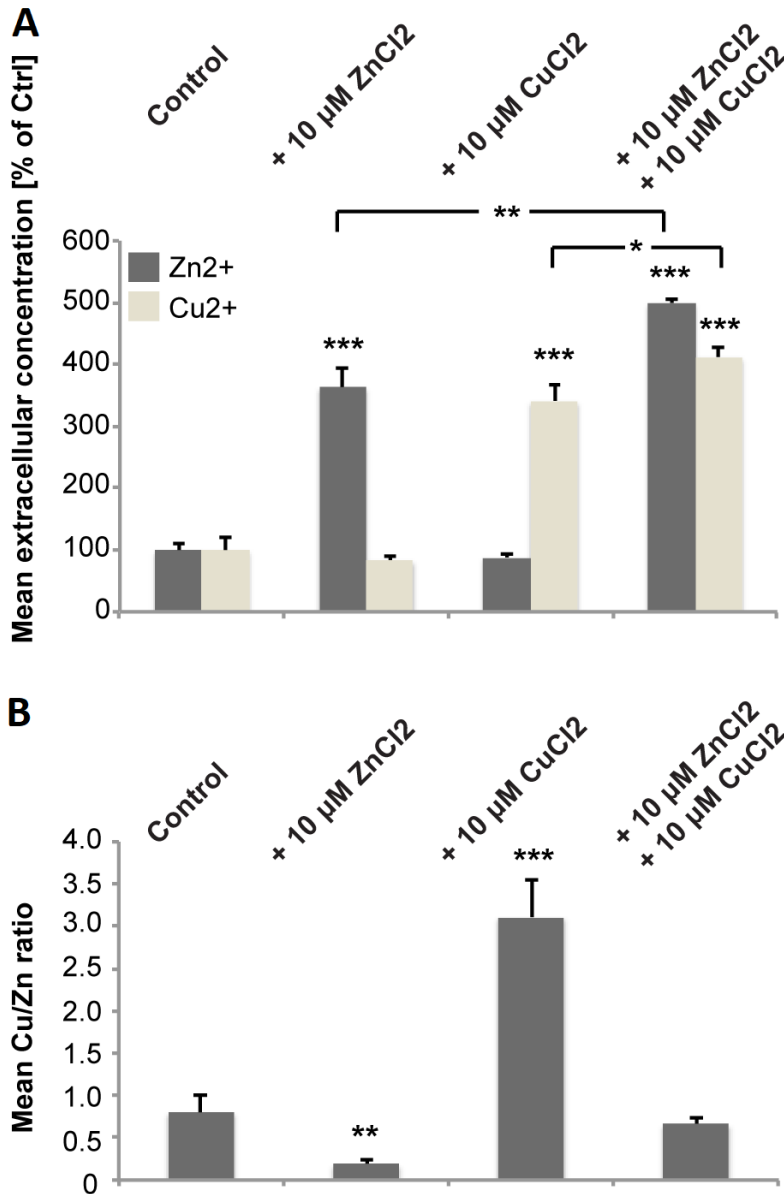
Figure 9: Extracellular zinc and copper concentrations of cell culture media from dishes (n = 6 per group) treated at DIV 14 for 1 h with 10 μM ZnCl$_2$, 10 μM CuCl$_2$ or 10 μM ZnCl$_2$ and 10 μM CuCl$_2$ were determined using atomic absorption spectrometry.

A) A significant increase in zinc and copper can be seen after treatment with ZnCl$_2$ and CuCl$_2$, respectively. Application of ZnCl$_2$ together with CuCl$_2$ also leads to significantly higher extracellular zinc and copper levels. However, this increase is again significantly higher compared to ZnCl$_2$ and CuCl$_2$ only treatment.

B) No significant change in the copper/zinc ratio between control cells and cells treated with ZnCl$_2$ together with CuCl$_2$ was seen underlining that both, zinc and copper uptake into cells is impaired by co-applications of both trace metals.

RESULTS

To confirm the results are not based on increased cell death in copper/zinc treated cells the number of cells per optic field was quantified by counting DAPI stained nuclei. No significant increase of cell death after the addition of zinc and/or copper was detected. Additionally, no alteration of dendritic branching was detected and no increase of swelling or pinching off of dendrites.
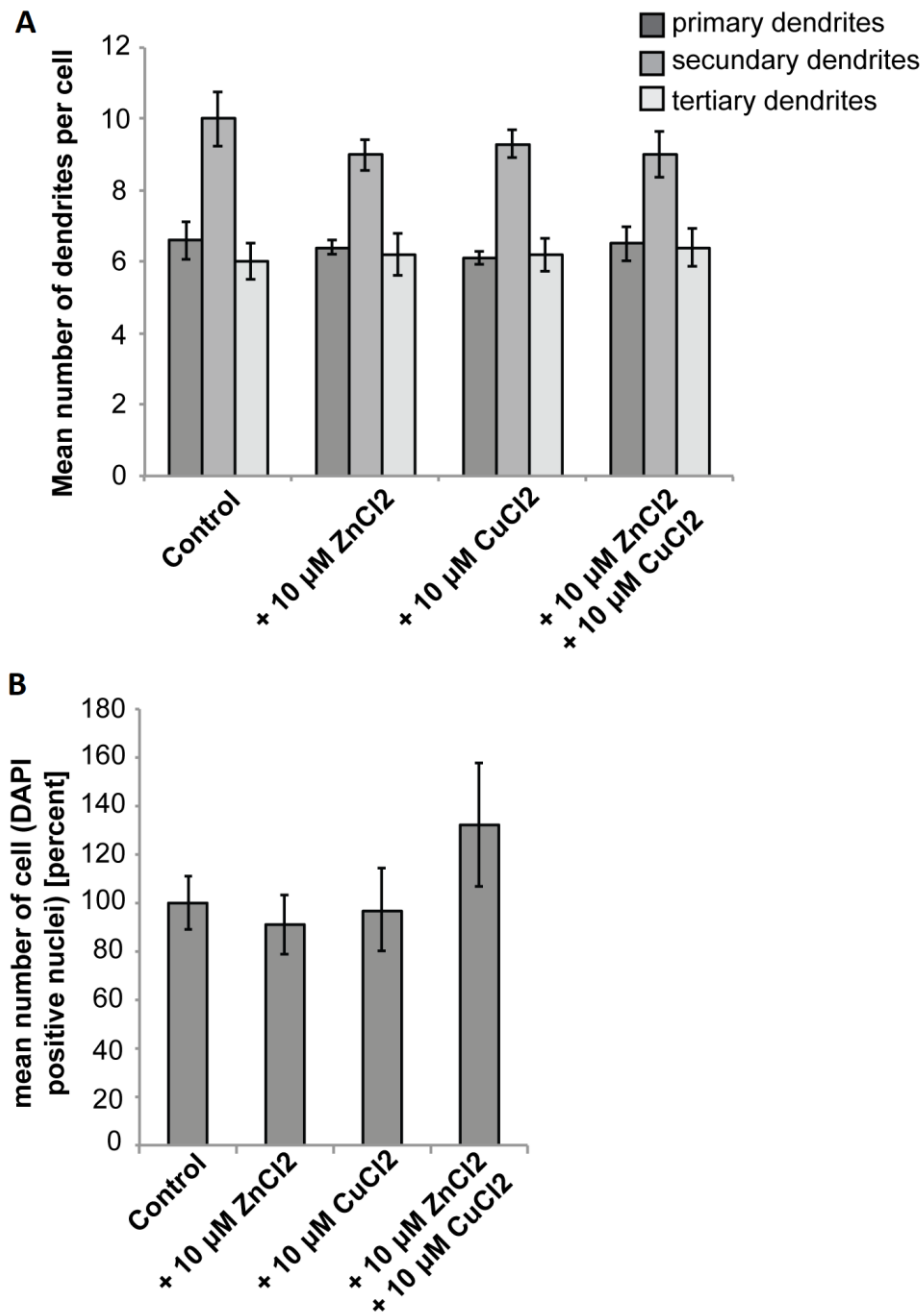
Figure 10: Supplementation of zinc and/or copper does not alter dendritic branching nor cell viability of primary hippocampal neurons: Treatment of hippocampal neurons at 14 DIV for 1 h with 10 μM ZnCl$_2$, 10 μM CuCl$_2$ or 10 μM

ZnCl$_2$ and 10 μM CuCl$_2$ does not significantly alter dendritic branching (A) nor causes significant cell death (B) given that the number of nuclei (DAPI positive) did not change after treatment.

3.2 PSD protein levels in vitro are influenced by altered copper concentration

ProSAP1/Shank3 and ProSAP2/Shank3 protein levels are upregulated in a zinc dependent manner (Grabrucker et al., 2014). To investigate whether this upregulation is altered in response to changed intracellular zinc concentration due to copper supplementation primary hippocampal neurons DIV 14 were treated with 10 μM ZnCl$_2$, 10 μM CuCl$_2$ or 10 μM ZnCl$_2$ and 10 μM ZnCl$_2$ at 37°C for 1 h. Subsequently, neurons were fixed and immunocytochemistry with antibodies against ProSAP1/Shank2 and ProSAP2/Shank3 was performed. Signal intensities of fluorescent puncta along dendrites from ten cells per condition were quantitatively analyzed using OpenView software. Synaptic levels of ProSAP1/Shank2 and ProSAP2/Shank1 were significantly increased after treatment of hippocampal neurons with ZnCl$_2$. This upregulation could not be seen when CuCl$_2$ was added and it was significantly inhibited after co-application of ZnCl$_2$ and CuCl$_2$.
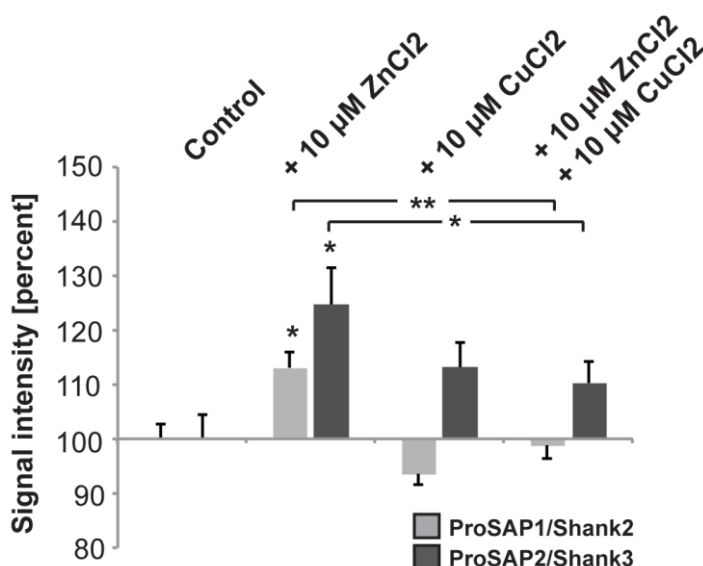
Figure 11: Supplementation of zinc leads to increased synaptic levels of ProSAP1/Shank2 and ProSAP2/Shank3 in primary hippocampal neurons while co-application with copper impairs this increase: Primary hippocampal neurons were treated at 14 DIV for 1 h with 10 μM ZnCl$_2$, 10 μM CuCl$_2$ or 10 μM ZnCl$_2$ and 10 μM CuCl$_2$ and stained for ProSAP1/Shank2 and ProSAP2/Shank3. Synaptic protein levels were analysed by quantification of fluorescence signal intensities of puncta along dendrites from n = 10 cells per group which reveals a significant increase in ProSAP1/Shank2

and ProSAP2/Shank3 due to zinc supplementation. This increase is blocked when copper is supplemented simultaneously.

Expression levels of Shank1, which does not bind zinc, Homer1b/c, mGluR5 and Gephyrin, which is a scaffold protein existing in inhibitory synapses, were analyzed under the same conditions as described above. Inhibitory synapses do not contain ProSAP/Shank proteins. The mentioned PSD protein levels did not show any general increase after supplementation with $ZnCl_2$ and/or $CuCl_2$. Immunofluorescence of synaptic mGluR5 receptors was increased after addition of $ZnCl_2$ as reported before (Grabrucker et al., 2014). However, when zinc was supplemented along with copper only a trend towards an elevation remains, which is no longer significant.
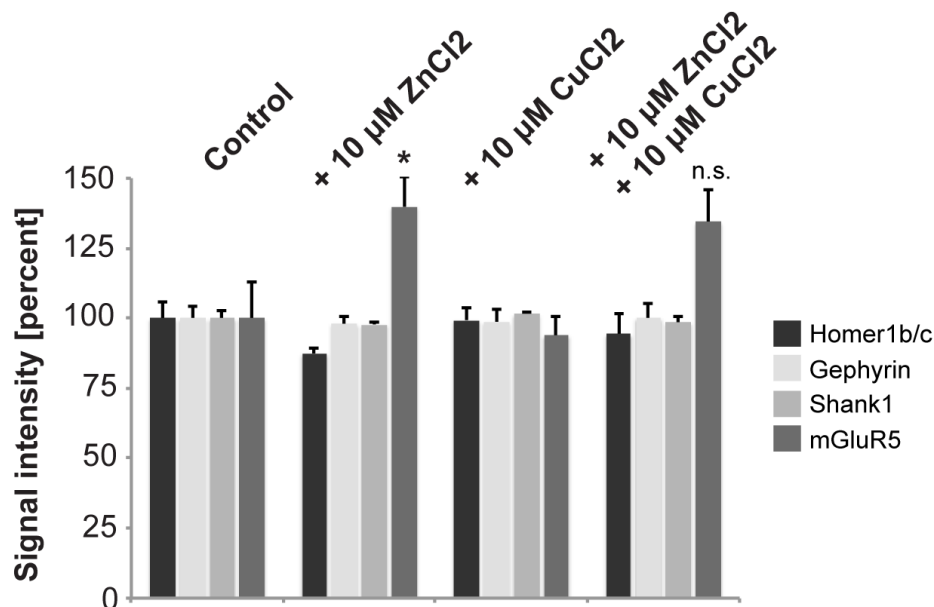
Figure 12: : Supplementation of zinc leads to increased synaptic levels of mGluR5 in primary hippocampal neurons while co-application with copper impairs this increase: Primary hippocampal neurons were treated at 14 DIV for 1 h with 10 μM $ZnCl_2$, 10 μM $CuCl_2$ or 10 μM $ZnCl_2$ and 10 μM $CuCl_2$ and stained for Homer1b/c, Gephyrin, Shank1 and mGluR5. The levels of Homer1b/c, Gephyrin, and Shank1 from treated cells show no significant differences compared to untreated neurons. The synaptic levels of mGluR5 are significantly increased after zinc supplementation, while supplementation of zinc along with copper only results in a trend towards an increase, however no longer significant ($p = 0.073$).

It was reported before that synaptic ProSAP1/Shank2 and ProSAP2/Shank3 levels are increased after neuronal activity (Grabrucker et al., 2014). Neuronal activity can be increased with HiK^+ (50 mM KCl). Primary hippocampal neurons were stimulated with HiK^+ for 1 min. Neurons were also incubated with 10 µM $CuCl_2$ at 37°C for 1 h. Subsequently, they were fixed and stained with antibodies against ProSAP1/Shank2 and ProSAP2/Shank3. HiK^+ indeed increases fluorescence intensities of synaptic ProSAP2/Shank3 and also, a bit less, of ProSAP1/Shank2 puncta under control conditions. However, after treatment with $CuCl_2$ the increase of synaptic ProSAP2/Shank3 and ProSAP1/Shank2 levels as response to neuronal activity was impaired. This is a hint that an increased copper burden might lead to a reduced bioavailability of zinc in general as ProSAP/Shank Proteins have SAM domains that are zinc sensitive, but show a low affinity for copper (Grabrucker et al., 2011a).
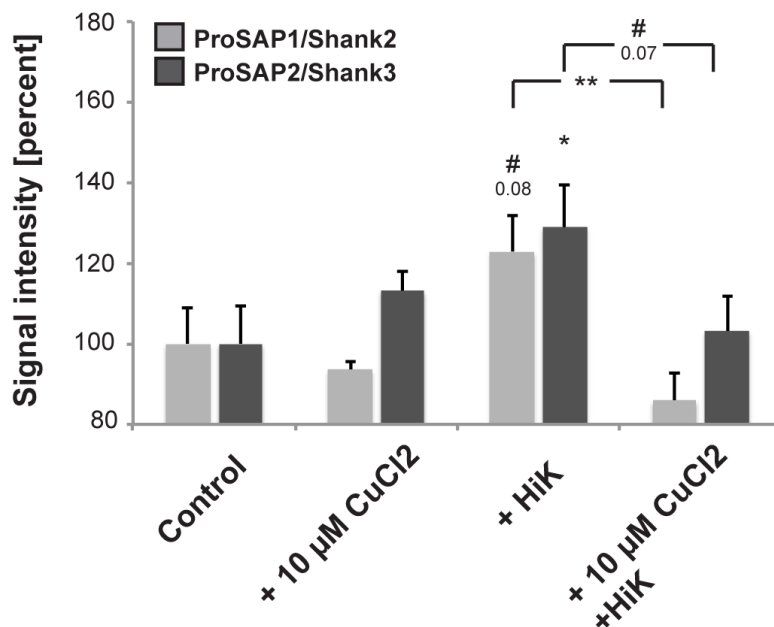
Figure 13: Treatment of primary hippocampal neurons with $CuCl_2$ impairs increase of synaptic ProSAP/Shank proteins due to HiK^+: Primary hippocampal neurons were stimulated with HiK^+ for 1 min, and/or incubated with 10 µM $CuCl_2$ for 1 h, and stained for ProSAP1/Shank2 and ProSAP2/Shank3. Synaptic protein levels were analyzed by quantification of fluorescence signal intensities of puncta along dendrites from n = 10 cells per group which reveals a significant increase ProSAP2/Shank3 and a trend towards an increase of ProSAP1/Shank2 (p=0.08). Treatment with $CuCl_2$ impairs this increase despite stimulation with HiK^+.

3.3 Alteration of COMMD1 concentration influences copper and zinc homeostasis as well as levels of ProSAP/Shank proteins *in vitro*

The next question was whether altered expression levels of COMMD1 might have an influence on intracellular levels of zinc. To this end, plasmids encoding myc-COMMD1, GFP-COMMD1, scrambled (scr)-shRNA and COMMD1-shRNA (also GFP expressing) were generated. NIH cells were transfected either with myc-COMMD1, COMMD1-shRNA or both to confirm the knockdown of COMMD1 with the generated shRNA. Western Blot analysis shows a significant knockdown of COMMD1 by COMMD1-shRNA compared to untransfected control cells. COMMD1 expression level was normalized to GAPDH. The COMMD1-shRNA demonstrates as well the ability to reduce COMMD1 overexpression induced by myc-COMMD1.
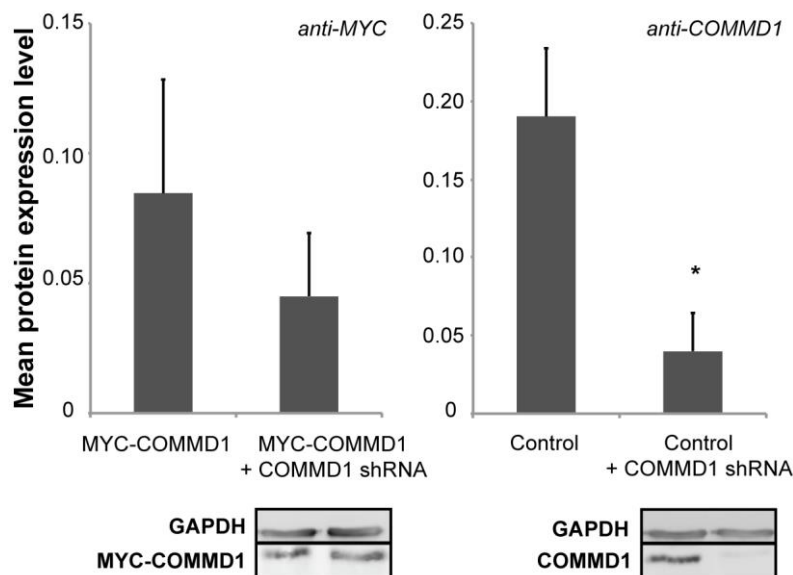
Figure 14: COMMD1-shRNA generated COMMD1 knockdown in NIH cells: Protein lysate from untransfected NIH 3T3 cells (control cells) as well as myc-COMMD1, myc-COMMD1 plus COMMD1 shRNA, or COMMD1 shRNA transfected cells was analyzed using Western Blot. The results show the average signal intensity of three lanes per group normalized against GAPDH. A significant knockdown effect of the shRNA plasmid can be seen.

Primary hippocampal neurons transfected with COMMD1-shRNA were additionally stained with a COMMD1 antibody for fluorescence analysis of the COMMD1 knockdown. The results show a reduced anti-COMMD1 labeling of shRNA transfected cells.
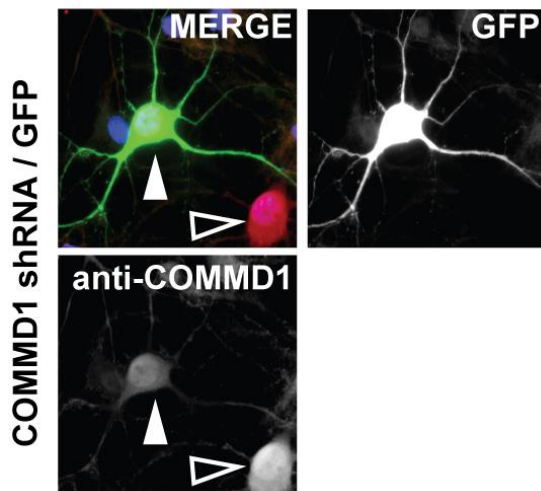
Figure 15: COMMD1-shRNA transfection leads to reduced COMMD1 levels analyzed by COMMD1 fluorescence levels:
Transfection of COMMD1 shRNA for three days results in reduced anti-COMMD1 labeling in shRNA expressing (GFP positive (full arrow)) cells compared to an untransfected cell (open arrow). Anti-COMMD1 signals were imaged with the same exposure time.

Additionally, endogenous COMMD1 in primary hippocampal neurons DIV14 was visualized together with ProSAP2/Shank3 to determine localization of COMMD1 inside neurons. The analysis shows that COMMD1 seems to be enriched at post-synapses.
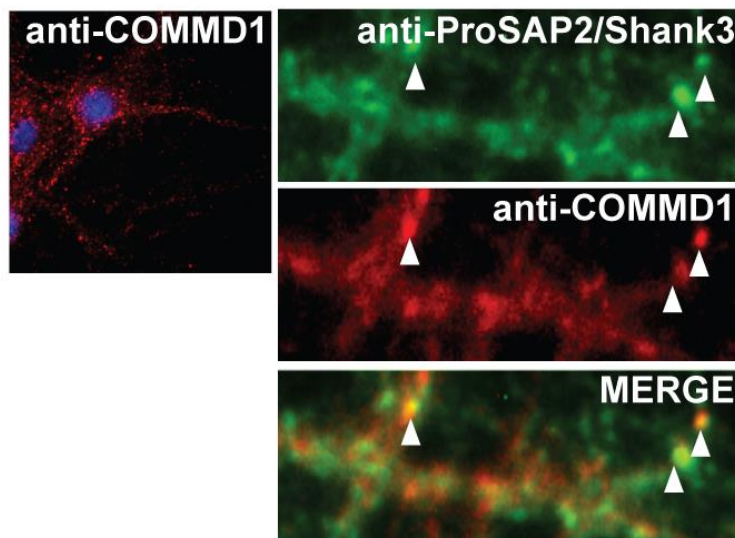
Figure 16: Endogenous COMMD1 shows enrichment at excitatory post-synapses labeled by ProSAP2/Shank3.

To verify the specificity of the used COMMD1 antibodies, HEK cells were transfected with GFP-COMMD1 or myc-COMMD1. The overexpressed protein was purified using magnetic beads. Western Blot analysis shows that the overexpressed COMMD1 protein can be

detected specifically with the used COMMD1 antibodies and also with antibodies against GFP or myc, respectively.
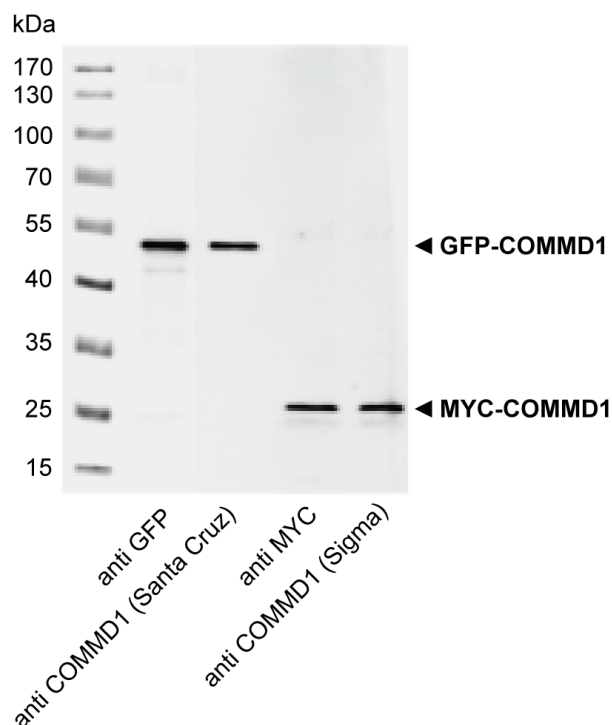
Figure 17: Used COMMD1 antibodies are specific for the COMMD1 protein: HEK293 cells were transfected with either GFP-COMMD1 or MYC-COMMD1 and the overexpressed COMMD1 protein purified using magnetic GFP-beads or Myc-beads, respectively. Western Blot analysis shows that both, the GFP- and Myc-tagged fusion proteins of COMMD1 can be detected by anti-GFP and anti-myc antibodies. Similarly, both anti-COMMD1 antibodies used in this study (from Santa Cruz and Sigma Aldrich) are able to specifically label the COMMD1 fusion-proteins.

Next, primary hippocampal neurons were transfected with GFP, GFP-COMMD1, scr-shRNA and COMMD1-shRNA. After 3 days the cells were incubated with $ZnCl_2$ and $CuCl_2$ at 37°C for 1 h. Immunocytochemistry was performed with Zinquin ethyl ester. Ratios of Zinquin fluorescence, correlating with intracellular zinc concentration, between transfected cells and untransfected cells next to them were determined. There is a significant increase of intracellular zinc levels in cells overexpressing COMMD1, whereas a decrease in neurons with COMMD1 knockdown can be seen.
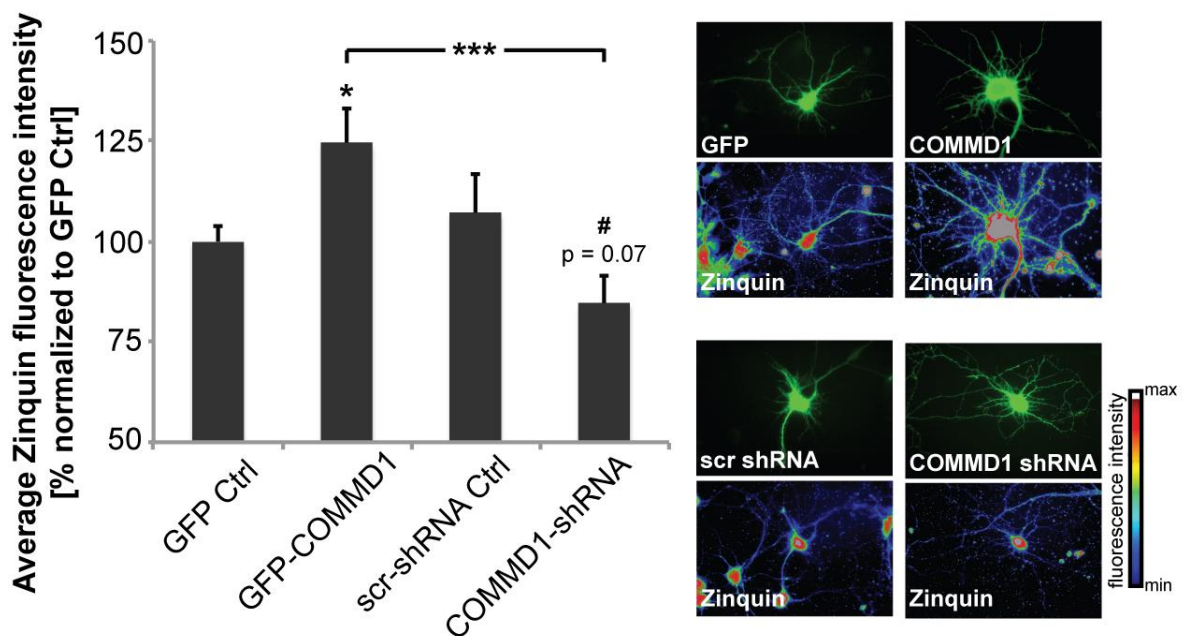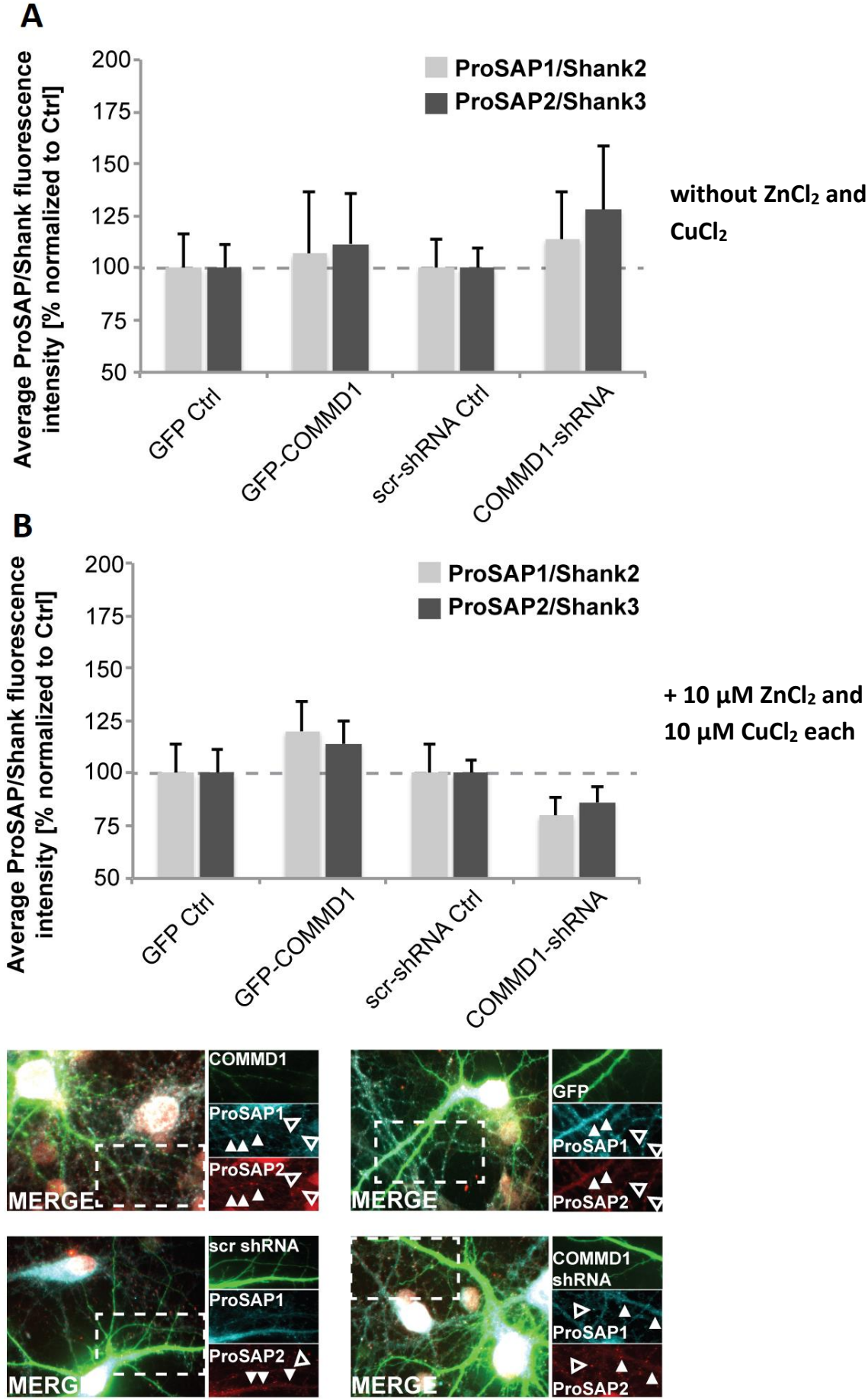
Figure 18: Neurons overexpressing COMMD1 after transfection with GFP-COMMD1 show an increase of intracellular zinc levels while neurons transfected with COMMD1-shRNA to create a knockdown of COMMD1 show a decrease: Primary hippocampal neurons were transfected at DIV10 with GFP, GFP-COMMD1. scrambled (scr) shRNA and COMMD1-shRNA followed by supplementation of $ZnCl_2$ and $CuCl_2$ for 1 h and the ratios of intracellular Zinquin ethyl ester fluorescence between transfected (GFP positive) and untransfected neighboring cells was calculated for ten cells per group. The values are shown normalized to GFP control transfected cells. A significant increase in intracellular zinc levels can be seen in cells overexpressing COMMD1, while levels in COMMD1 knock-down cells show a decrease.

To investigate whether an alteration of COMMD1 concentration also influences ProSAP/Shank protein levels at synapses, primary hippocampal neurons were transfected with GFP, GFP-COMMD1, scrambled (scr) shRNA and COMMD1-shRNA with and without supplementation of $ZnCl_2$ and $CuCl_2$. The cells were stained with ProSAP1/Shank2 and ProSAP2/Shank3 antibodies to determine fluorescence levels at synapses. Comparison shows that there is no significant change of synaptic ProSAP1/Shank2 or ProSAP2/Shank3 levels after altered COMMD1 concentration. However, there is a trend towards an increase of the two mentioned ProSAP/Shank proteins in cells overexpressing COMMD1 and a reduction in COMMD1 knockdown cells when they were supplemented with zinc and copper.

Figure 19: Effect of COMMD1 expression levels on ProSAP1/Shank2 and ProSAP2/Shank3 protein concentration in synapses of primary hippocampal neurons: Overexpression or knockdown of COMMD1 by transfection of GFP-COMMD1 and COMMD1 shRNA encoding plasmids, respectively, does not alter synaptic ProSAP1/Shank2 or ProSAP2/Shank3 levels in hippocampal neurons without treatment with $CuCl_2$ and $ZnCl_2$. However, there is a trend towards an increase of the two mentioned ProSAP/Shank proteins in cells overexpressing COMMD1 and to reduction in COMMD1 knockdown cells when the neurons were supplemented with zinc and copper. For the quantification, a puncta by puncta analysis of fluorescence intensities of immunoreactive signals was performed on 5-10 cells per group after 3d of transfection at DIV 14. The values are shown in percent normalized to the respective controls (GFP expression (GFP Ctrl) and scrambled (scr) shRNA (scr-shRNA Ctrl)). For each cell, the ratio between the fluorescence intensity of the transfected cell and a neighboring untransfected cell was calculated.

To investigate whether the change of synaptic ProSAP/Shank levels regarding COMMD1 expression level depends on the treatment with $ZnCl_2$ and $CuCl_2$, the ratio of copper/zinc treated cells to untreated cell was calculated and fluorescence intensities with high and low COMMD1 expression levels between treated and untreated conditions were compared. A significant change of synaptic ProSAP1/Shank2 and ProSAP2/Shank3 protein levels dependent on the expression level in treated vs. untreated cells can be seen.
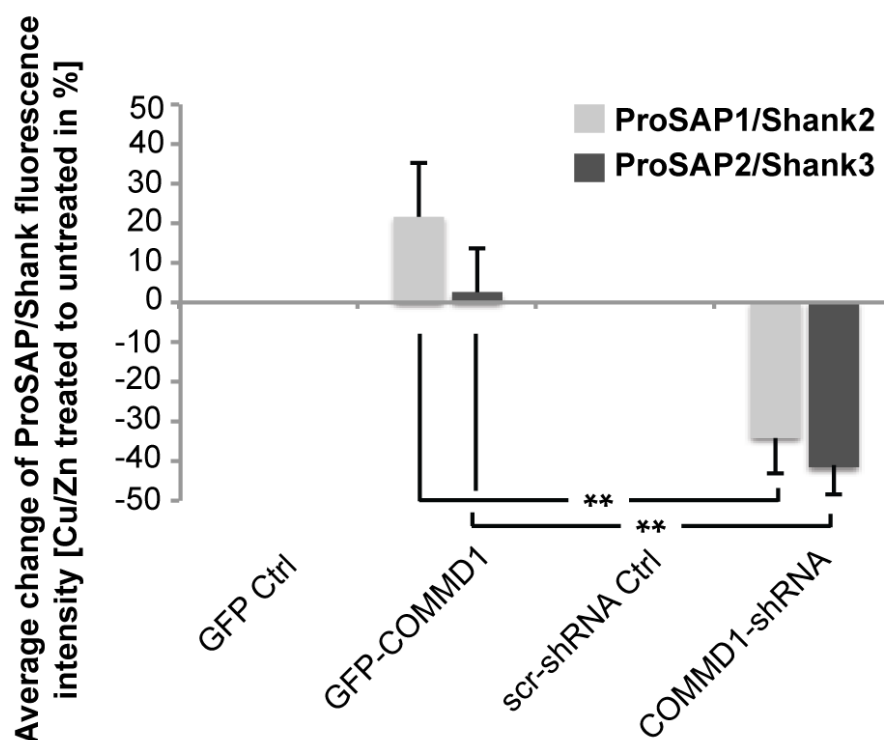
Figure 20: Synaptic ProSAP/Shank protein levels show a significant difference between cells with overexpression or knockdown of COMMD1 after treatment with CuCl₂ and ZnCl₂: Comparing synaptic ProSAP1/Shank2 and ProSAP2/Shank3 levels in transfected cells with and without application of CuCl₂ and ZnCl₂ reveals a significant difference between COMMD1 overexpressing and knockdown cells.

There was no influence of COMMD1 level observed for other synaptic proteins like mGluR5 and Bassoon as no significant changes could be detected.
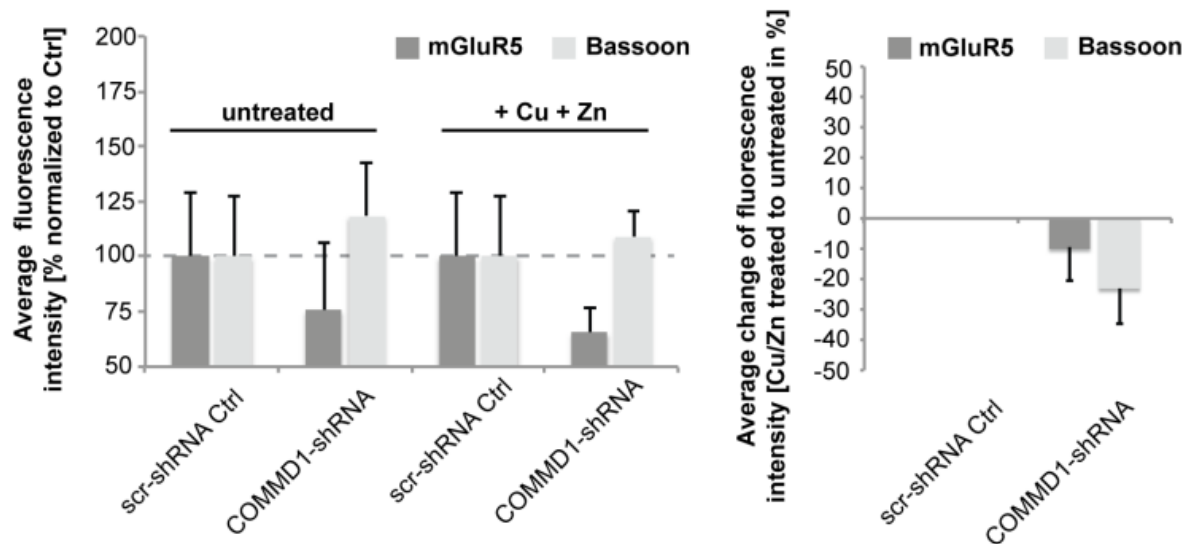

Figure 21: No significant alteration of synaptic mGluR5 and Bassoon levels after knockdown of COMMD1 independent of treatment with copper/zinc in primary hippocampal neurons: Knockdown of COMMD1 by transfection of a COMMD1 shRNA encoding plasmid does not significantly alter synaptic levels of mGluR5 or Bassoon in primary hippocampal neurons with and without treatment with $CuCl_2$ and $ZnCl_2$ (left panel). Comparing mGluR5 or Bassoon levels in transfected cells without and with application of $CuCl_2$ and $ZnCl_2$ also reveals no significant alteration (right panel).

COMMD1 is expressed during development of the CNS and in adult mice which could be shown by Western Blot analysis of P2 fractions of the brain regions cortex, cerebellum, hippocampus and striatum of mice 32 days or 18 months of age using an antibody against COMMD1. Therefore, it is likely that loss of COMMD1 could increase copper levels, which could in turn have an influence on zinc levels and synaptic concentrations of ProSAP/Shank proteins also *in vivo*.
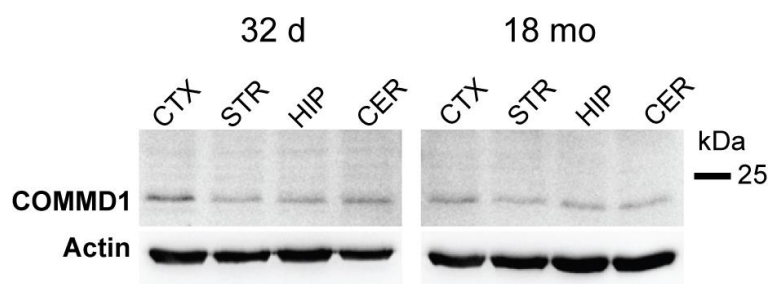

Figure 22: Expression of COMMD1 during CNS development and in adult mice brains: Western Blot analysis of protein homogenate from cortex (CTX), striatum (STR), hippocampus (HIP) and cerebellum (CER) of wild type mice at two different time points (32 days and 18 months of age) reveals expression of COMMD1 in all mentioned brain regions.

3.4 Acute copper overload influences zinc homeostasis *in vivo*

To investigate the effects of an altered copper concentration *in vivo* and its influence on zinc homeostasis, copper overload in mice was induced by feeding mice with a copper enriched diet for 5 weeks. The mice were examined and weighed every day and also the food consumption of the mice was determined.

There were no significant differences in body weight of the adult mice after 32 days. From day 21 to day 25 the copper supplemented mice weighed significantly more than the mice from the control group even though they did not consume more food. In week 5 the mice in the group with the copper enriched diet consumed significantly more food, otherwise there we no differences in food consumption. The copper treated mice did not show any signs of disease. However, after 5 weeks of copper supplementation the mice displayed hind limb clasping.
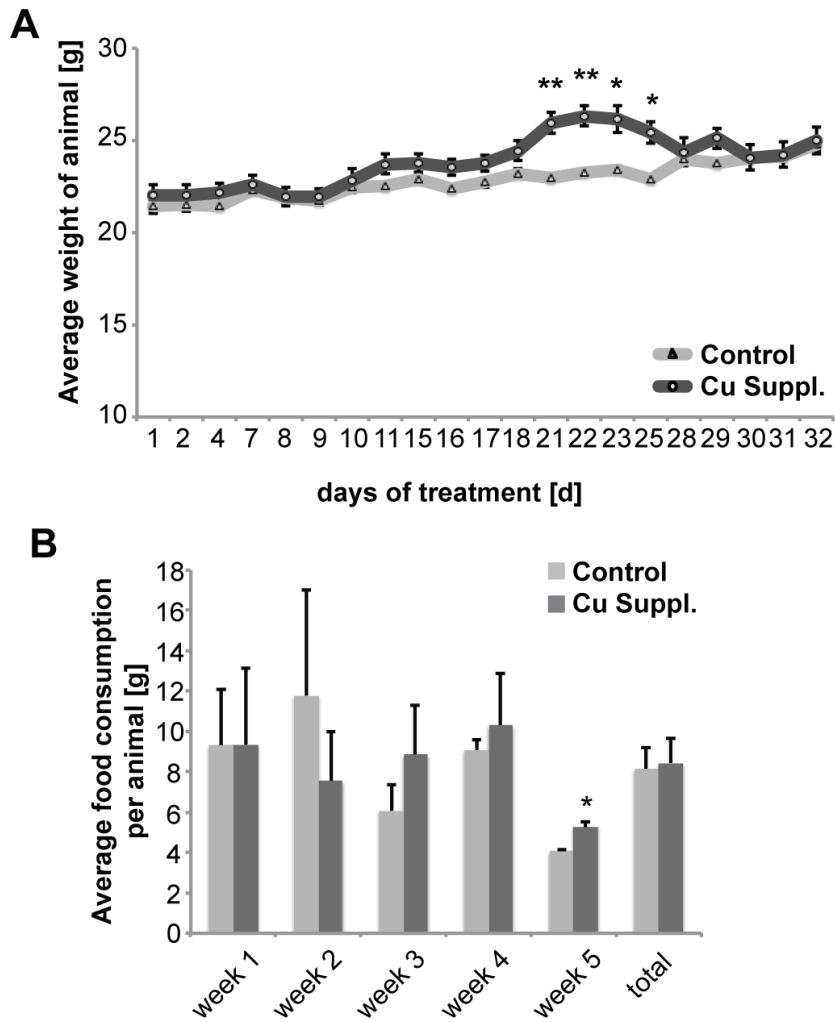
Figure 23: No significant differences between animal weight and food consumption of mice on a copper supplemented diet compared to control mice after 5 weeks of treatment: A) Mice on a copper- supplemented diet for 5 weeks show an increase in body weight after 21-25 days of treatment compared to control animals fed a normal diet. However, after 5 weeks both, supplemented and control mice had similar weights on average. B) During the 5th week of treatment, mice fed a copper supplemented diet consumed more food compared to control animals. However, averaging over the whole time of treatment, no significant difference in food consumption was visible.

The brains of all mice were also weighed and the body/brain weight ratio was determined. After 5 weeks of copper supplementation, the brain weight of the mice was significantly reduced and the body/brain weight ratio was significantly increased compared to mice with a normal diet.
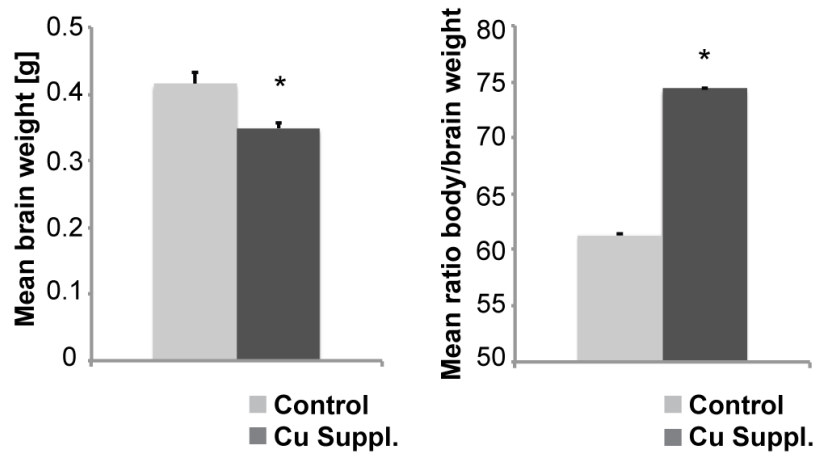

Figure 24: Significant decrease of brain weight and increase of body/brain ratio of copper supplemented mice: Mice on a copper supplemented diet for 5 weeks show a significant decrease in brain weight and an increase in the ratio of body/brain weight compared to control mice.

To investigate whether there is cell loss in brains of treated mice, especially in cortex and hippocampus, DAPI positive nuclei were counted in defined optic fields of several brain sections. The analysis of cell density shows a clear trend towards a reduction in cortex of mice fed with a copper enriched diet.
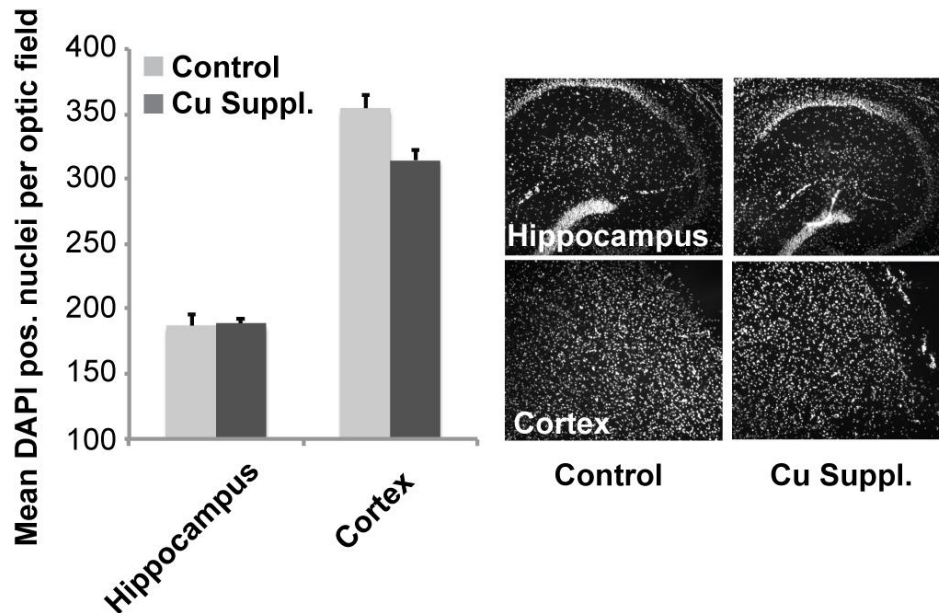

Figure 25: Analysis of cell density by DAPI staining shows a trend towards a reduction of cells in the Cortex of copper supplemented mice: Analysis of cell density by DAPI staining of nuclei in the two brain regions (hippocampus and cortex) reveals a clear trend towards a decrease (p = 0.053, n = 3) in cortex.

Next it was investigated whether a copper supplemented diet changes copper and zinc concentrations in brain and body using AAS, ICP-MS, Timm staining and fluorophores specific for zinc ions. Copper content was significantly higher in urine and feces of mice fed with a copper enriched diet. Interestingly, zinc levels were also increased in feces of those mice.

Copper levels in the serum were slightly increased in copper supplemented mice compared to control mice (0.66 g/g vs. 0.62 g/g) and serum levels of zinc were decreased (0.88 µg/g vs 0.92 µg/g). It is thus possible that the increased copper content in the diet leads to a decreased uptake of zinc.
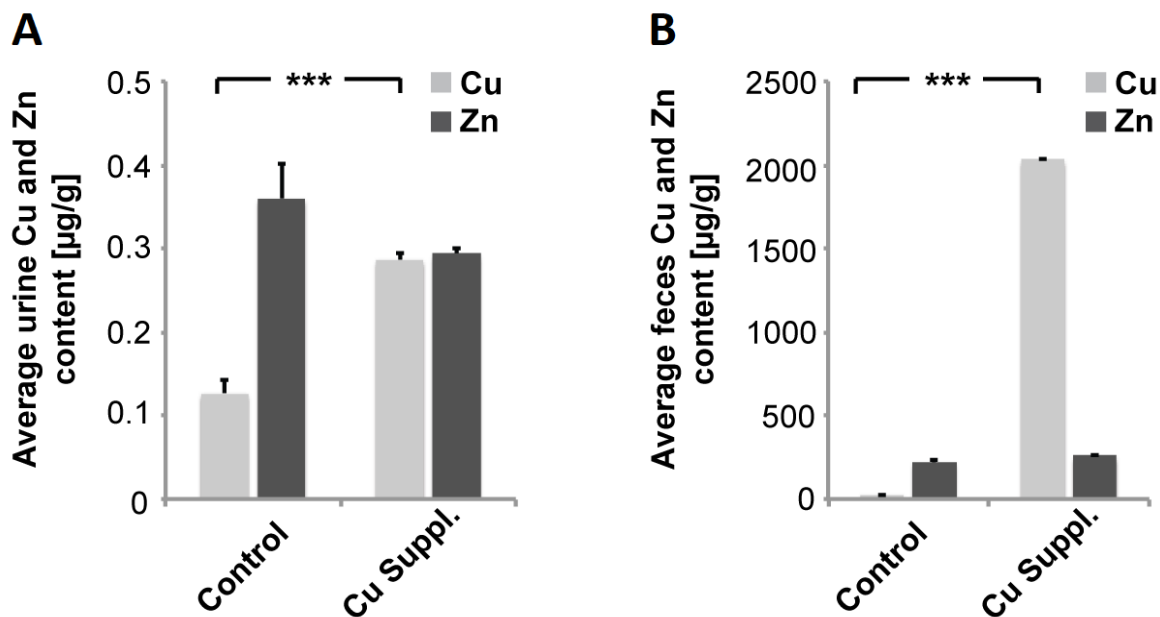

Figure 26: Urine and feces of copper supplemented mice contain significantly more copper: A) ICP-MS analysis shows that animals fed a copper enriched diet contain on average a significantly higher amount of copper in their urine. B) Copper supplemented mice also have a higher amount of copper in their feces. Zinc levels were slightly increased as well (p = 0.18; n = 3).

Liver and kidney were investigated for zinc content as well using AAS. There was no significant difference between the livers of both groups of mice, but the zinc content of the kidneys of copper supplemented mice was significantly decreased compared to animals fed a normal diet.
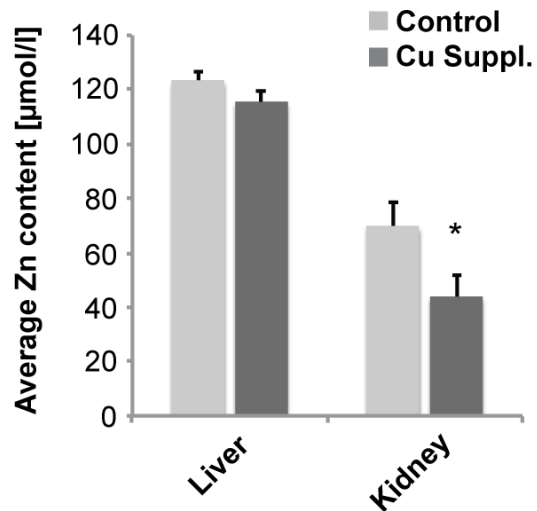

Figure 27: Kidneys of mice on a copper diet contain significantly less zinc: Analysis of zinc concentrations in liver and kidney from three different animals per group using atomic absorption spectrometry indicates slightly reduced zinc concentrations in blood and liver and a significantly reduced zinc concentration in kidneys of animals fed a copper-supplemented diet.

Total brain copper and zinc content was analyzed using ICP-MS. A significant decrease of zinc concentration in total brain was detected. Copper levels were unchanged.
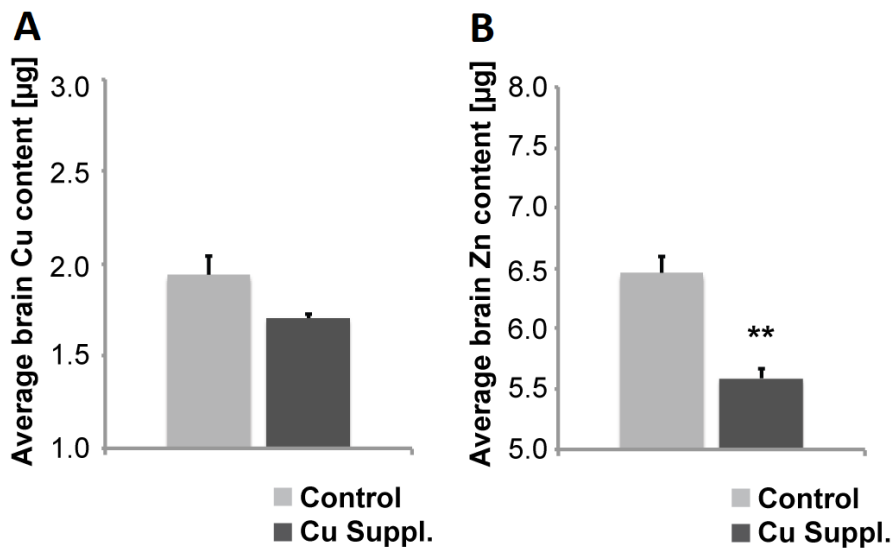

Figure 28: Brains of copper supplemented mice contain significantly reduced zinc concentrations while the copper content was unchanged: A) Analysis of copper and zinc concentrations using ICP-MS from three animals per group shows no difference in average brain copper levels. B) Average brain zinc levels were significantly reduced in animals fed a copper- supplemented diet.

To investigate where in the brain zinc levels had been decreased, zinc ions in cortex and Hippocampus of mice were visualized with Zinpyr-1. To verify that Zinpyr-1 detects zinc sensitively, TPEN was used to form chelates with zinc. After immunohistochemistry with Zinpyr-1 no signal could be detected with Zinpyr-1 anymore, which verifies that Zinpyr-1 detects specifically zinc. Subsequently, the mean signal intensity of Zinpyr-1 was analyzed in cortex and Hippocampus. There was a significant decrease of zinc in the hippocampus and a trend towards a decrease in the cortex of mice supplemented with a copper enriched diet.
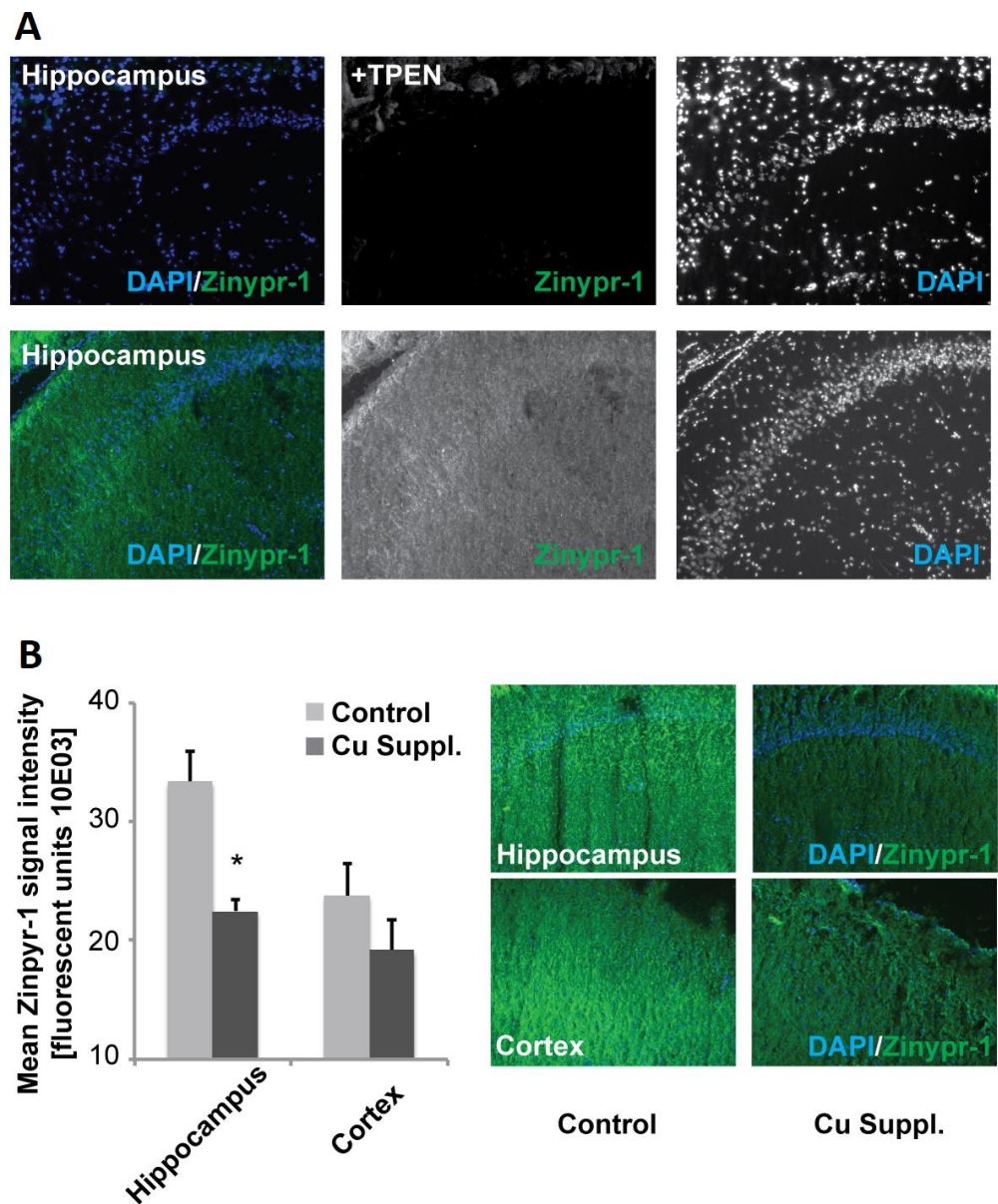
Figure 29: Zinpyr-1 fluorescence analysis shows a significant decrease of zinc concentrations in hippocampi of mice fed with a copper supplemented diet: A) Hippocampal brain sections treated with 15 μM TPEN for 10 min before Zinpyr-1 staining (upper panel) show almost no detectable fluorescence levels compared to untreated sections (lower panel). B) Brain region specific analysis of zinc using Zinpyr-1 in at least three optic fields of view per brain region from three different animals shows a significant reduction in Zinpyr-1 staining correlating with zinc concentrations in hippocampus.

To verify this finding, Timm staining was used detecting a similar significant decrease of zinc in the hippocampus. Interestingly, copper levels in the brain were not significantly altered in the brain of mice fed with a copper supplemented diet.

Based on these results it is likely that changes of copper levels in the body lead to zinc deficiency in the brain that is more pronounced in regions that are usually rich in zinc as the hippocampus.

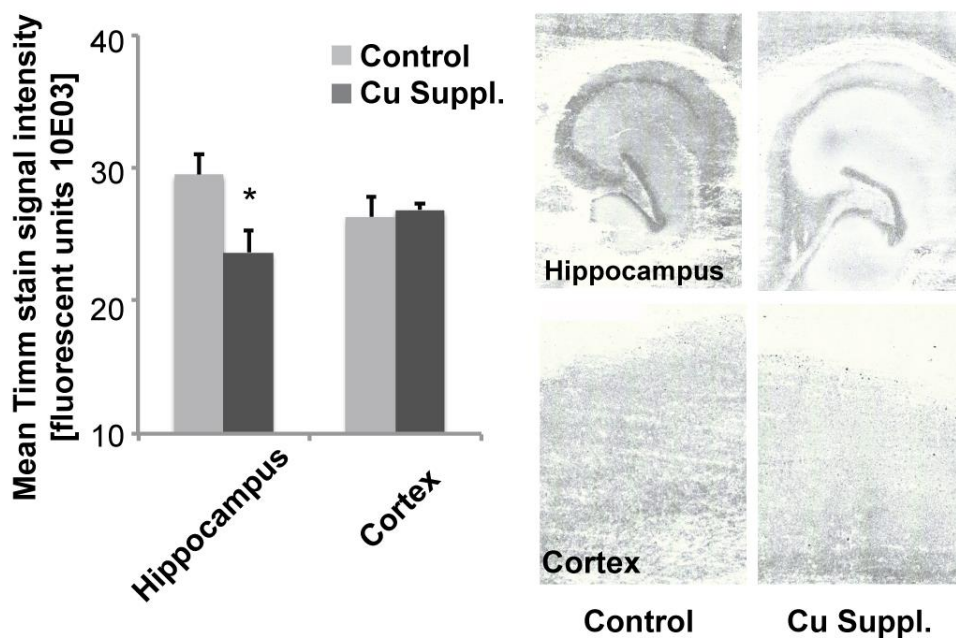**Figure 30: Analysis of Timm staining shows a significant decrease of zinc concentrations in hippocampi of mice fed with a copper supplemented diet:** Similarly to Figure x22B), a brain region specific analysis of zinc (using Timm stained intensity within on optic field from different brain regions) from three different animals shows a significant reduction of staining intensity in the hippocampus.

3.5   Influence of zinc deficiency due to copper overload on PSD proteins
in vivo

To compare expression on protein level, whole brain P2 fractions from mice on a copper supplemented diet were prepared in order to compare protein levels using Western Blot analysis. Whole brain lysates did not show any significant alteration of ProSAP/Shank protein levels nor in proteins involved in metal homeostasis.
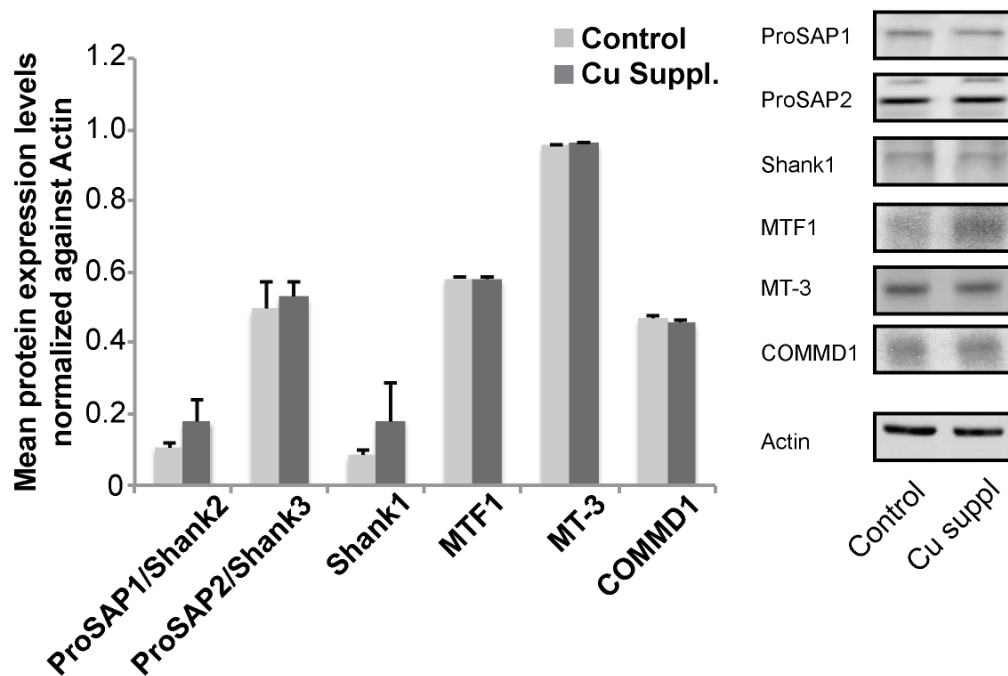

Figure 31: Analysis of whole brain lysate of mice on a copper diet reveals no differences on expression level of investigated proteins: Western Blot analysis of P2 fractions from whole brain lysate using control animals and copper supplemented mice (n = 3). No significant differences can be detected in ProSAP/Shank proteins or MTF-1, MT-3 and COMMD1.

Given that zinc deficiency could only be detected in specific brain regions in copper supplemented mice, the same analysis was done using homogenate from four different brain regions (cortex, cerebellum, hippocampus and striatum) to investigate protein levels in a brain region specific manner. The results of the analysis show a trend towards a decrease of ProSAP/Shank proteins in hippocampus, but no significant alterations.
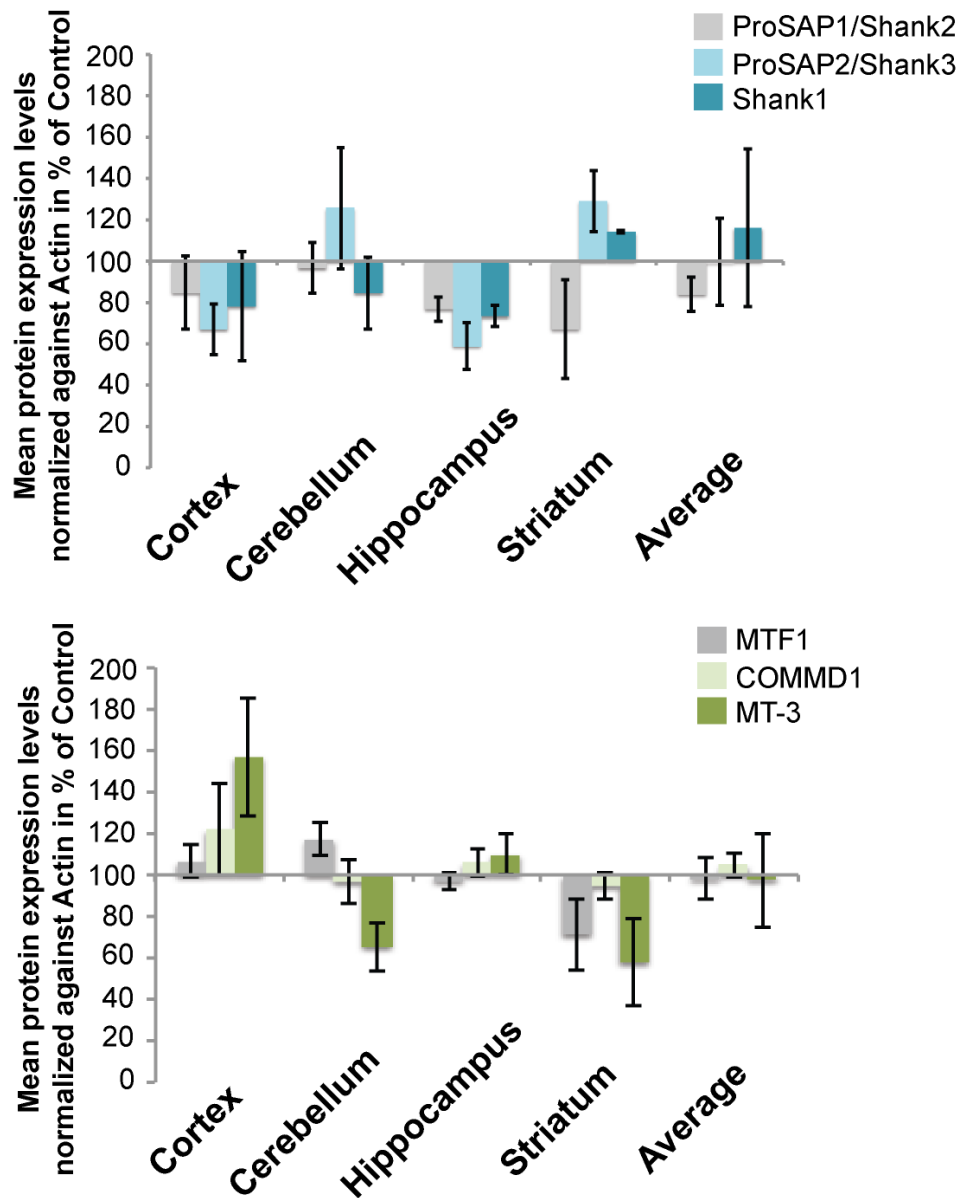
Figure 32: Analysis of P2 fractions from four brain regions of mice on a copper diet shows a trend towards a decrease of ProSAP/Shank proteins in hippocampus: Western Blot analysis of ProSAP/Shank proteins (left panel) in P2 fractions from different brain regions using control animals and copper supplemented mice (n = 3 pooled, 3 technical replicates). The results show a trend towards a decrease especially in hippocampus, however, no significant changes could be detected. Analysis of MTF1, COMMD1 and MT-3 (right panel) similarly revealed no significant alterations.

Additionally, ProSAP/Shank levels were analyzed using immunohistochemistry. The results show that the decrease of ProSAP2/Shank3 seen by Western Blot analysis is confirmed, as a significant reduction of ProSAP2/Shank3 can be observed here. There were no significant alterations seen for ProSAP/Shank proteins other than this one in the four brain regions.
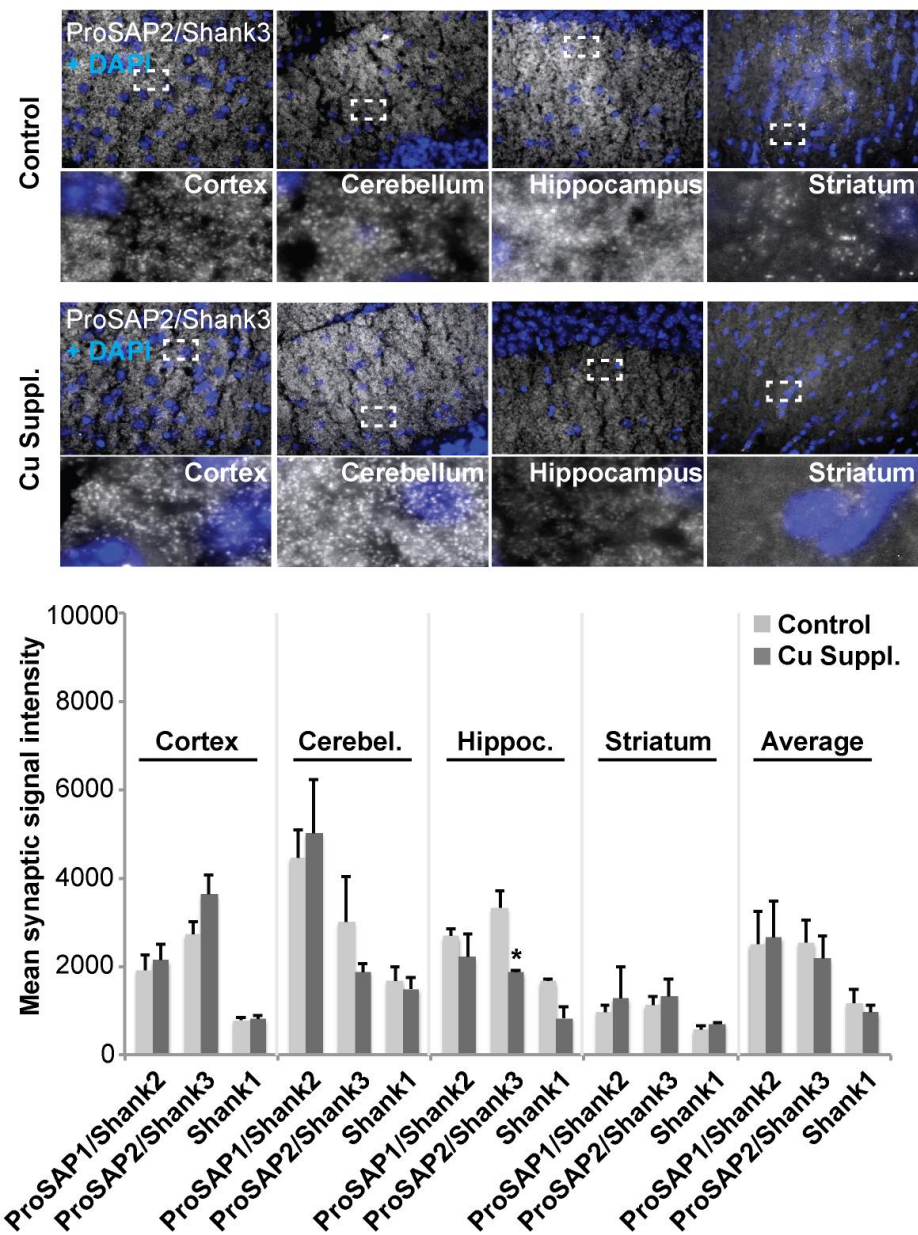
Figure 33: Immunohistochemistry shows a significant decrease of ProSAP2/Shank3 in the hippocampus of mice fed a copper supplemented diet: Sections of three animals from control and copper supplemented mice were stained for ProSAP1/Shank2, ProSAP2/Shank3 and Shank1. The signal intensity of synaptic ProSAP/Shank puncta was evaluated. A slight but significant decrease can be seen for ProSAP2/Shank3 in the hippocampus. Upper panels show exemplary sections for ProSAP2/Shank3.

Synapse number in the four brain regions and in general was also investigated measuring the average number of signals within a determined optic field. No change could be detected in synapse numbers.

Figure 34: Copper supplementation does not lead to alterations in synapse density: The average signal density (all ProSAP/Shanks combined) per optic field was determined from sections of three animals per group. No significant changes in excitatory synapse density were detected.
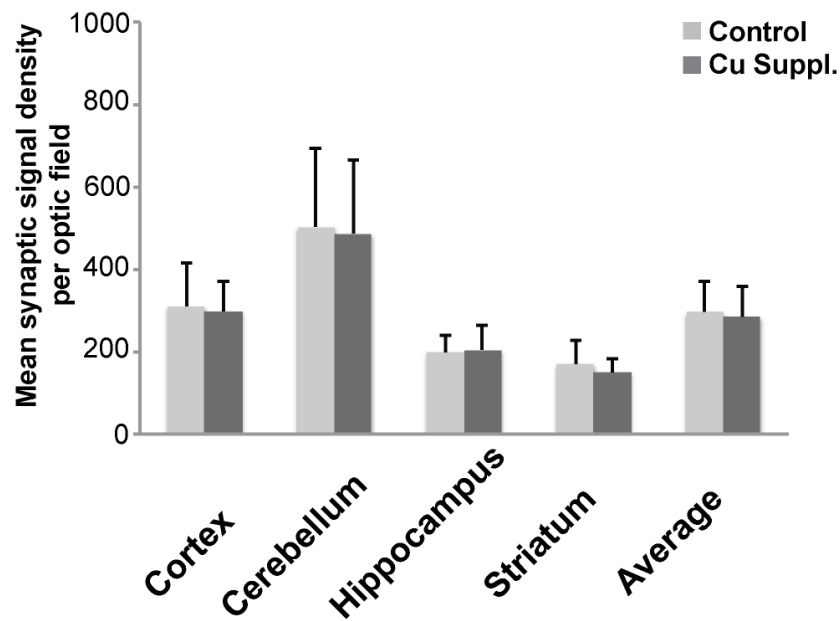
3.6 Pups of copper supplemented mice show reduced zinc levels in the brain

To investigate whether the copper enriched diet of the mother had any effect on zinc levels in the brain of the pups, brain sections of pups were stained using Zynpyr-1 and Timm staining to label zinc in the cortex and the hippocampus. A significant decrease of zinc in the cortex and a trend towards a decrease in the hippocampus can be seen after the staining with Zynpyr-1. Additionally, there is a significant decrease of zinc on average in all brain regions.
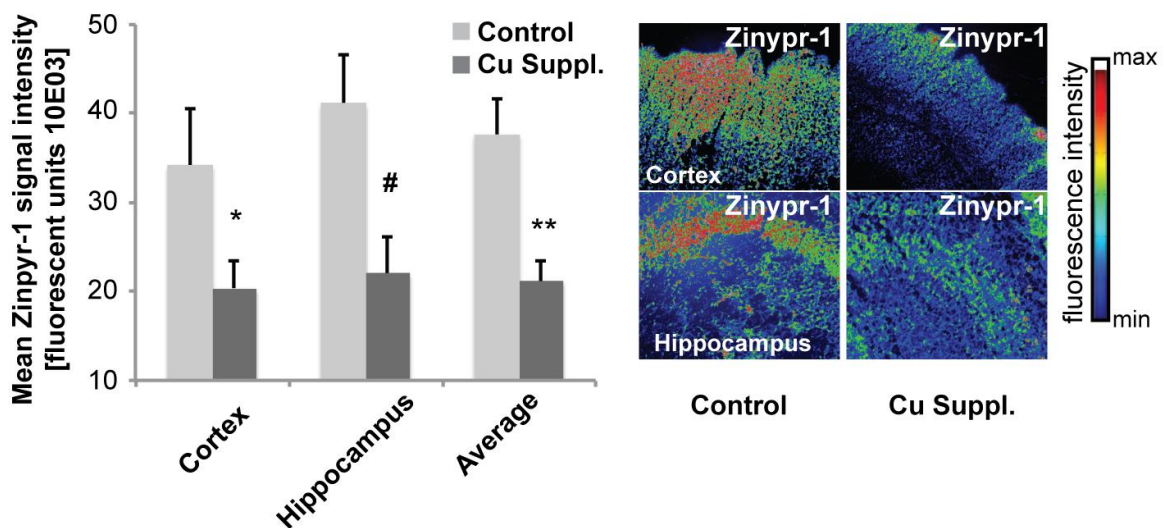
Figure 35: Zinpyr-1 staining shows a significant reduction of the zinc concentration in the cortex and in the brain on average of pups from mice on a copper supplemented diet: Brain region specific analysis of zinc (using Zinpyr-1) using at least three optic field of view per brain region from three different animals shows a significant reduction in Zinpyr-1 staining correlating with zinc concentrations in cortex and on average across all analyzed brain regions in pups from mice on a copper supplemented diet.

The analysis of the Timm staining shows a significant decrease of zinc concentration on average in all brain regions. However, here, a significant decrease can be shown in the hippocampus and only a trend in the cortex.
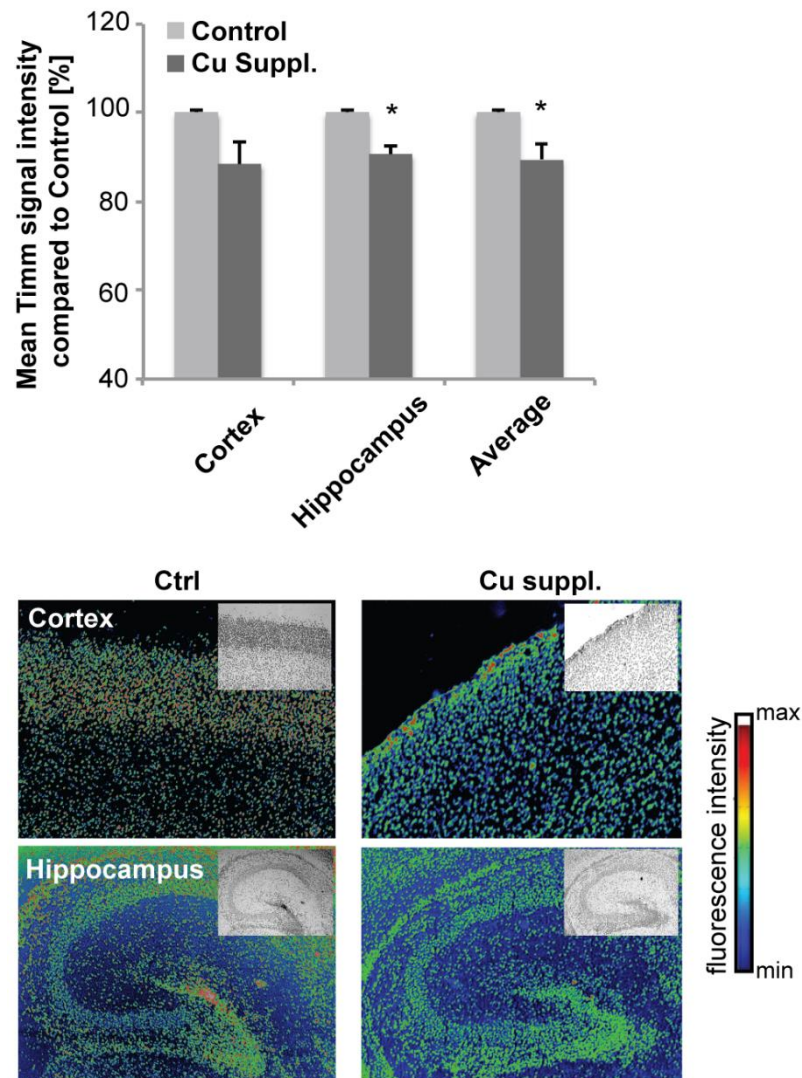
Figure 36: Zinc concentration analyzed by Timm staining is significantly reduced in hippocampus of pups from mice treated with copper: A brain region specific analysis of zinc (using Timm staining) from different brain regions from three different pups per group shows a significant reduction of staining intensity in the hippocampus and averaged across hippocampus and cortex. Right panel: Exemplary images of Timm staining for pups PD3 from wild type controls and mice on a copper enriched diet. Signal intensity is shown also in color code.

To analyze general brain morphology, the number of DAPI positive nuclei was counted in determined optic fields of cortex and hippocampus. The brains of pups of mice on a copper enriched diet did not show any differences of brain morphology in any brain region compared to mice on a normal diet.

Figure 37: DAPI staining shows no difference in cell density in brains of pups from mice with a copper enriched diet: Analysis of cell density by DAPI staining of nuclei in the two brain regions (hippocampus and cortex) of pups from control mice and mice on a copper enriched diet. At least three optic fields (red boxes) from three sections each of three animals per group were measured. No significant differences were detected.

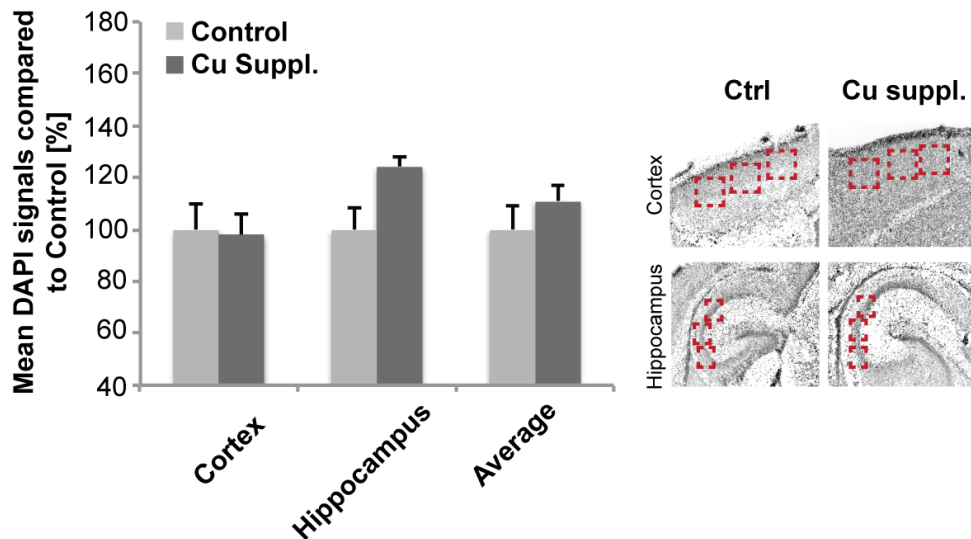## 3.7 Prenatal zinc deficiency caused by copper overload influences synaptic ProSAP/Shank levels *in vivo*

As ASDs are disorders of neurodevelopment, it is of interest to investigate the influence of prenatal copper overload on glutamatergic synapses. Zinc deficiency caused by copper overload could contribute to defects that can be observed in ProSAP/Shank associated disorders of the brain like ASD.

To compare expression on protein level, whole brain P2 fractions from pups from mothers fed with a copper supplemented diet were prepared. Protein levels were compared using Western Blot analysis. A significant decrease of ProSAP1/Shank2, all three major isoforms of ProSAP2/Shank3 and COMMD1 (normalized against Actin) can be seen in pups from copper supplemented mice compared to pups from control mice.

It has been reported that the loss of ProSAP2/Shank3 is associated with a decrease in NMDA receptor subunit 1 (GluN1) and AMPA receptor subunit 2 (GluA2) in ProSAP2/Shank3 knockout animals (Bozdagi et al., 2010; Wang et al., 2011, Won et al., 2012). Therefore, we also determined synaptic levels of GluN1 and GluA2 in the pups. The results show a significant reduction of GluN1 levels in pups from mothers on a copper enriched diet.
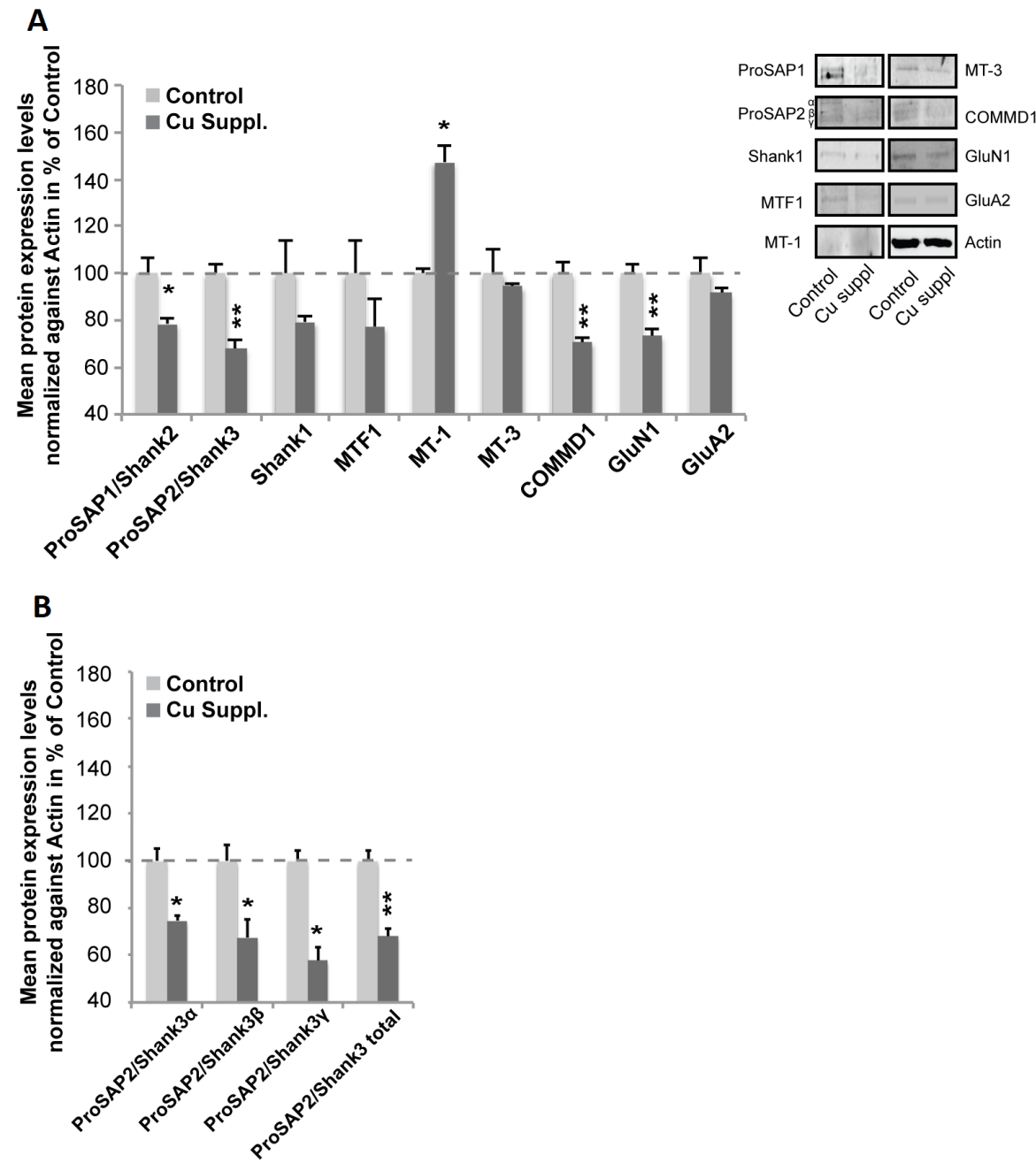
Figure 38: Whole brain protein analysis is of pups from copper supplemented mice using Western Blot analysis shows a significant decrease of ProSAP1/Shank2, ProSAP2/Shank3 (all isoforms), COMMD1 and GluN1: Western Blot analysis of P2 fractions from whole brain lysate using control pups and pups (PD 3) from copper supplemented mice (n = 3). A) A significant decrease of ProSAP1/Shank2, ProSAP2/Shank3, COMMD1, and GluN1 can be seen normalized to Actin. Additionally, an increase in MT-1 levels is visible. B) The decrease in ProSAP2/Shank3 comprises all three major isoforms.

Another question was whether this alteration in ProSAP/Shank is caused by loss of scaffold proteins per synapse or by loss of synapses in general. To this end, mean ProSAP/Shank levels per synapse were measured using immunohistochemistry and

synapse density in the brain regions cortex and hippocampus was determined. No significant changes in the mean synaptic fluorescence intensities of ProSAP/Shank proteins can be found in brain regions in brain slices of pups from mice with copper overload or from control mice. There is a trend towards a decrease of ProSAP/Shank proteins per synapse in hippocampus though.
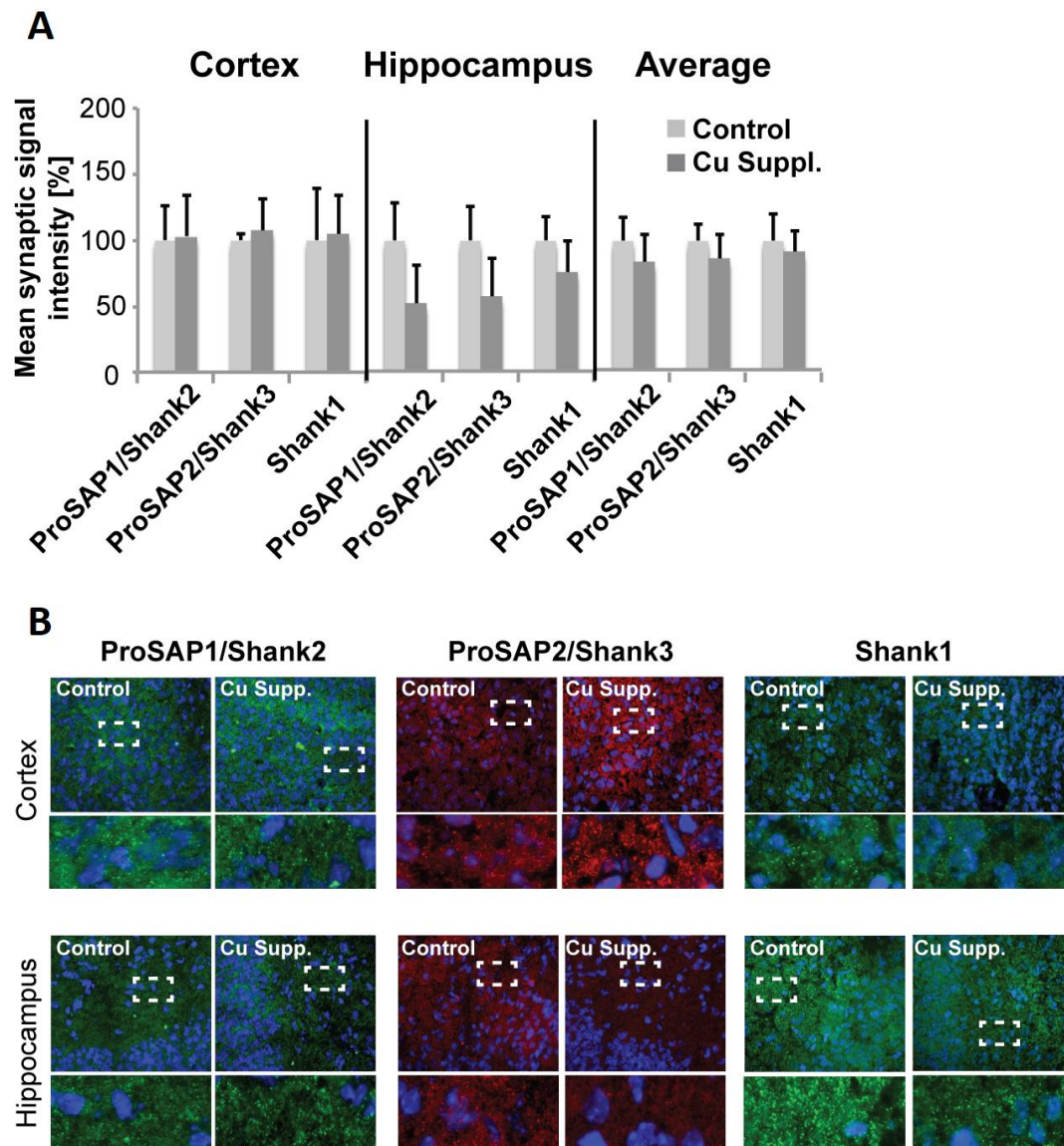
Figure 39: A trend towards a decrease of ProSAP/Shank proteins per synapse in hippocampus of pups from mice that were supplemented could be detected using immunohistochemistry: Sections of three pups from control and three pups from copper supplemented mice were stained for ProSAP1/Shank2, ProSAP2/Shank3, Shank1 and Bassoon. The signal intensity of synaptic ProSAP/Shank puncta was evaluated. Although a trend towards a decrease in the mean fluorescence intensity per synapse can be seen in hippocampus, no significant decrease of ProSAP/Shanks could be detected.

RESULTS

Subsequently, cumulative histograms were generated to visualize pools of synapses with lower and higher ProSAP1/Shank2 fluorescence intensities. The analysis shows a significant decrease of ProSAP1/Shank2 levels in the pool of synapses with high fluorescence. It is likely that this pool of synapses represents mature synapses with strong scaffolds in the PSD.
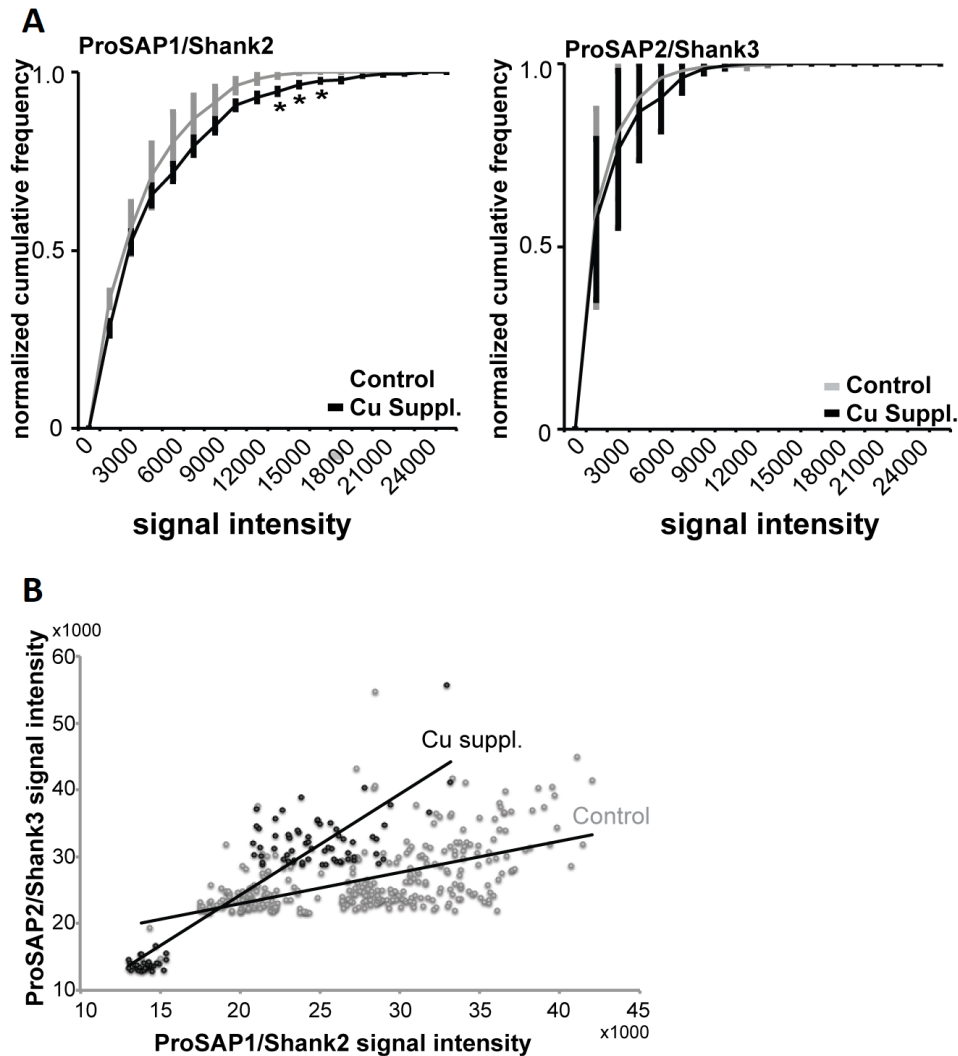
Figure 40: ProSAP1/Shank2 levels are decreased in a population with high fluorescence intensities in hippocampus of pups from mice on a copper enriched diet: A) Cumulative histograms for hippocampal ProSAP1/Shank2 (left) and ProSAP2/Shank3 (right) illustrate that the puncta intensity values are significantly shifted across the highly fluorescent populations of ProSAP1/Shank2 puncta in sections of control pups versus pups from copper supplemented mice. B) Sections of three pups from control and three pups from copper supplemented mice were stained for ProSAP1/Shank2 and ProSAP2/Shank3. The signal intensity of synaptic puncta for both ProSAP/Shank isoforms was evaluated simultaneously per synapse. A correlation analysis reveals a positive correlation for ProSAP1/Shank2 and ProSAP2/Shank3 signal intensities. Note the shift in slope of the correlation in pups from copper supplemented mice due to the loss of ProSAP1/Shank2 from a pool of synapses with strong ProSAP1/Shank2 and ProSAP2/Shank3 scaffold.

To determine general synapse density in hippocampus and cortex, the mean number of synaptic signals (positive Bassoon and ProSAP/Shank) within a determined optic field was analyzed. A slight decrease in synapse density in the hippocampus of pups from mice with copper overload can be found.

This decrease together with the decrease in fluorescence intensities underlines the result of decreased ProSAP1/Shank2 and ProSAP2/Shank3 levels in the brains of pups from mothers on a copper enriched diet. In conclusion, the reduced level of ProSAP1/Shank2 on protein level seems to be based on both, a slight decrease in number of synapses and a weaker scaffold of synapses especially in synapses with a strong ProSAP1/Shank2 PSD scaffold.

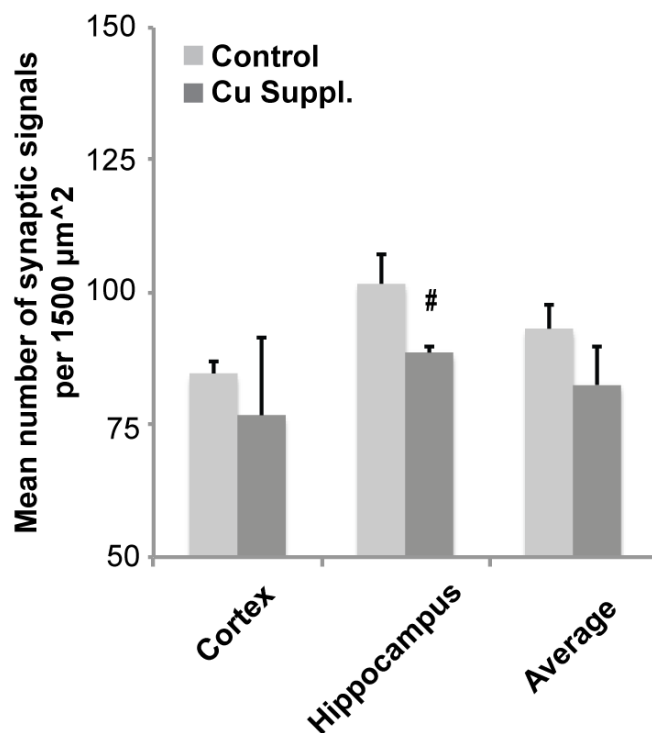**Figure 41: A decrease in the number of synapses in the hippocampus of pups from copper supplemented mice can be seen:** The number of synaptic signals (ProSAP/Bassoon positive) per 1500 μm^2 was assessed in three optic fields of brain sections from three pups per group. A clear trend towards a decrease is visible in hippocampus (# p = 0.07).

4 DISCUSSION

4.1 Pathomechanisms that can lead to ASD

Autism spectrum disorders are neurodevelopmental disorders and so far the pathomechanisms that can lead to ASD are not well understood. However, there seems to be a strong genetic component. A lot of genes associated with ASD are involved in different neuronal developmental processes like neuronal migration and synaptic and dendritic formation (Pardo and Eberhart, 2007).

Mutations or deletions associated with ASD have been found in several genes like neurexins, neuroligins or ProSAP/Shanks (Betancur et al., 2009; Grabrucker et al., 2011c). Autism-associated mutations in all three ProSAP/Shank family members have been reported (Durand et al., 2007; Moessner et al., 2007; Gauthier et al., 2009; Berkel et al., 2010; Pinto et al., 2010; Sato et al., 2012). ProSAP/Shank proteins are the "master" scaffold proteins at the PSD of glutamatergic synapses (Sheng and Kim, 2000; Boeckers et al., 2001). They play an important role in organizing the PSD. ProSAP/Shank proteins can regulate the synaptic assembly, stability and plasticity, which are necessary for learning and memory formation (Boeckers et al., 2006; Grabrucker et al., 2011a). ProSAP2/Shank3 is the most important ProSAP/Shank isoform associated with neurodevelopmental deficits or neurobehavioral disorders (Durand et al., 2007; Moessner et al., 2007; Gauthier et al., 2009). Disturbances at synapses may lead to learning disabilities (Durand et al., 2012). Mutations in the ProSAP1/Shank2 gene have also been found in ASD patients (Berkel et al., 2010; Pinto et al., 2010).

Mutations or deletions that are associated with ASD can be placed in a common synaptic pathway at excitatory glutamatergic synapses (Bourgeron, 2009; Delorme et al., 2013). However, this pathway may not only be influenced by the participating proteins/genes themselves, but by factors that affect the environment in which these proteins function. Part of this environment may be zinc among others as it is able to directly modulate the structure and the function of the PSD by binding to the SAM domain of ProSAP/Shank proteins (Baron et al., 2006; Gundelfinger et al., 2006).

Other defect genes that were associated with ASD are coding for proteins involved in copper or zinc homeostasis like metallothioneins or COMMD1 (Serajee et la., 2004; Tórsdóttir et al., 2005; Koumura et al., 2009; Betancur et al., 2009; Levy et al., 2011). A disruption of the homeostasis of biometals like zinc or copper has been found in many ASD patients (Lakshmi Priya and Geetha, 2011; Faber et al., 2009). It was suggested that infantile zinc deficiency or copper overload might contribute to the pathogenesis of autism (Yasuda et al., 2011). A connection between zinc deficiency and synaptic malfunctioning has already been found (Grabrucker et al., 2014). Here, the connection between environmental factors like nutritional availability of copper or zinc and the proposed common synaptic pathway at excitatory glutamatergic synapses was investigated.

4.2 Interaction of zinc and copper homeostasis *in vitro* and their influence on ProSAP/Shank proteins

Usually the ratio of copper to zinc in the blood is close to 1:1. (Faber et al, 2009; Van Weyenbergh et al., 2004). At the mucosa of the intestine zinc and copper influence each other's absorption and it is known that in the body zinc and copper have antagonistic roles whereas an overload of copper leads to zinc deficiency (Underwood, 1977; Hall et al., 1979). The absorption of zinc is impaired as metallothioneins bind copper within intestinal cells (Russo and DeVito, 2011). Here, it could be shown that supplementation of zinc *in vitro* leads to a significant increase of intracellular zinc concentrations. This increase is blocked when copper is co-applied. Supplementation of both, zinc and copper, results in elevated extracellular levels of zinc and copper which in turn influence intracellular zinc levels *in vitro*. This means that the uptake of zinc is impaired by copper overload. Interestingly, ASD patients often show changes in the intestinal function and flora which can lead to malabsorption of zinc, maybe also due to copper overload (de Theije et al., 2011; Finegold et al., 2012).

ProSAP/Shank proteins have SAM domains that are zinc sensitive, but they only show a low affinity for copper (Grabrucker et al., 2011a). ProSAP1/Shank2 and ProSAP2/Shank3

protein levels are upregulated zinc dependently and zinc deficiency leads to decreased levels of these two ProSAP/Shank proteins (Grabrucker et al., 2014). The zinc-dependent upregulation of ProSAP1/Shank2 and ProSAP2/Shank3 could be confirmed in this study by zinc supplementation, but this upregulation is impaired when copper is applied as well. This shows again that copper overload can have an influence on zinc homeostasis. By influencing zinc levels elevated extracellular copper levels also have an impact on zinc-sensitive proteins like ProSAP1/Shank2 and ProSAP2/Shank3. Shank1 levels are not influenced by altered zinc and/or copper concentrations. This is not surprising as it acts zinc-independently (Grabrucker et al., 2011a).

Homer binds to ProSAP/Shank and can also cluster group 1 metabotropic receptors like mGluR5 (Tu et al., 1999). Homer levels are not changed by zinc/copper supplementation, which was seen before (Grabrucker et al., 2014). mGluR5 levels are increased when zinc is applied. The cause for the increase might be increased ProSAP2/Shank3 levels, as the expression of mGluR5 seems to be regulated by ProSAP2/Shank3 (Verpelli et al., 2011). However, not all ProSAP23/Shank3 isoforms are able to interact with mGluRs. The newly discovered complexity of ProSAP2/Shank3 makes it difficult to describe concrete effects of ProSAP2/Shank3 on other proteins at glutamatergic synapses (Wang et al., 2014).

Inhibitory synapses do not contain ProSAP/Shank proteins (Naisbitt et al., 1999). A changed zinc homeostasis does not affect inhibitory synapses (Grabrucker et al., 2014). Here, it is confirmed that altered zinc, copper and/or ProSAP/Shank levels have no influence on protein levels of inhibitory synapses like Gephyrin levels. However, the interaction of altered excitatory synapses and unaltered inhibitory synapses may lead to an imbalance of excitation and inhibition which might be an important factor in neurological diseases such as ASD (Rubenstein and Merzenich, 2003).

Synaptic ProSAP1/Shank2 and ProSAP2/Shank3 levels are increased as response to neuronal activity (Grabrucker et al., 2014). *In vitro* increase of neuronal activity can be achieved with HiK$^+$ (50mM KCl). After treatment with copper the usual increase of synaptic ProSAP2/Shank3 and ProSAP1/Shank2 levels was impaired. An increased copper burden might lead in general to a reduced bioavailability of zinc.

4.3 Alteration of COMMD1 concentration influences copper and zinc homeostasis as well as ProSAP/Shank levels at synapses *in vitro*

COMMD1 is another protein associated with ASD being homozygous deleted in some ASD patients (Levy et al., 2011). COMMD1 plays an important role in controlling copper homeostasis and above all in copper excretion (deBie et al., 2007; Vonk et al., 2011). There exists an inverse correlation between the COMMD1 protein and the intracellular level of copper. Therefore COMMD1 deficiency can lead to intracellular copper overload (Burstein et al., 2004). Here, it could be shown that when cells overexpress COMMD1 intracellular zinc levels increase and knockdown of COMMD1 leads to a zinc decrease when the cells are supplemented with zinc and copper. This implies that non-functional COMMD1 can lead to zinc deficiency probably due to intracellular copper overload.

Here, it was indicated that COMMD1 is expressed during CNS development and in adult mice. Therefore, it is likely that loss of COMMD1 leads to increased copper levels which in turn affect zinc levels and also influences the concentrations of synaptic ProSAP/Shank proteins. There is a significant difference of ProSAP1/Shank2 and ProSAP2/Shank3 levels when COMMD1 is knocked down and enough zinc and copper are available compared to COMMD1 overexpression.

4.4 Influence of acute copper overload on zinc homeostasis *in vivo*

To investigate the effects of excess copper *in vivo* and its influence on zinc homeostasis, mice were fed with a copper enriched diet.
Copper levels in feces and urine of the mice were increased which confirms the uptake and therefore excretion of more copper than usual. However, no increased copper levels in the brains could be found. The blood-brain-barrier and the blood-cerebrospinal fluid play an important role in the regulation of copper homeostasis in the brain (Choi and Zheng, 2009). The entry of copper into the brain is still poorly understood, but the main entrance for copper to get access into the brain is the blood-brain-barrier. Several transporters have been suggested to be responsible for the transport of copper over the cell membrane like ATP7B, CTR1 and DMT1 (Li and Zheng, 2005). CTR1 seems to play a

role at the entry of copper into the brain through the blood-brain-barrier whereas ATP7A and ATP7B are likely responsible for the efflux of copper (Choi and Zheng, 2009; Fu et al., 2014). The blood-cerebrospinal fluid seems to be important to maintain copper homeostasis in the extracellular fluid of the brain (Choi and Zeng, 2009). Copper in high concentrations is neurotoxic. Therefore its distribution is tightly regulated and almost all copper in the brain is bound to proteins (Que et al., 2008; Lutsenko et al., 2010). In the brain high copper levels can inhibit AMPA and NMDA receptors. AMPA receptor-mediated synaptic transmission can also be impaired (Weiser and Wienreich, 1996; Peters et al., 2011). High levels of copper lead to an inhibition of hippocampal LTP formation at NMDA receptors, which is important for learning and memory (Doreulee et al., 1997; Lynch, 2004). Other described neurotoxic effects are depression, fear, nervousness and perhaps Alzheimer's disease (Bjørklund, 2013; Madsen and Gitlin 2007; Brewer 2012). Thus it is likely that the brain is protected from too high copper concentrations by controlled uptake and efflux of copper and therefore no increased copper levels could be found in the brain of mice fed with a copper supplemented diet.

The mice also showed a slight increase of zinc levels in the feces and a significant decrease in kidneys. Therefore it is possible that zinc uptake was already impaired in the mice's gastrointestinal tract due to a competition between copper and zinc at the intestinal mucosa. It is known that zinc and copper influence each other's absorption (Underwood, 1977). 98% of copper excretion is via the bile, the rest via the kidneys (Wijmenga and Klomp, 2004). In this thesis it could also be shown that the body of copper supplemented mice tried to get rid of too much copper as copper concentrations in excretion products like urine, the ultrafiltrate of blood, and feces of these mice were significantly elevated. In line with this, slightly increased copper and decreased zinc serum levels were found in copper supplemented mice.

Additionally, significant zinc deficiency was detected within the brain of the copper supplemented mice, which was especially pronounced in the hippocampal region. Apparently zinc deficiency observed in mice fed with a copper supplemented diet was not severe enough to lead to lots of changes in the whole brain of mature mice regarding protein levels. However, in hippocampus at synapses, a significant decrease of

ProSAP2/Shank3 was seen. This change goes in line with zinc deficiency that was detected in hippocampus as well. In this study only four specific brain regions were examined. The brain consists of lots of other parts. Therefore, it is not possible to draw a final conclusion how much zinc levels are changed in all regions of the brain caused by acute copper overload.

Copper supplemented mice which therefore were zinc deficient also showed less brain weight and cell density was reduced. This significant reduction of cell density in cortical regions was confirmed with Nissl staining which is not shown in this thesis (Baecker et al., 2014). Zinc is important for the functioning of many enzymes and transcription factors (Williams, 1989; Prasad, 2012; Oteiza and Mackenzie, 2005). Therefore it is important for the proliferation of cells, DNA and RNA metabolism, differentiation and growth and it is also involved in gene expression and apoptosis (Chesters, 1978; MacDonald, 2000). Disturbances of zinc homeostasis might lead to cell loss.

4.5 Influence of prenatal zinc deficiency caused by copper overload on zinc homeostasis and synaptic ProSAP/Shank levels *in vivo*

A dysfunctional biometal homeostasis with elevated intracellular levels of copper has been found in hair and nail samples of many ASD patients (Lakshmi Priya and Geetha, 2011). The copper/zinc ratio is increased in the blood serum of patients with autism and it was suggested to use this ratio as biomarker for patients with ASD (Faber et al., 2009). The mentioned patients, especially children, either showed a zinc deficiency or a copper toxicity. There exists a significant correlation between zinc concentration and age in the autistic patients. In the youngest children, aged 0-3 years, the lowest zinc levels in hair samples have been found (Yasuda et al., 2011; Yasuda et al., 2013; Lakshmi Priya and Geetha, 2011). The copper level in the examined autistic children could also be correlated with the severity of their symptoms. The highest copper levels were found in children with the most severe symptoms (Lakshmi Priya and Geetha, 2011). Given that ASD is a neurodevelopmental disorder, a critical time window during brain development may exist in which infantile copper overload or zinc deficiency might contribute to the pathogenesis

of autism (Yasuda et al., 2011).

Wilson's disease is characterized by high levels of copper in the liver and in the brain which in turn can lead to neurodegeneration and cognitive impairment (Bost et al., 2012; Huster, 2010). This disease where ATP7B is non-functional is quite rare. However, there exists also a form where ATP7B is only heterozygous deleted which means that these people are carriers of one defect copy. This form is not so rare (Das and Ray, 2006). High copper and low zinc levels during pregnancy can affect a developing fetus. Early development of the brain is influenced by this altered biometal homeostasis later manifesting in autism (Johnson, 2001). Interestingly, Walker et al. reported a case of a woman who, without having Wilson's disease, suffered from unexplained unusually high copper levels during two pregnancies. The woman already gave birth to two boys who had elevated copper levels. They were both diagnosed with autism later. At her third pregnancy the woman suffered again from high copper levels and also from zinc deficiency. Therefore she was treated with zinc supplements during pregnancy, which resulted in normalized zinc levels. The boy she delivered was not diagnosed with autism so far (Walker et al., 2011 and personal correspondence of Andreas M. Grabrucker with the author). This shows that copper overload/zinc deficiency may be treated with zinc supplementation.

High placental zinc concentrations lead to altered fetal copper concentrations (Barone et al., 1998). Zinc deficiency in mouse mothers induced by a zinc deficient diet leads to zinc deficiency in their offspring (Grabrucker et al., 2014). Here, it is shown that a high concentration of copper in mouse mothers also leads to abnormal fetal zinc concentrations in the brain, above all in cortex and hippocampus. The supplemented copper decreases the total amount of zinc in the brain of these pups.

In the pups' brains no increased cell loss due to necrosis, apoptosis or developmental deficits could be detected. However, there was an alteration of the molecular composition of the synapses in these pups. In zinc deficient pups a decrease of PSD proteins like ProSAP1/Shank2 and ProSAP2/Shank3 can be seen (Grabrucker et al., 2014). In this study a significant reduction of the zinc binding proteins ProSAP1/Shank2 and ProSAP2/Shank3 was observed as well in pups of copper supplemented mice. Zinc ions that are released from synaptic terminals of mossy fibers are important for the induction

of LTP (long-term potentiation) in the CA3 region of the hippocampus (Li et al., 2001). The distribution suggests that a normal zinc homeostasis in the brain is necessary for development, synaptic plasticity, neurotransmission and proper cognitive function (Li et al., 2001; Sensi et al., 2009). Therefore zinc deficiency, especially during early development, may lead to brain dysfunction and can impair thinking, attention, learning and memory (Takeda, 2000; Bhatnagar and Taneja, 2001; Grabrucker et al., 2011a).

Changed MT-1 and COMMD1 levels are possibly a compensatory regulation of changed metal ion homeostasis. In ProSAP2/Shank3 knockout animals the loss of ProSAP2/Shank3 is associated with a decrease in NMDA receptor subunit 1 (GluN1) (Bozdagi et al., 2010; Wang et al., 2011). A similar loss has been reported for prenatal zinc deficient mice and ProSAP1/Shank2 knockout mice (Grabrucker et al., 2014, Won et al., 2012). In pups from copper supplemented mice mothers we could also see a significant reduction of GluN1 levels.

Zinc directly modulates structure and function of the PSD by stabilizing the platforms built by ProSAP/Shank proteins. A depletion of zinc leads to a loss of post-synaptic localisation of ProSAP/Shank proteins and therefore to a disintegration of immature PSDs (Grabrucker et al., 2009). The reduced ProSAP/Shank protein levels seen in Western Blot analysis here seems to be caused by both, a loss of ProSAP/Shank per synapse in which ProSAP/Shank is highly expressed and a slight overall reduction of synapses. Probably mature synapses with strong PSD scaffolds are more affected. At different developmental stages all three ProSAP/Shank family members can be detected in various tissues. While ProSAP1/Shank2 and ProSAP2/Shank3 appear early at the synapse and therefore are important for synapse formation, the zinc-insensitive form Shank1 appears there last and seems to be more important for maturation (Grabrucker et al., 2011a). Therefore, probably synapse formation of the pups from copper supplemented mice was disturbed.

4.6 Conclusion

Based on the results of the work in this thesis the following conclusions can be drawn: Extracellular copper overload influences the uptake of zinc and therefore intracellular zinc levels, which in turn affect the zinc-dependent upregulation of ProSAP1/Shank2 and ProSAP2/Shank3.

The knockdown of COMMD1, a protein that is important for copper excretion, also leads to zinc deficiency, which has an influence on the synaptic scaffold proteins of the ProSAP/Shank family.

The result of acute copper overload is zinc deficiency in the brain, which can also cause a decrease of ProSAP2/Shank, above all in hippocampus. Prenatal copper overload leads to zinc deficiency in the brain of pups as well, which in turn results in a decrease of ProSAP1/Shank2 and all ProSAP2/Shank3 isoforms. Moreover, the reduced level of ProSAP1/Shank2 on protein level seems to be based on both, a slight decrease in number of synapses and a weaker scaffold of synapses especially in synapses with a strong ProSAP1/Shank2 PSD scaffold, which are probably mature synapses.

Taken together, altered zinc homeostasis by copper overload or changed levels of COMMD1 affect the autism-associated pathway at glutamatergic synapses in which ProSAP/Shank proteins and lots of other ASD candidate genes are involved. Therefore, the deregulation of important members of this pathway may represent major genetic risk factors for ASD. The interplay of zinc and copper may also be a core regulatory component of this disrupted pathway in ASD patients. Additionally, there seems to be a critical developmental time window for the effects of copper on the ASD-associated pathway by influencing zinc homeostasis.

4.7 Future perspectives

In this thesis it was shown that copper supplemented mice had significantly higher copper levels in their feces. It is not totally clear whether these high copper levels are a result of non-absorption or higher copper excretion via the bile. Therefore in future experiments it would be informative to test copper levels in intestine and liver. Most intracellular metal

ions like zinc or copper are bound to metal-binding proteins like metallothioneins (Maret, 2000). For this reason it would also be interesting to see if there is any change of MT levels in the intestine.

Repetitive behavior is one of the characteristics of autistic-like behavior (Rapin, 1997; Abrahams and Geschwind, 2008). Some of the copper supplemented mice showed hind-limb clasping as a sign of repetitive behavior. Mouse models of Rett syndrome, which belongs to the autism spectrum disorders, or ProSAP1/Shank2 knockout mice showed this behavior as well (Schmeisser et al., 2012, Won et al., 2012). Therefore it would be interesting to observe the behavior of the copper supplemented mice and of their pups more intensively. Others signs of autistic-like behavior would be abnormalities in social and vocal behaviors (Schmeisser et al., 2012; Abrahams and Geschwind, 2008).

For this thesis the pups were not tested for the total amount of zinc or copper in their blood, brains or other organs like the liver. In the future this could be tested as well. The copper or zinc content of mother's milk of copper supplemented mice compared to controls has also not been checked so far.

In the small study of Walker et al. the woman who suffered from unusually high copper levels and low zinc levels during her pregnancies was treated with zinc supplementation in her third pregnancy. The results were normalized zinc levels in the boy she delivered and he was not diagnosed with autism so far (Walker et al., 2011 and personal correspondence of Andreas M. Grabrucker with the author). This shows that zinc supplementation may rescue someone from copper overload/zinc deficiency. It would be interesting to see if such a rescue is also possible for the pups of copper supplemented mice in the beginning of their lives maybe by receiving mother's milk from mice with a normal copper/zinc ratio in the blood. Zinc and copper levels in blood, excretion products or various organs could be tested in these rescued pups as well as ProSAP/Shank or other protein levels.

5   SUMMARY

Autism spectrum disorders (ASD) are a heterogeneous group of neurodevelopmental disorders. Several mutations/deletions that have been found to be associated with ASD can be placed in a common synaptic pathway at excitatory glutamatergic synapses. Among them are mutations/deletions in genes coding for proteins from the ProSAP/Shank (proline-rich synapse-associated protein/Src homology 3 domain and ankyrin repeat-containing) family, which are important scaffold proteins at excitatory glutamatergic synapses. ProSAP/Shank proteins build platforms at the postsynaptic density (PSD), which are stabilized with the help of zinc. Zinc and copper have antagonistic roles in the body and also influence each other's absorption. Extracellular copper overload influences the uptake of zinc and therefore intracellular zinc levels *in vitro*. Furthermore, zinc-dependent upregulation of zinc-sensitive proteins like ProSAP1/Shank2 and ProSAP2/Shank3 is impaired by copper overload *in vitro*. Increased levels of copper might lead to a generally reduced bioavailability of zinc as *in vitro* they impair the usual increase of synaptic ProSAP1/Shank2 and ProSAP2/Shank3 due to neuronal activity. Acute copper overload *in vivo* results in zinc deficiency in the brain, above all in hippocampus. However, the brain is protected by the blood-brain-barrier from copper overload. In adult mice copper overload shows little influence on synaptic proteins in the whole brain, but it can lead to a decrease of ProSAP2/Shank in hippocampus. Prenatal copper overload also leads to zinc deficiency in the brain *in vivo*. This in turn results in a decrease of ProSAP1/Shank2 and all ProSAP2/Shank3 isoforms. Moreover, the reduced level of ProSAP1/Shank2 on protein level seems to be based on both, a slight decrease in number of synapses and a weaker scaffold of synapses especially in synapses with a strong ProSAP1/Shank2 PSD scaffold, which are probably mature synapses. This also indicates that there exists a critical developmental time window for the effects of copper on the ASD associated pathway. COMMD1 (Copper-metabolism gene MURR1-domain; MURR1: mouse U2af1-rs1 region 1), which is expressed in adult mice and also during CNS development, plays an important role in copper excretion and it has been found to be homozygous deleted in some ASD patients. Knockdown of COMMD1 leads to intracellular copper overload and zinc decrease *in vitro*

which in turn results in a decrease of ProSAP1/Shank2 and ProSAP2/Shank3 when there is enough zinc and copper available compared to COMMD1 overexpression. Therefore, COMMD1 can be seen as a member of the common synaptic pathway at excitatory glutamatergic synapses that was mentioned before. This pathway is also influenced by environmental factors like zinc or other metal ions like copper that in turn affect zinc homeostasis.

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ACKNOWLEDGEMENTS

Die Danksagung wurde aus Gründen des Datenschutzes entfernt.

CURRICULUM VITAE

Der Lebenslauf wurde aus Gründen des Datenschutzes entfernt.