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Developmental stage-dependent role of the COP9 signalosome in the brain

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ABSTRACT/ZUSAMMENFASSUNG

Developmental stage-dependent role of the COP9 signalosome in the brain

The COP9 signalosome (CSN) is a multifunctional protein complex that regulates protein degradation through removal of the ubiquitin-like modifier Nedd8 from cullin-based E3 ubiquitin ligases. E3 ubiquitin ligases conjugate ubiquitin to target proteins, which labels them for degradation by the proteasome. By controlling protein turnover, E3 ubiquitin ligases are involved in numerous cellular processes such as cell-cycle progression, signal transduction, transcriptional regulation, receptor down-regulation, and endocytosis. In neuronal biology the ubiquitin-proteasome system was found critically involved in axon and dendrite growth, synaptogenesis, presynaptic function, postsynaptic plasticity and postsynaptic receptor trafficking. Several studies have established that CSN is a regulator of cell cycle progression in proliferating cells. Although the significance of the ubiquitin-proteasome system as a key regulator of neuronal biology is well known, the role of CSN in neurons, i.e. postmitotic cells, has remained unexplored. In this study, we comprehensively analyzed the impact of CSN loss of function *in vitro* and *in vivo* in different periods of neuronal development by conditionally knocking down its catalytic subunit CSN5 which harbours the de-neddylase activity, via the *Cre-loxP* system. The *Cre-loxP* system is a genetic tool that enables the disruption of a target gene in a spatially and temporally restricted manner. Briefly, *loxP* sites are inserted into the gene of interest via homology-based gene targeting. The *Cre* recombinase then recognizes and efficiently catalyzes the recombination between two pairs of *loxP* sites leading to excision of the DNA fragment. By driving *Cre* expression through specific promoters it is possible to induce gene inactivation in a particular tissue or subpopulation of cells and/or a specific developmental stage. Important in the context of this work, it was previously shown that the constitutive knockdown of CSN5 is early embryonic lethal. We therefore induced *Cre* expression in a tissue and developmental stage-dependent manner by placing it under control of neuron-specific promoters. We found that early

embryonic knockdown of CSN5 restricted to proliferating neuroblasts by *Nestin-Cre* is early embryonic lethal. Interestingly, knockdown in early postmitotic neurons induced by *Nex-Cre* proved to be lethal at postnatal day 1 (P1). Extending the capabilities of the *Cre-loxP* system, additional temporal control over gene inactivation can be obtained by employing a tamoxifen-inducible *CreERT2* recombinase that is fused to the ligand-binding domain of the estrogen receptor. For a timed knockdown in mature neurons we used the tamoxifen-inducible *CaMKIIαCreERT2* which, under control of Ca2+/calmodulin-dependent kinase type II promoter, mediates loss of CSN5 in principal neurons of the forebrain. In neurological disease, both dendrites and dendritic spines, small protrusions from the dendritic tree forming synapses, are often found compromised. Consequently we analyzed these structures *in vivo*. Adult *CaMKIIαCreERT2* mice featured minor but significant reduction of dendritic length and arborization, whereas spine density on apical dendrites remained unaffected. In an additional behavioral screening including locomotor activity, hippocampus-dependent spatial learning, fear conditioning and stress-coping behavior the knockout had only minor or no effect. No conspicuous alterations of lifespan or brain anatomy were observed. To further determine the relevance of CSN for neuronal development during the embryonic stage, we induced knockdown of CSN5 in primary neurons of the developing cortex *in utero*, via the *in utero* electroporation technique. This method introduces the *Cre* recombinase into a subset of developing neuronal precursors and therefore overcomes the limitations of constitutive *Cre* expression in the very early stages of embryonic brain development. Our experiment revealed altered neuronal migratory behavior during cortex formation and stronger reduction of dendritic length and arborization compared to the previously observed effects in the adult stage. The viability of these neurons, however, was not affected. Taken together, our findings suggest that cullin-based and thus CSN-reliant regulatory mechanisms may exhibit developmental stage-dependent activity patterns in the brain.

Entwicklungsstadium-abhängige Rolle des COP9 Signalosoms im Gehirn

Das COP9 Signalosom (CSN) ist ein multifunktionaler Proteinkomplex, der den Proteinabbau mittels Entfernung des Ubiquitin-ähnlichen Proteins Nedd8 von Ubiquitin-E3 Ligasen reguliert. Ubiquitin-E3 Ligasen konjugieren Ubiquitin mit Zielproteinen, wodurch diese für den Abbau durch das Proteasom markiert werden. Weil Ubiquitin-E3 Ligasen den Proteinabbau kontrollieren sind sie an vielfältigen intrazellulären Prozessen beteiligt, wie der Zellzyklus-Progression, der Signaltransduktion, der Regulierung von Transkriptionsfaktoren und Rezeptoren und der Endozytose. Im Bereich der Neurobiologie hat das Ubiquitin-Proteasom System besondere Bedeutung für das Wachstum von Axonen und Dendriten, die Ausbildung von Synapsen, für die Funktion der Präsynapse und die postsynaptische Plastizität, sowie für die postsynaptische Verarbeitung von Rezeptoren.

Mehrere Arbeiten haben gezeigt, dass CSN ein wichtiger Regulator des Zellzyklus in proliferierenden Zellen ist. Obwohl die Bedeutung des Ubiquitin-Proteasom Systems als Schlüsselregulator in der Neurobiologie bekannt ist, blieb die Rolle von CSN in Nerven-, und damit postmitotischen Zellen noch immer unerschlossen. In dieser Arbeit haben wir ausführlich *in vitro* und *in vivo* die Auswirkungen des Funktionsverlustes von CSN in verschiedenen Phasen der neuronalen Entwicklung untersucht, indem wir seine katalytische Untereinheit CSN5 mit Hilfe des *Cre-loxP* Systems ausgeschaltet haben. Das *Cre-loxP* System ist ein genetisches Werkzeug, mit dem man ein bestimmtes Gen in einer räumlich und zeitlich festgelegten Weise ausschalten kann. Kurz zusammengefasst: Es werden *loxP*-Sequenzen in ein bestimmtes Zielgen durch homologe Rekombination eingebracht. Daraufhin erkennt eine *Cre* Rekombinase diese Sequenzen und katalysiert deren Rekombination, was zur Entfernung des DNA Fragments führt. Indem man die Expression der *Cre* durch einen gewebespezifischen Promotor steuert, kann man Gene sowohl innerhalb eines spezifischen Gewebes als auch eines bestimmten Entwicklungsstadiums ausschalten. Bedeutsam im Kontext dieser Arbeit wurde im Vorfeld gezeigt, dass sich der konstitutive Knockout von CSN5 im Embryonalstadium frühzeitig letal auswirkt. Aus diesem Grund haben wir die die Expression der *Cre* in einer Gewebetyp- und Entwicklungsstadium-abhängigen Weise herbeigeführt und unter die Kontrolle von Neuron-spezifischen Promotern gesetzt. Hierbei zeigte sich der

embryonale Knockout von CSN5 in proliferierenden Neuroblasten, induziert durch *NestinCre*, als frühzeitig embryonal letal. Der Knockout in frühen postmitotischen Neuronen, welcher durch *NexCre* vermittelt wurde, zeigte sich interessanterweise letal am ersten Tag post partum (P1). Man kann die Möglichkeiten des *Cre-LoxP* Systems dahingehend erweitern, indem man eine durch Tamoxifen induzierbare *CreERT2* verwendet, die mit dem Liganden-bindenden Bereich des Östrogenrezeptors verbunden ist, was eine zeitlich genau steuerbare Genausschaltung ermöglicht. In dieser Arbeit nutzten wir die Tamoxifen-induzierbare *CaMKIIαCreERT2,* welche unter der Kontrolle des Ca^{2+}/Calmodulin-abhängige Proteinkinase II Promotors steht, so dass der Verlust von CSN5 in adulten Projektionsneuronen des Vorderhirn resultiert. Bei neurologischen Erkrankungen zeigen sich sowohl Dendriten als auch dendritische Dornen häufig beeinträchtigt. Aus diesem Grund wurden jene Merkmale *in vivo* untersucht. Erwachsene *CaMKIIαCreERT2* Mäuse zeigten eine geringe aber signifikante Verminderung der Dendritenlänge und Verzweigung bei unveränderter Zahl der Dornen an apikalen Dendriten. Verhaltenstests zur Prüfung von Bewegungsaktivität, Hippocampus-abhängigem räumlichen Lernen, Angstkonditionierung und Stressbewältigung ergaben geringe oder keine Unterschiede. Die Mäuse zeigten darüber hinaus keine augenfälligen Veränderungen der Lebensspanne oder Neuroanatomie. Um die Bedeutung von CSN für neuronale Komplexität in früheren Entwicklungsphasen zu untersuchen, induzierten wir den Verlust in neuronalen Vorläuferzellen *in utero* mittels *in-utero*-Elektroporation. Bei dieser Methode wird die *Cre* in eine Subpopulation neuronaler Vorläuferzellen eingebracht, wodurch man die Negativeffekte einer globalen Expression der *Cre* in den sehr frühen Embryonalstadien der Hirnentwicklung umgeht. In diesem Experiment beobachteten wir ein verändertes neuronales Migrationsverhalten während der Cortexentwicklung und eine stärker ausgeprägte Reduktion der Dendritenlänge und Verzweigung als im Erwachsenenstadium. Das Überleben der Zellen war indes nicht beeinträchtigt. Zusammenfassend legen unsere Ergebnisse nahe, dass Cullin-basierte und somit vom CSN abhängige Regelmechanismen im Gehirn Aktivitätsmuster aufweisen, die vom jeweils entsprechenden Entwicklungsstadium abhängig sind.

ABBREVIATIONS

aIPC	Astrocytic intermediate progenitor cell
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
APC	Anaphase-promoting complex
ATP	Adenosine triphosphate
BBS	BES-buffered saline
BES	N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid
Bp	Basepair
BRSK2	Brain-specific kinase 2
CA1	Cornu ammonis area 1
CA3	Cornu ammonis area 3
$CaCl_2$	Calcium chloride
CaMKIIα	Calcium/calmodulin-dependent protein kinase IIα
CD	Cluster of differentiation
cdc20	Cell-division cycle protein 20
cDNA	Complementary desoxyribonucleic acid
cm	Centimeter
COP9	Constitutive photomorphogenesis 9
CP	Cortical plate
c.p.m.	Counts per minute
CPNE1	Copine1
CRL	Cullin-RING ligase
cRNA	Complementary ribonucleic acid
Cre	Cyclization recombination
CSN	COP9 signalosome
CUL	Cullin
Dab1	Disabled-1
DAPI	4',6-diamidino-2-phenylindole
dB	Decibel
DCX	Double cortin
DEN1	Deneddylase 1
DG	Dentate gyrus
DIV	Day(s) *in vitro*
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
dNTP	Desoxyribonucleotide triphosphate
DUB	Deubiquitinating enzymes
E	Embryonic day
EC	Entorhinal cortex
EDTA	Ethylendiamintetraacetate
eGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
ERT2	Tamoxifen-inducible estrogen receptor 2

E6AP	E6 associated protein (ubiquitin ligase)
FC	Fear conditioning (test)
G	Gravitational constant
GABA	Gamma-aminobutyric acid
GFP	green fluorescent protein
h	Hour
HBSS	Hank's Balanced Salt Solution
HCL	Hydrogen chloride
HECT	Homologous to the E6AP carboxyl terminus
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HPO_4	Phosphoric acid
HRP	Horseradish peroxidase
Hz	Herz (unit)
H_2O	Water
i*Cre*	Inducible *Cre*
IgG	Immunoglobulin G
IκB	Inhibitor of NFκB
i.p.	Intraperitoneal (injection)
ISH	*in situ* hybridization
IZ	Intermediate zone
Jab1	c-Jun activation domain-binding protein-1
JAMM	Jab1/MPN/Mov34 metalloenzyme
KCl	Potassium chloride
kHz	Kiloherz
KH_2PO_4	Monopotassium phosphate
KO	Knockout
LGE	Lateral ganglionic eminence
LIS1	Lissencephaly 1
loxP	Locus of crossover [x] of P1
LTP	Long term depression
Lys	Lysine
M	Mole (unit)
MAP	Microtubule-associated protein
mg	Milligramme
$MgCl_2$	Magnesium chloride
MGE	Medial ganglionic eminence
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
ms	Milisecond(s)
mTOR	Mammalian target of rapamycin
MZ	Marginal zone
µm	Micrometer
NA	Numerical aperture
NaCl	Sodium chloride
NAE	Nedd8-activating enzyme
NaOH	Sodium hydroxide

NE	Neuroepithelium
Nedd8	neural precursor cell expressed, developmentally down-regulated
NEDP1	Nedd8-specific protease 1
NFκB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
nIPC	neuronal intermediate progenitor cell
nm	Nanometer
NSC	Neural stem cell
n.s.	Not significant
OF	Open field (test)
oIPC	Oligodendrocytic intermediate progenitor cell
o.n.	Overnight
OTU	Ovarian tumor (superfamily of ubiquitin isopeptidases)
P	Postnatal day
p	P-value (for statistical analysis)
PBS	Phosphate buffered saline
PBS(-T)	Phosphate buffered saline (with Triton X-100)
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
pH	*Potentia Hydrogeni* (value of acidity)
PINK1	PTEN-induced kinase 1
PP	Preplate
PS	Pial surface
PSD	Post-synaptic density
PSD 95	Post-synaptic density protein 95
PVC	Polyvinyl chloride
PVDF	Polyvinylidene fluoride
RFP	Red fluorescent protein
RGC	Radial glial cell
RING	Really interesting new gene
RIPA	Radioimmunoprecipitation assay (buffer)
RM-ANOVA	Repeated measures analysis of variance
RNA	Ribonucleic acid
RT	Room temperature
s	Second
SAD	Synapses of amphids defective
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	Second
SEM	Standard error of the mean
SENP8	Sentrin-specific protease 8
SP	Subplate
SSC	Saline-sodium citrate buffer
SVZ	Subventricular zone
TAE	Tris acetate with EDTA
TAM	Tamoxifen
TBS(-T)	Tris buffered saline (with Tween)
Thy1	Thymocyte differentiation antigen 1
Tris	Tris[hydroxymethyl]aminomethane

TUBA1A	Tubulin α 1A
U	Unit(s)
Ub	Ubiquitin
Uba	Ubiquitin-activating enzyme
Ubc	Ubiquitin-conjugating enzyme
UCH	Ubiquitin C-terminal hydrolase
UPS	Ubiquitin proteasome system
USP	Ubiquitin-specific protease
UTP	Uridine-triphosphate
UV	Ultraviolet
v	Volt
v/v	Volume per volume
VZ	Ventricular zone
WB	Western blot
WCM	Water-cross maze
WT	Wild-type
w/v	Weight per volume

1. INTRODUCTION

1.1 Neuronal morphology is changed in neurological and psychiatric disease

The ability of neurons to form distinct processing pathways provides the fundament of the brain`s integrative function. The finding that neurons pass on information through unidirectional flow of electrical activity upon establishment of specific connections among each other has shaped our concept of neuronal circuits. Axons and dendrites, elaborate multiform processes arising from the cell body, constitute the structural links in these circuits. Accordingly, damage or misformation of these structures may impair the functioning of the brain as a whole. Both dendrites and dendritic spines, small protrusions from the dendritic tree forming synapses, have been found compromised in disorders associated with intellectual disability (Kaufmann and Moser 2000; Fiala, Spacek, and Harris 2002; Penzes et al. 2011; Emoto 2011). Dendritic alterations and spine loss occur early in Alzheimer´s disease, were found to correlate with symptoms and were reproducible in animal models (Knobloch and Mansuy 2008; Scheff et al. 2006; Spires et al. 2005; Tsai et al. 2004; Luebke et al. 2010; Duyckaerts, Potier, and Delatour 2008). Similar findings were made in Parkinson disease (Day et al. 2006). In Huntington´s disease and its animal models, both dendritic and spine morphology was found to be altered (Ferrante, Kowall, Richardson, 1991; Spires et al., 2004). Typical findings include altered dendritic branching patterns, reduced dendritic diameters, decreased dendritic length and immature, stubby spines (Sorra and Harris 2000; Swann et al. 2000; Martone, Hu, and Ellisman 2000; Nimchinsky, Oberlander, and Svoboda 2001). These observations, among others, have established the analysis of dendritic and spine morphology as an important method to determine the contribution of genetic defects to altered neuronal circuitry found in disease or injury.

1.2 Development of dissociated neurons in culture

Modelling neuronal development *in vitro* has become an essential and widely used approach. Artificially induced changes in the environment in which neurons are left to grow (Oorschot 1989; Shen and Schwartzkroin 1988; Fields, Yu, and Nelson 1991; Yair, Weichsel Jr., and de Vellis 1986; Fallon 1985) and manipulations performed on the cell directly (Goslin and Banker 1989; Carlos G. Dotti and Banker 1987) give insights into the adaptability of neurons. Genetic manipulations on cells such as transfection experiments or virus-mediated gene transfer reveal a lot about gene expression (Zeitelhofer et al. 2007; Anliker et al. 2010; Ailles and Naldini 2002; Yuzhi Zhang et al. 2006; Jiang and Chen 2006), whereas patch clamp techniques and electrophysiological recordings allow for study of their electrophysiological properties (Segal 1983; van Pelt et al. 2004; Maeda et al. 1998; Segev et al. 2002; Gross and Kowalski 1999; Jimbo, Tateno, and Robinson 1999; Beggs and Plenz 2003). Approaches to modelling neuronal development *in vitro* include the preparation of brain slices (Schwartzkroin 1975; Schwartzkroin 1977; Caeser, Bonhoeffer, and Bolz 1989; Wenzel et al. 1994; Dailey and Smith 1996) and the establishment of cell cultures (Banker and Cowan 1977; Banker and Cowan 1979; Huettner and Baughman 1986; Peacock, Rush, Daphne, and Mathers 1979; Kaech and Banker 2006). In the brain slice which is between 70 and 400 µm thick and usually obtained from an adult brain some of the intrinsic circuitry of the brain is preserved, thus a more realistic model of the brain is represented. However, the complete visualization of an individual cell can be difficult and remains a challenge. Cell cultures, on the other hand, allow for convenient visualization of individual cells up to the subcellular localization of their cell organelles and proteins. The method of growing dissociated hippocampal neurons in a primary neuronal cell culture established by Banker & Cowan (1977) proved especially useful for modelling neuronal development *in vitro* and now serves as a blueprint for generation of most primary neuronal cell cultures.

Primary neuronal cell cultures are based on post-mitotic, developing neurons that were previously extracted from the mouse or rat embryonic brain. The most common extraction site is the hippocampus or the cortex because of its rather uniform cellular

composition, consisting primarily of glutamatergic pyramidal neurons (~90%). Interneurons account only for a minority (~10%) and glial cells appear in very low numbers (Bayer 1980a; Bayer 1980b; Soriano et al. 1994). *In situ* pyramidal neurons are characterized by an elaborate dendritic tree which features a robust apical-basal polarity, high spine density and distinct domains for synaptic input (Spruston 2008). Pyramidal neurons of the cortex and hippocampus, once cultivated *in vitro*, develop a characteristic polarized morphology with wide dendritic networks following similar developmental steps as their counterparts *in vivo*, which makes them of great appeal for studies of neuronal development (Yavin and Yavin 1977; Huettner and Baughman 1986; Ogura et al. 1987; Carlos G. Dotti, Sullivan, and Banker 1988; Ichikawa et al. 1993; Dailey and Smith 1996). Primary neuronal cell cultures have been in use for over 20 years and thus constitute reliable, extensively studied systems for modelling neuronal development *in vitro.* Dotti et al. (1988) identified five developmental stages in which an undifferentiated, post-mitotic pyramidal neuron, grown in culture, polarizes and develops a distinct somatodendritic and axonal domain (Figure 1).

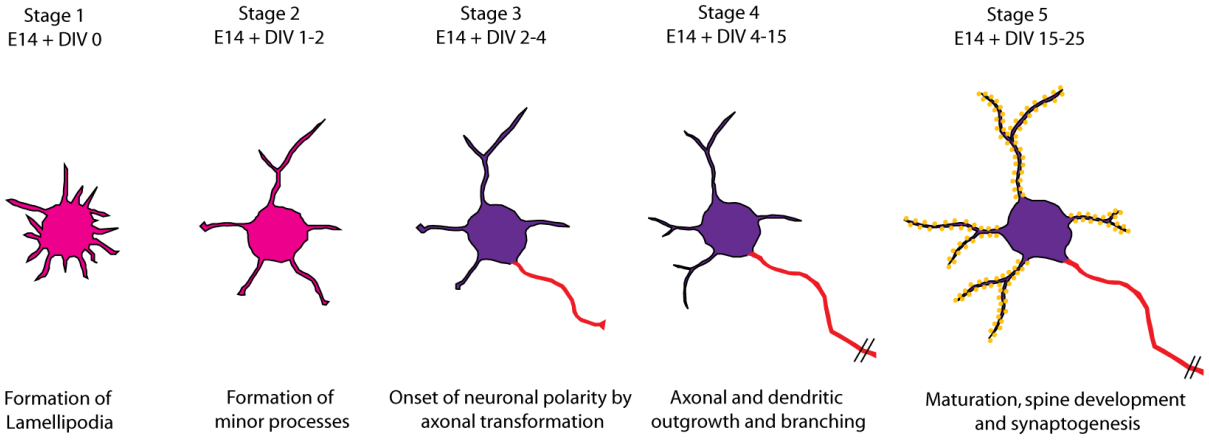


Figure 1: Development and polarization of pyramidal neurons *in vitro*

Postmitotic primary neurons that were extracted at embryonic day 14 (E14) from mouse or rat hippocampus or cortex and left to grow in culture develop a characteristic polarized morphology by transitioning though 5 stages.
Stage 1 (day in vitro (DIV) 0): briefly after attachment to the substrate, lamellipodia which are short and dynamic protrusions from the soma form around the cell´s periphery.
Stage 2 (DIV 1-2): some of the lamellipodia transform into distinct immature minor processes.
Stage 3 (DIV 2-4): establishment of neuronal polarity by transformation of one minor process into the axon (red).
Stage 4 (DIV 4-15): rapid axonal and dentritic outgrowth and branching.
Stage 5 (DIV 15-25): maturation, increased branching, establishment of dentritic spines (orange dots) and functional synapses.
(Adapted from Dotti et al., 1988; Barnes and Polleux, 2009)

Stage 1 is characterized by lamellipodia which are short and dynamic protrusions from the soma that form around the cell´s periphery. During stage 2, some of the lamellipodia transform into distinct minor processes, extending to a length of 10 – 15 µm and remain stable once they have attained this size. Stage 3 marks the onset of neuronal polarity as one minor process begins to grow at much higher speed (5 – 10 times) than the other processes of the cell. That process becomes the future axon whereas the remaining stationary processes will become dendrites. The beginning of dendritic outgrowth characterizes stage 4 and occurs 2-3 days after axonal outgrowth. Stage 5 represents the maturation of the neuron characterized by increased branching of the axon and dendrites and by the establishment of dendritic spines and functional synapses.

Dendritic growth is different from axonal growth. First, dendrites elongate much slower, at rates of 12 µm per day whereas axons grow up to 60 µm per day. Second, dendritic growth is continuous, while but axons grow at varying speed. Also, axons are stationary for a more considerable amount of time than they are actively growing (Carlos G. Dotti, Sullivan, and Banker 1988).
Dendritic and axonal branching patterns, too, differ in several respects. Dendrites emerge from rather broad bases, branch in small, Y-shaped angles and continuously taper towards their endings, with every daughter branch being significantly smaller in diameter than its parent. Axons on the other side usually emerge from thin bases, branch in right angle collaterals and show little decrease in diameter towards their tips. Their length often reaches multiples of an individual dendrite (Bartlett and Banker 1984).

Both dendrites and axons feature prominent and highly motile growth cones at their tips. These bulbous structures contain sensor proteins that translate extracellular guidance cues into intracellular messaging pathways, thus steering the extension of the neurites (Dent, Gupton, and Gertler 2011; Landis 1983; O'Donnell, Chance, and Bashaw 2009; Polleux and Snider 2010; Gordon-Weeks 2003; Gallo and Letourneau 2004; Smalheiser 1990; Goodman 1996).

These observations have proven that the establishment of neuronal polarity, i.e. the asymmetric organisation of structural components and functional properties within the cell, takes place early in cellular development. Neuronal polarity provides the fundament of the unidirectional flow of information within neurons and determines cellular features as diverse as cell organelles (Horton & Ehlers 2003; Ye et al. 2007; Horton et al. 2005), cytoskeletal proteins (Craig and Banker 1994; Lewis, Courchet, and Polleux 2013; Baas, Black, and Banker 1989) and signalling cascades (Barnes and Polleux 2009; Polleux and Snider 2010). By using antibodies and taking advantage of the characteristic distribution of the respective components it has become possible to identify and characterize different cellular compartments (C. G. Dotti, Banker, and Binder 1987). Furthermore, it has become possible to visualize the complete morphology of specific neuronal subtypes and glial cells *in situ* by employing genetic constructs containing endogenously expressed fluorescent proteins (Zhuo et al. 1997; G. Feng et al. 2000; Nolte et al. 2001; Suzuki et al. 2003).

Dendrites constitute the recipients in neuronal signalling. Their morphology proves crucial for proper integration of the axonal input they receive (London and Häusser 2005). Dendritic geometry acts in concert with voltage-gated ion channel density and kinetics for action potential propagation (Vetter, Roth, and Häusser 2001). Similarly, dendritic morphology exerts significant influence on firing modes of pyramidal cells (van Elburg and van Ooyen 2010). The ramified dendritic trees are established and maintained through a complex regulation by transcription factors and intrinsic and extrinsic cues (Parrish et al. 2007).

1.3 Dendritic spines

Spines are bulbous structures that appear along dendrites during neuronal development *in vivo* and *in vitro* (Figure 2). They function as recipients for the input from axonal terminals when neurons establish contact among each other via the formation of excitatory synapses. Inhibitory synapses do not exhibit spine formation, although sometimes both inhibitory and excitatory connections are made onto the same spine. Dendritic spines protrude from the post-synaptic membrane of the receiving dendrite. They typically comprise a bulbous spine head which contains the post-synaptic density (PSD), an electron-dense region accumulating many neurotransmitter receptors, scaffold and signalling proteins (Kennedy 1998; Walikonis et al. 2000; Racca et al. 2000; Ziff 1999; Jee Hae Kim and Huganir 1999). Their head is separated from the dendrite by a thin spine neck. Nevertheless, shapes often vary including thin, stubby, mushroom-like spines as well as filopodia (Parnass, Tashiro, and Yuste 2000; Harris and Stevens 1989; Harris, Jensen, and Tsao 1992). There have been promoted three models of spine formation along dendritic shafts (Yuste and Bonhoeffer 2004). In the Sotelo model, spines emerge independently of the axonal terminal from the dendritic shaft. In the Miller/Peters Model the axonal terminal actively induces the formation of the spine whereas in the filopodium model, dendritic filopodia capture an axonal terminal and, once the contact is maintained, mature into spines (Fiala et al. 1998; Ziv and Smith 1996; Petrak, Harris, and Kirov 2005). In fact, it was shown that dendritic spines are highly motile and dynamic structures that can rapidly change their shape and number during lifetime (Harris 1999; Sala, Cambianica, and Rossi 2008; W. Zhang and Benson 2000; Smart and Halpain 2000). Although not present in all neuronal subtypes they proved characteristic for pyramidal neurons of the cortex and hippocampus and are thought to be the morphological basis for synaptic plasticity associated with learning and memory formation (Segal 2005; Yuste and Bonhoeffer 2001).

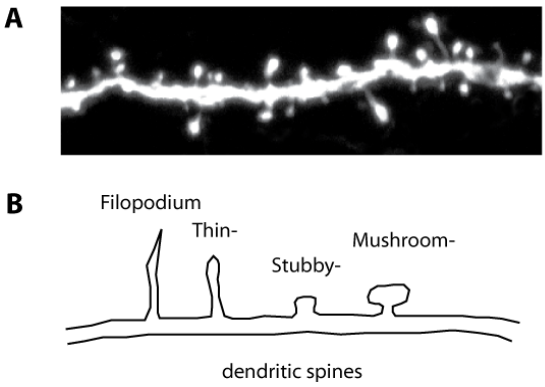


Figure 2: Dendritic spines

A) Representative image depicting spines of a cortical layer V pyramidal neuron acquired with confocal microscopy.
B) Dendritic spines exhibit a variety of shapes including filopodia, thin-, stubby- and mushroom-shaped spines.

1.4 Organisation of the neocortex

The mammalian neocortex constitutes the most peripheral region of the brain and covers the two cerebral hemispheres. In rodents such as mice, the neocortex is smooth, whereas in primates it is folded into a gyrencephalic configuration, which increases its surface area (Hofman M A, 1985). Exclusively found in mammals, it is after archicortex and paleocortex the evolutionary most recently acquired brain structure (Medina & Abellán, 2009).

The neocortex receives inputs from the thalamus, the contralateral hemisphere and other structures. Its outputs target the neocortex, basal ganglia, thalamus, pontine nuclei, and the spinal cord. The cellular composition and organization of the neocortex differs significantly from subcortical regions: neurons make up the majority of the cells in the neocortex, with glial cells accounting only for a minority (Bayer 1980a; Bayer 1980b; Soriano et al. 1994). Based on morphology only, i.e. the distribution of their dendrites and axons, over 40 different neuronal cell types have been identified (Lorente de Nó R, 1949). Broadly classified, they comprise two groups: principal (projection) neurons or local interneurons (Nieuwenhuys 1994). Projection neurons make up roughly 80% of all cortical neurons and typically have pyramid-shaped cell bodies. They carry many spines along their dendrites and use glutamate as their primary transmitter. Outside the cortex they appear in the hippocampus and the amygdala. Interneurons constitute approximately 20% of the cortical neurons and have axons that remain close to their cell body. They employ

the inhibitory neurotransmitter γ-aminobutyric acid (GABA), feature a diverse morphology but do not carry spines on their dendrites.

As opposed to subcortical regions that have a nuclear organization, the neocortex is organised in layers (Figure 3). This laminar organisation efficiently structures the complex input-output relationships as each layer receives inputs from and sends outputs to specific cortical and subcortical regions. Based on the thickness of individual layers, their cell size and packing characteristics the neocortex can be subdivided into over 50 areas (Brodmann K, 1909).

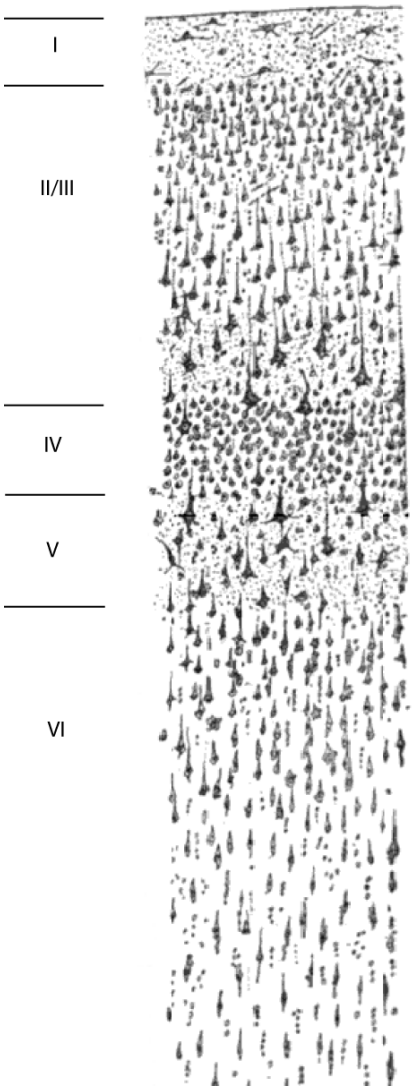


Figure 3: Laminar organisation of the neocortex

Drawing of neocortical layers adopted from Ramón y Cajal (Histologie du système nerveux de l'homme et des vertébrés 1909) based on Nissl staining of the human parietal cortex.

Layer I, the *molecular layer,* contains the dendrites and axons of deeper residing neurons that travel within this layer to form intracortical connections. No pyramidal neurons are found in this layer.

Layer II, the *external granular cell layer,* is composed of mainly small pyramidal or spherical neurons that lie tightly packed next to one another.

Layer III, the *external pyramidal cell layer*, contains pyramidal neurons that are larger than those in layer II and gradually increase in size. Axons emerging from layers II and III project locally as well as to other cortical areas, comprising the majority of association and commisural fibers.

Layer IV, the *internal granular cell layer*, contains many pyramidal and non-pyramidal cells and represents the main recipient of thalamocortical afferent fibers, being most prominent in primary sensory areas. Primary motor areas are almost absent in this layer.

Layer V, the *internal pyramidal cell layer*, is characterized by large pyramidal neurons that send projections to other cortical areas as well as to subcortical structures, constituting the major output pathway of the cortex. Layer V is most prominent in the primary motor cortex.

Layer VI, the *multiform layer*, is heterogeneously composed and marks the border between grey and white matter. Its neurons, mostly small pyramidal and non – pyramidal cells, send projections to other cortical areas, the thalamus and other subcortical regions.

In addition to its laminar organisation, the neocortex features columns that are approximately 300 – 600 µm thick, transverse cortical layers and reach far into the white matter, constituting a local processing network (Mountcastle 1997; E. G. Jones 2000; Oberlaender et al. 2012; Helmstaedter, Sakmann, and Feldmeyer 2009).

1.5 Embryonic development of the neocortex

The embryonic development of the neocortex is driven by the proliferation of neural stem and progenitor cells and their subsequent differentiation into neurons and glial cells (Borrell & Reillo, 2012; Götz & Huttner, 2005; Hansen, Lui, Parker, & Kriegstein, 2010; Kriegstein & Alvarez-Buylla, 2009; Lui, Hansen, & Kriegstein, 2011; Martínez-Cerdeño, Noctor, & Kriegstein, 2006; Noctor, Martínez-Cerdeño, & Kriegstein, 2008; Noctor, Martínez-Cerdeño, Ivic, & Kriegstein, 2004; Taverna, Götz, & Huttner, 2014) (Figure 4).

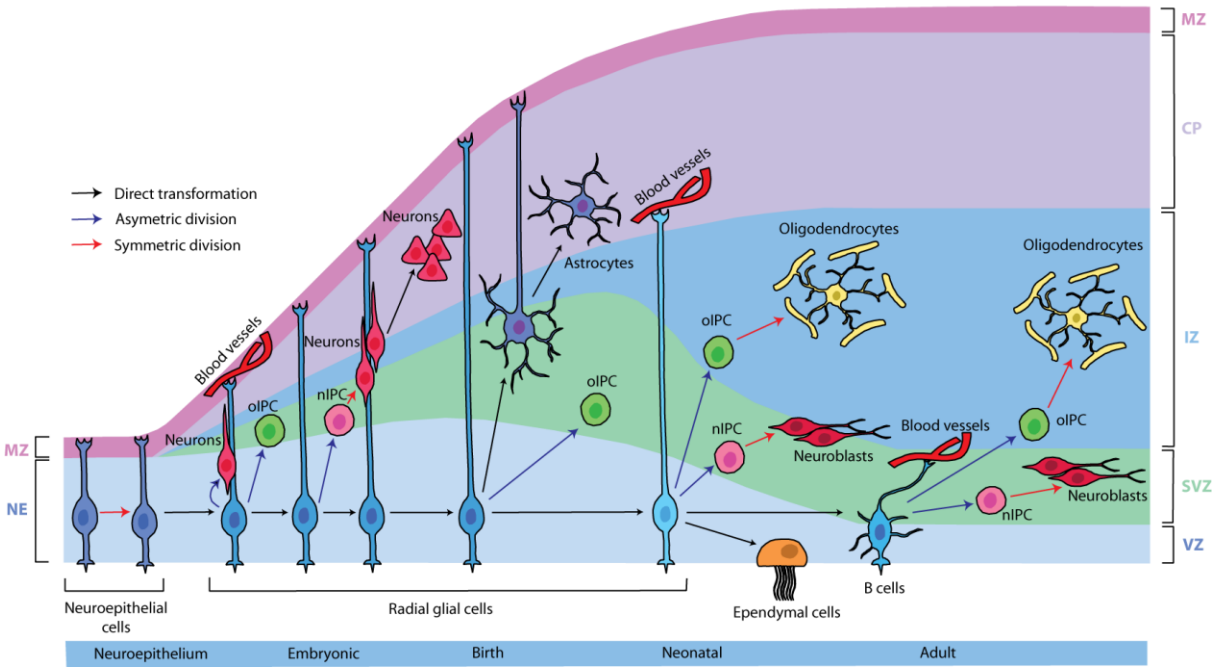


Figure 4: Neurogenesis during cortical development

Early in cortical development the thickness of the neuroepithelium increases through symmetric division of neuroepithelial cells.
Once a critical thickness is reached, some of these cells elongate and convert into radial glial cells (RGCs).
RGCs function as neural stem cells (NSCs) which devide asymetrically to generate neurons directly or indirectly through neurogenic intermediate progenitor cells (nIPCs) in the ventricular (VZ) and subventricular zone (SVZ), the cell-dense germinal center of the developing cortex.
Neurons migrate along RGC processes into the cortical plate (CP). RGCs feature an apical – basal polarity, making contact with the ventricle (apical) and the meninges, basal lamina and blood vessels (basal).
They also generate oligodendrocytes through oligodendrocytic intermediate progenitor cells (oIPCs) and astrocytes through direct transformation or through astrocytic intermediate progenitor cells (aIPCs, not shown).
In the neonate stage, some RGCs continue to generate neurons and oligodendrocytes, some convert into ependymal cells and some tranform into B cells (SVZ astrocytes) that continue to function as NSCs in the adult. B cells feature apical contact at the ventricle and basal endings on blood vessels.
Colors depict symmetric, asymmetric, or direct transformation. IPC, intermediate progenitor cell; CP, cortical plate; IZ, intermediate zone; MZ, marginal zone; NE, neuroepithelium; nIPC, neurogenic progenitor cell; oIPC, oligodendrocytic progenitor cell; RG, radial glia; SVZ, subventricular zone; VZ, ventricular zone. (Adapted from Kriegstein and Alvarez-Buylla, 2009.)

In the early stages of embryonic development, the thickness of the neuroepithelium increases through symmetric division of neuroepithelial cells which are of ectodermal origin. Once a critical thickness is reached, some of these cells elongate and convert into radial glial cells (RGCs). RGCs function as neural progenitor or neural stem cells (NSCs) and, by asymmetric division, generate both neuronal and glial cells in the ventricular (VZ) and subventricular zone (SVZ), the cell-dense germinal center of the developing cortex. They feature an apical – basal polarity, making contact with the ventricle (apical) and the meninges, basal lamina and blood vessels (basal). During most of the time their cell bodies remain close to the ventricles in the ventricular zone. During proliferation they undergo interkinetic nuclear migration, a manoeuvre likely involved in regulating neurogenesis by modulating the exposure of RGC nuclei to neurogenic or proliferative signals. To maintain ventricular zone integrity they establish adherent junctions, a feature which also carries importance for RGC behaviour.

RGCs feature two modes of cell division: (a) symmetric, which increases the cellular density of the ventricular zone by generation of two daughter cells that share the same identity with one another and (b) asymmetric, which generates two cells of different identities. If one daughter cell shares the same identity with its mother cell, the division is called self-renewing. In consumptive divisions the daughter cells acquire identities different from their mother cell (Figure 5).

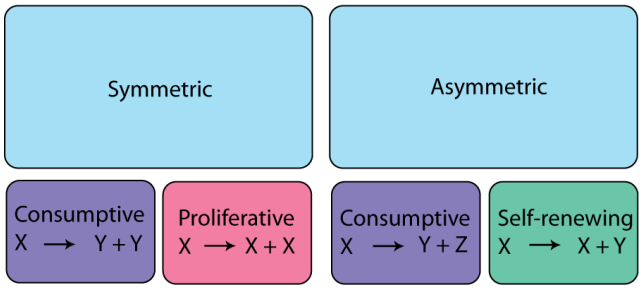


Figure 5: Modes of cell division of neural stem and progenitor cells

Symmetric divisions, either proliferative or consumptive, generate two daughter cells that share the same identity with each other. Asymmetric divisions, either self-renewing or consumptive, generate daughter cells of different identities (adapted from Taverna, Götz and Hutter, 2014).

These modes of division play an important role in increasing the size and thickness of the cortical plate and in increasing the variety of cell types such as intermediate progenitor cells (IPCs) which further on transform into neurons and glial cells.

After their generation from RGCs, neurons start migrating along RGC processes to reach their final position in the cortex. Upon reception of a signal from Cajal–Retzius cells they stop migration, distribute horizontally in the cortical plate and begin their differentiation into mature neurons. The arrangement of neurons in cortical layers occurs in an inside-out fashion, which means neurons reach and build up the lower layers first and the upper layers last (Figure 6). The correct execution of these steps critically relies on a genetic programme (Gupta, Tsai, and Wynshaw-Boris 2002). One of the best characterized genes in this process encodes the extracellular protein reelin, which is secreted from Cajal-Retzius cells. Its absence is associated with a characteristic and reproducible cortical malformation, an outside to inside gradient of the cortical layers (Lakomá, Garcia-Alonso, and Luque 2011; Lambert de Rouvroit and Goffinet 2001; Bar and Goffinet 1999; Tissir and Goffinet 2003; Hartfuss et al. 2003).

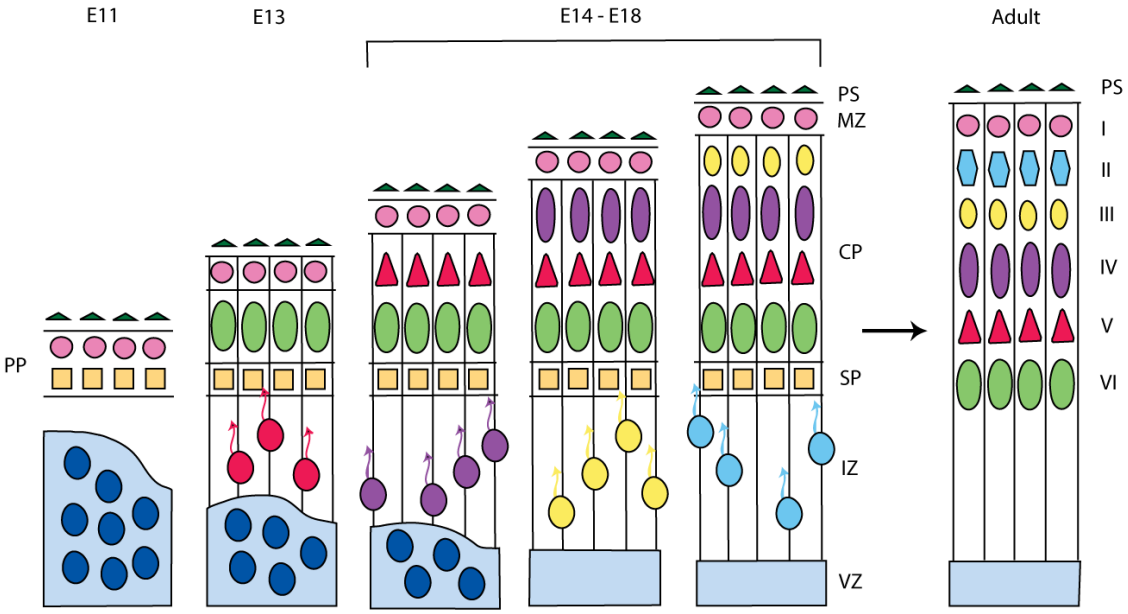


Figure 6: Layer formation in the developing neocortex

The organisation of the adult neocortex into distinct neuronal layers. Key developmental stages (embryonic day, E) of the radial component of neocortical layer formation are shown, which occurs mainly by migration along radial glia (shown as vertical bars). At E11, the preplate (PP) is established by a postmitotic wave of neurons that has migrated from the ventricular zone (VZ) to the pial surface (PS). By E13, a second postmitotic neuronal wave has migrated through the intermediate zone (IZ) and splits the PP into the marginal zone (MZ) and subplate (SP), creating the cortical plate (CP). During E14–E18, subsequent waves of neurons expand the CP in an inside-out fashion, as each wave of neurons passes its predecessors to settle underneath the MZ. In adulthood, the SP degenerates, leaving behind a six-layered neocortex (layers I - VI). (Adapted from Gupta et al., 2002.)

Principal neurons and interneurons, although resting in close proximity with one another in the adult cortex, originate in different locations: the dorsal forebrain and the medial (MGE) and lateral (LGE) ganglionic eminence of the ventral telencephalon, respectively. Their modes of migration, however, were shown to vary greatly (Figure 7). Principal neurons migrate radially whereas interneurons migrate tangentially into the cortex (Tan et al. 1998; Marín and Rubenstein 2003; Hippenmeyer 2014).

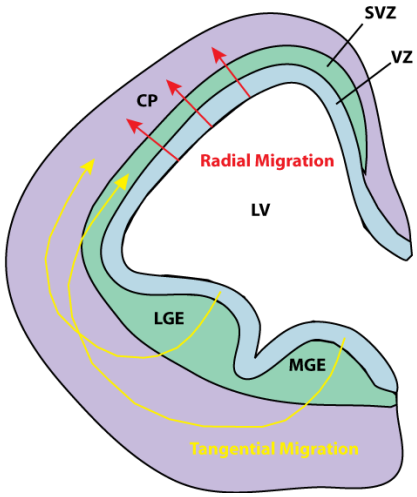


Figure 7: Diagram of the complex migratory pathways of excitatory and inhibitory neurons in the developing brain

Schematic drawing of an E14 mouse embryo forebrain hemisection. Excitatory projection neurons arise from the dorsal regions of the ventricular zone (VZ) adjacent to the lateral ventricle (LV) and migrate radially into the cortical plate (CP).
GABA-ergic interneurons are born in the VZ and subventricular zone (SVZ) of the medial (MGE) and lateral (LGE) ganglionic eminence and migrate tangentially into the CP. The paths of the radial migrants are shown in red, those from the tangential migrants are shown in yellow. (Adapted from Nguyen et al., 2014.)

The embryonic development of the cerebral cortex is a complex process and tightly regulated by a series of gene expression cascades (Guillemot et al. 2006). After migration is completed a complex series of apoptotic and synaptogenic events finely regulates the number of mature neurons and their connections, which ultimately shapes neuronal circuitry.

1.5.1 Disruption of cortex formation causes neurological disease

Mutations in key genes controlling neuronal proliferation, migration and postmigrational development have been linked to a number of neurological diseases (Barkovich, Guerrini, Kuzniecky, Jackson, & Dobyns, 2012; Bozzi, Casarosa, & Caleo, 2012; Morris, Efimov, & Xiang, 1998; Valiente & Marín, 2010).

For instance, it has been shown that mutations in lissencephaly 1 (LIS1), double cortin (DCX) and tubulin α 1A (TUBA1A) cause lissencephaly, a congenital malformation of the cortex characterized by a smooth or misfolded brain surface and a loosely arranged four-layer cortex (Kerjan & Gleeson 2007; Pilz, Matsumoto, et al. 1998; Keays et al. 2007; Des Portes et al. 1998; Reiner et al. 1993; Pilz, Macha, et al. 1998; Dobyns et al. 1996; Dobyns & Truwit 1995).

Furthermore, it was found in some cases of idiopathic epilepsy that congenital migration defects lead to the development of cortical heterotopias which constitute locations of origin for seizures (Bozzi et al. 2012). These findings underline the importance of sound cortical development for the functioning of the brain as a whole.

1.6 The hippocampus: a brain structure well suited for studies of morphology and behavior

The hippocampus is a brain structure that features a highly ordered cytoarchitecture composed of well-confined cell types: spine-rich pyramidal neurons in CA1 and CA3 regions, granule neurons in the dentate gyrus and few interneurons (Bayer 1980a; Bayer 1980b; Benson et al. 1994; Soriano et al. 1994). By employing fluorescent dyes and high resolution microscopy it allows for studying neuronal morphology *in vivo*. Its well characterized tri-synaptic circuit (Figure 8) constitutes a local processing network (Amaral and Witter 1989) and is involved in memory storage and spatial navigation (Reed and Squire 1997; Rempel-Clower et al. 1996; Milani et al. 1998; Neves et al. 2012). These cognitive functions can be evaluated through specific behavioral tests, as done in this work.

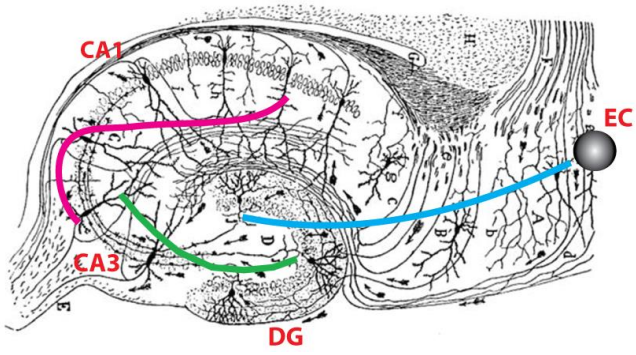


Figure 8: Organisation of the hippocampus

Pyramidal neurons reside in the CA1 and CA3 regions whereas the dentate gyrus, a neighboring region, consists mostly of granule neurons.
The tri-synaptic hippocampal circuit is made up of three excitatory projections and involves the CA1 and CA3 regions, the dentate gyrus (DG) and the entorhinal cortex (EC). The perforant path (blue) emerges from the EC to innervate the DG. The mossy fibers (green) connect the DG with the CA3 region of the hippocampus and the Schaffer collaterals (pink) excite the CA1 region. CA1 cells project outside the hippocampus, through the subiculum and entorhinal cortex to several cortical and subcortical areas.
(Adapted and modified from Ramón y Cajal, 1911.)

An intact hippocampus was shown to be a crucial requirement for spatial flexibility in mice. Spatial navigation is essential for rodents to locate food, social partners, and shelter. It results from two very different strategies: (1) place learning which enables for flexible navigation using environmental information and (2) response learning that employs a more rigid “route following” (Kleinknecht et al. 2012).

Place learning is a hippocampus-dependent navigation strategy, characterized by the incorporation of environmental cues into a cognitive map to locate a target (Morris et al. 1982; O´Keefe et al. 1975; Dupret et al. 2010; Gutiérrez-Guzmán et al. 2011). In an experimental setup, it is independent of the starting position of the subject and is thus considered flexible. Response learning, in contrast, is based on stimulus-response guided navigation, depends on the starting position (therefore considered less flexible) and requires intact basal ganglia (Mcdonald and White 1994; Packard and McGaugh 1996; Brioni, Nagahara, and McGaugh 1989; Jacobson, Gruenbaum, and Markus 2012; Tzavos, Jih, and Ragozzino 2004).

1.7 The ubiquitin proteasome system

Cellular protein turnover and function is controlled in a pathway known as ubiquitylation. Ubiquitin is a small, 76-amino-acid peptide named for its universal biological presence. It is attached to target proteins as a post-translational protein modification in an evolutionary conserved and tightly regulated enzymatic cascade and thereby either changing the target´s function or causing its proteasome-mediated degradation (reviewed in Hershko & Ciechanover 1998).

Interest in its role in the brain was originally sparked by the discovery of ubiquitin in proteinaceous deposits in neurodegenerative disorders (Kuzuhara et al. 1988; DiFiglia et al. 1997; Ross and Poirier 2004; Ross and Pickart 2004; Q. Ding, Cecarini, and Keller 2006). Neurofibrillary tangles of Alzheimer´s disease, Lewy bodies of Parkinson´s disease, and Pick bodies of Pick´s disease all proved to be immunopositive for ubiquitin (Lennox et al. 1988; Lowe et al. 1988; H. Mori, Kondo, and Ihara 1987). Since that time the ubiquitin-proteasome system (UPS) has been found altered in numerous diseases of the central nervous system (Ciechanover and Brundin 2003; Tai and Schuman 2008; M. Ding and Shen 2008; Schwartz and Ciechanover 2009; Zheng et al. 2016). The UPS was shown to critically control neuronal development, axon and dendrite growth, synaptogenesis, presynaptic function, postsynaptic plasticity and postsynaptic receptor trafficking (reviewed in Yi & Ehlers 2005; Kawabe & Brose 2011; Yi & Ehlers 2007; Segref & Hoppe 2009). Neurons in particular, given their highly polarized cellular organization, rely on the UPS for creating, maintaining and dismantling their cellular protein subdomains.

1.7.1 Components of the ubiquitin proteasome system

Ubiquitin is synthesized as an inactive precursor, processed and then transferred to its substrates in a pathway involving three classes of enzymes (called E1, E2 and E3) (Figure 9). The procession of the ubiquitin precursor involves specific proteases called deubiquitinating enzymes (DUBs) that expose the glycine carboxylate which is the site of substrate conjugation at its carboxyl terminus (Jung Hwa Kim et al. 2003;

Amerik and Hochstrasser 2004). Subsequently, a specific activating enzyme (E1) uses ATP to adenylate ubiquitin at its carboxyl terminus. The resulting high-energy mixed anhydride bond attracts the sulfhydryl group of the E1 active-site cysteine, forming a high-energy thioester bond between the E1 and ubiquitin, expelling AMP. Ubiquitin is then passed to the active-site cysteine of an ubiquitin conjugating enzyme (E2), likewise forming a thioester bond. It is then conjugated to its substrate with help of an E3 ubiquitin ligase, resulting in the covalent isopeptide bond of the ubiquitin carboxyl terminus to the ε-amino group of a lysine in the substrate. The target specificity of ubiquitylation is warranted by large number E3 ligases (e.g. more than 600 in humans), most of which belong to two major families, distinguished by homologous to E6-AP carboxyl terminus (HECT) or really interesting new gene (RING) domains (Deshaies and Joazeiro 2009; Petroski and Deshaies 2005; Rotin and Kumar 2009; Glickman and Ciechanover 2002). If the E3 enzyme belongs to the RING finger family of ligases, ubiquitin is transferred directly from the E2 enzyme to the target substrate. In the case of a HECT domain-containing ligase, the activated ubiquitin moiety is transferred first to the E3, to generate yet another high-energy thiol ester intermediate, before it is transferred to the E3-bound target substrate.

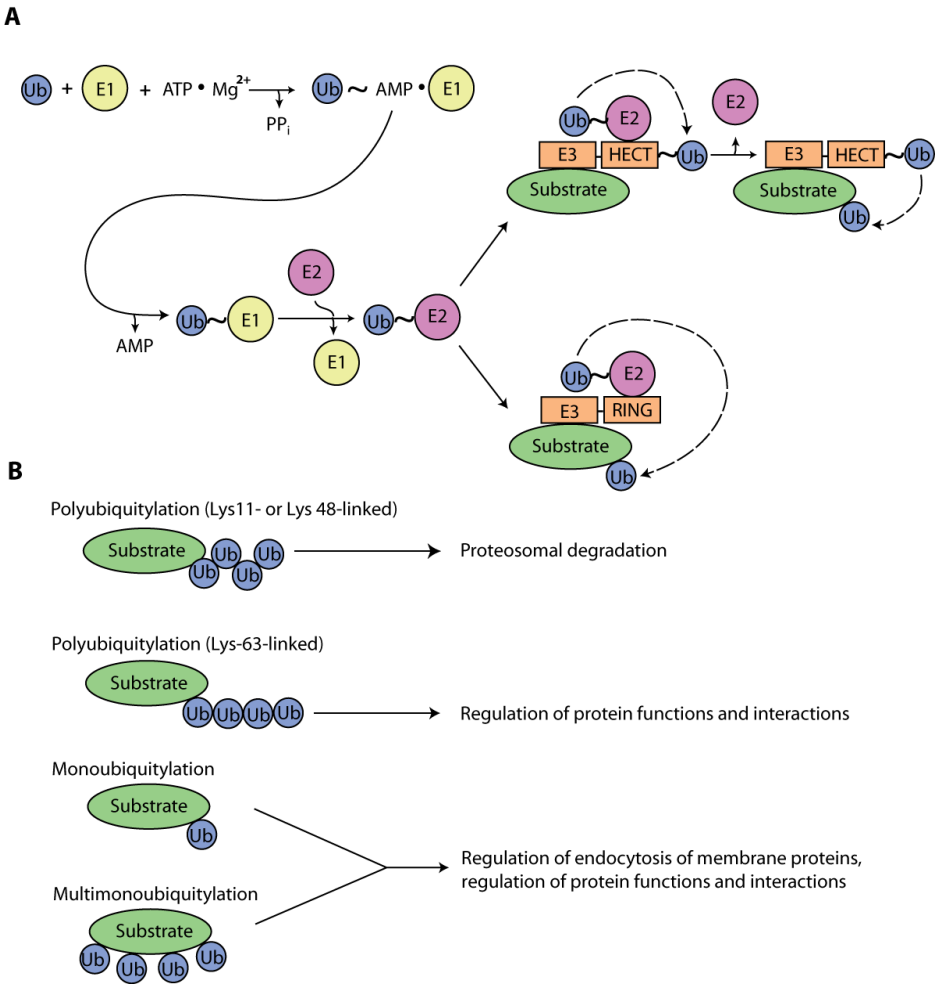


Figure 9: The protein ubiquitylation pathway

(**A**) Ubiquitylation is a sequential reaction mediated by three classes of enzymes (E1, E2 and E3). A ubiquitin-activating enzyme (E1) is conjugated with a free ubiquitin moiety through a thioester bond (~). This reaction uses ATP•Mg^{2+} to form a ubiquitin adenylate intermediate, in which ubiquitin and adenosine monophosphate (AMP) are conjugated by a high-energy thioester bond. This intermediate is first coupled to the E1 through a non-covalent bond (•). Ubiquitin activated in this manner is then transferred to a cysteine residue of the E1 enzyme. Active ubiquitin conjugated to the E1 enzyme through a high-energy thioester bond is subsequently transferred to a ubiquitin conjugating enzyme (E2) that, in turn, is recognized by a ubiquitin ligase (E3), of which there are two major types — homologous to E6AP carboxyl terminus (HECT)-type and RING finger-type ligases. HECT-type ligases receive the active ubiquitin from the E2 enzyme (shown by a dashed arrow), generating yet another high-energy thioester bond, and subsequently transfer it to a lysine residue in the ultimate ubiquitylation substrate protein (shown by a dashed arrow), which is recognized by the substrate recognition domain of the E3 ligase. By contrast, the RING finger-type ligases transfer the active ubiquitin directly from the E2 enzyme to the final ubiquitylation substrate protein without forming a covalent thioester bond (shown by a dashed arrow). The human genome encodes two E1, approximately 30 E2 and about 600 E3 enzymes. (**B**) Functional consequences of protein ubiquitylation. Lys48-linked polyubiquitin chains and probably also Lys11-linked polyubiquitin chains are directly recognized by the proteasome an lead to degradation of the substrate. Lys63-linked polyubiquitin chains and also head-to-tail- linked linear polyubiquitin chains regulate protein function. Monoubiquitylation or multi-monoubiquitylation regulate the function or endocytosis of many proteins. PPi, pyrophosphate; Ub, ubiquitin. (Adapted from Kawabe and Brose, 2011).

The functional consequences of ubiquitylation depend on the number of ubiquitin molecules that are attached to the substrate and the conjugation variety. Attachment of a single ubiquitin molecule (known as monoubiquitination) induces changes of the substrate´s function, for example cell surface expression of membrane proteins, endocytosis or protein interaction (Hicke 2001). The same applies when a substrate carries several ubiquitin residues on different sites (multimonoubiquitylation). The attachment of ubiquitin chains (referred to as polyubiquitylation) in which the C-terminus of each ubiquitin unit is linked to a specific lysine residue (most frequently Lys48 and probably also Lys11) of the previous ubiquitin results in proteasome-mediated degradation of the respective target. In contrast, ubiquitin chains that are Lys63-linked regulate protein function and interactions (Thrower et al. 2000; Chau et al. 1989; Glickman and Ciechanover 2002). In addition to substrate linked ubiquitin, it was shown that unanchored free ubiquitin chains, too, constitute physiologically relevant signalling components (Zeng et al. 2010). Ubiquitylation can be reversed by deubiquitinating enzymes (DUBs) which remove a single ubiquitin from its substrate or remodel polyubiquitin chains on target proteins. These ubiquitin molecules may be recycled and replenish the cellular ubiquitin pool (Hershko and Ciechanover 1998). Nearly 100 DUBs are known in the human genome and comprise 5 families: the ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), Machado-Josephin domain-containing ubiquitin peptidases, ovarian tumor superfamily of ubiquitin isopeptidases (OTUs) and the JAMM (JAB1/MPN/Mov34 metalloenzyme) family of proteases. The first four families are cysteine proteases, while the JAMM family constitutes zinc metalloproteases (D'Andrea and Pellman,1998; Reyes-Turcu et al., 2009; Kim et al. 2003).

1.8 Ubiquitin-like proteins

Ubiquitin-like proteins (UBLs) are small proteins that share many similarities such as sequence homology and tertiary structure with ubiquitin and function as post-translational modifiers. They exist either in free form or attached to substrate proteins. Their mode of attachment follows a similar tri-enzymatic pathway like ubiquitin and includes an activating E1, a conjugating E2 and a ligating E3 enzyme. UBLs are often highly conserved between species. The tertiary structure of UBLs is usually very similar to ubiquitin and characterized by a compact globular beta-grasp fold, the “ubiquitin fold”, and many UBLs expose a conserved glycine-residue at their carboxyl terminus, identical to ubiquitin. However, although most UBLs share all these features, their physiological functions can vary greatly (Hochstrasser 2009; Herrmann, Lerman, and Lerman 2007).

1.8.1 The Nedd8 pathway and its role in the brain

Neural precursor cell expressed developmentally downregulated protein 8 (Nedd8) was initially discovered in the developing brain and belongs to the family of ubiquitin-like proteins (UBLs). It features ~60% sequence homology with ubiquitin (Kumar, Yoshida, and Makoto 1993; Kumar, Tomooka, and Noda 1992; Kamitani et al. 1997) and was found necessary for the viability of most model organisms such as mouse, *Drosophila*, *Caenorhabditis elegans* and *Schizosaccharomyces pombe* (Tateishi et al. 2001; D. Jones and Candido 2000; Hansen et al. 2010; Ou et al. 2002; Dharmasiri et al. 2003; Kurz et al. 2002). Similar to ubiquitin, Nedd8 is synthesized as a precursor that must be processed at it carboxyl terminus by deneddylating enzymes (such as DEN1/NEDP1) in order to expose a glycine-glycine motif that allows for attachment to target substrates (Kamitani et al. 1997).

In a sequence called neddylation which is very similar to the ubiquitin pathway, Nedd8 is activated by an E1 enzyme (NAE (Nedd8 activating enzyme); a heterodimer of NAE1 and UBA3 subunits), transferred to an E2 enzyme (Ubc12, also known as UBE2M) and then conjugated to target substrates by an E3 enzyme.

Nedd8 was found to control ubiquitylation by targeting the cullin family of proteins (Rabut & Peter 2008; Petroski & Deshaies 2005; Deshaies & Joazeiro 2009; Xirodimas 2008). Cullin proteins serve as core scaffolds to a subclass of ubiquitin E3 ligases, the cullin-RING ligases (CRLs) and covalent modification of the cullin protein by Nedd8 controls ubiquitin ligase activity. For example, neddylation of cullin proteins, by controlling important downstream targets such as nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB), NF-κB inhibitor alpha (IκBα), $p27^{Kip1}$ and cyclin E, was shown to regulate important cell cycle progression and signaling cascades (Podust et al. 2000; Read et al. 2000; Ou et al. 2002; Amir, Iwai, and Ciechanover 2002; Herrmann, Lerman, and Lerman 2007). Apart from the cullin family of proteins, ribosomal proteins have been found to be targets of NEDD8 (Xirodimas et al. 2008) and, more recently, a role in tumorigenesis has been established (Lisha Zhou et al. 2018; X. Zhou et al. 2016; Abidi and Xirodimas 2015; Yao et al. 2014). Cancers such as glioblastoma, leukemia, lymphoma, melanoma, osteosarcoma, cholangiocarcinoma, cervical carcinoma, Kaposi sarcoma, ovarian cancer, lung cancer, gastric cancer, liver cancer, breast cancer and prostate cancer exhibit altered neddylation patterns (Mansouri and Zadeh 2015; Hua et al. 2015; Han et al. 2016; Paiva et al. 2015; Godbersen et al. 2015; Y. Wang et al. 2015; Nawrocki et al. 2013; Li et al. 2014; Cheng et al. 2014; Chen et al. 2016; Q. Zhang et al. 2015; Yi Zhang et al. 2016; Chen et al. 2015; Gao et al. 2014; Lin et al. 2015; T. Zhu et al. 2016; X. Wang et al. 2014; Hughes et al. 2015). In many of these studies MLN4924, an inhibitor of neddylation, was employed. MLN4924 disrupts cullin-RING ligase-mediated protein turnover by selectively inhibiting NAE activity and was shown to increase apoptotic tumor cell death *in vitro* and in *vivo* (Soucy et al. 2009; Brownell et al. 2010). It has recently been tested in multiple phase I clinical trials as an anticancer drug (Swords et al. 2010; Bhatia et al. 2016; Nawrocki et al. 2012; Shah et al. 2016; Sarantopoulos et al. 2016). Apart from tumorigenesis, Nedd8 was also found to play an important role in the immune response by regulating dendritic cell function (Mathewson et al. 2013) and promoting stress granule assembly (Jayabalan et al. 2016). It was shown to be required for interferon beta production in the context of viral infections (X. Zhang et al. 2016; Song et al. 2016). Important in the context of this work, the neddylation pathway has been implicated in a number of neurological

diseases and Nedd8 has recently been established as a regulator of neuronal development. Notably, it was proven that Nedd8 conjugation increases during postnatal brain development and that neddylation is essential for the formation of spines, the maintenance of synapses and higher cognitive function (Vogl et al. 2015).

1.8.2 Deneddylating enzymes

Like ubiquitin, Nedd8 can be removed from substrates by deneddylating enzymes. Two well-characterized deneddylating enzymes are known: the human deneddylase 1 (DEN1), also known as Nedd8-specific protease 1 (NEDP1), and the COP9 signalosome. DEN1 is a member of the SENP family and was initially named SENP8 (Gan-Erdene et al., 2003; Mendoza et al., 2003). DEN1, apart from processing the Nedd8 precursor (pNedd8), was found *in vivo* to deneddylate primarily proteins other than cullins (Chan et al. 2008; Broemer et al. 2010; Mergner et al. 2015; Rabut and Peter 2008; Lihong Zhou and Watts 2005). A function in deneddylating cullins was shown *in vitro,* but requires unphysiologically elevated DEN1 concentrations (K. Wu et al. 2003). The COP9 signalosome (constitutive photomorphogenesis mutant 9) is a zinc metalloprotease that effectively removes Nedd8 from cullin proteins and thus regulates ubiquitin E3 ligase activity. The COP9 signalosome is subject to research in this work.

1.9 The COP9 signalosome

The COP9 signalosome is an evolutionary conserved protein complex whose principal known function is Nedd8 isopeptidase activity (Y. Y. Choo et al. 2011a; Cavadini et al. 2016; Cope and Deshaies 2003; Schwechheimer 2004). It targets cullin proteins primarily (Lyapina et al. 2001; Cope and Deshaies 2003; Hori et al. 1999). The cullin family of proteins comprises CUL1, 2, 3, 4A, 4B, 5, and 7, PARC, a parkin E3 ubiquitin ligase, and Apc2 (Petroski and Deshaies 2005; Deshaies and Joazeiro 2009). Given that CRLs ubiquitinylate a large number of proteins, the COP9 signalosome is implicated in the control of a considerable portion of the proteome, including protooncogenes, tumor-suppressor genes, and other important cellular protagonists (Wei, Serino, and Deng 2008). The attachment of Nedd8 induces conformational rearrangements of the cullin carboxyl terminus and the RING domain, accelerating the formation of the E2-E3 complex, which stimulates protein polyubiquitylation (K. Wu, Chen, and Pan 2000; Kawakami et al. 2001). Thus, cullin neddylation causes ubiquitylation activity of CRLs to increase and thereby stimulates proteasome-mediated degradation of CRLs substrates (Saha and Deshaies 2008; Y. Y. Choo et al. 2011b).

Structurally, the COP9 signalosome is composed of 8 heteromeric subunits designated CSN1-8. The presence and proper arrangement of its subunits is critical for normal function of the complex as a whole (Wei and Deng 1999; Wei and Chamovitz 1994; Yan et al. 2003). The protease function of the complex is harboured in its CSN5 subunit, also known as Jab-1 (JUN activation binding protein 1) (Echalier et al. 2013; Kotiguda et al. 2012). CSN5 contains an embedded JAMM motif (also termed an MPN+ motif) which acts as catalytic center for the CSN (Cope et. al 2002). CSN5 is the only subunit that can stably exist outside of the CSN and participate in important biological functions, both as part of the CSN holocomplex and independently (Wei & Deng 2003; Tomoda et al. 2005; Cope & Deshaies 2006; Tanguy et al. 2008; Hallstrom & Nevins 2006). The CSN promotes cullin activity, indicating that cycles of neddylation and deneddylation are required for normal cullin function *in vivo* and for the assembly of multisubunit ubiquitin E3s

(Mosadeghi et al. 2016; Bosu and Kipreos 2008; Cope and Deshaies 2003; Pintard et al. 2003). As CSN inactivation destabilizes many subunits of cullin-based ubiquitin ligases it is thought that, at least in some cases, the deneddylating activity of the CSN protects components of cullin-based ubiquitin ligases from autocatalytic degradation. (Wu et al 2006; Bosu & Kipreos 2008).

Apart from CSN as a protease, several other functions, such as a transcriptional repressor or regulator of DNA repair, have been established (D. A Chamovitz, 2009). CSN was found to control cell cycle, DNA stability, and exerts influence during tumorigenesis (Cope and Deshaies 2003; Schwechheimer 2004; Wolf, Zhou, and Wee 2003; Richardson and Zundel 2005). Recently, it was found that loss of CSN5 was associated with exacerbated atherosclerotic lesion formation (Asare, Ommer, Azombo, Alampour-Rajabi, & Sternkopf, 2017). Most organisms require the CSN for viability. For instance, absent of CSN5, mice die early *in utero*. In their embryoblasts, higher levels of p27, p53 and cyclin E are associated with impaired proliferation and accelerated apoptosis (Tomoda, Yoneda-Kato, Fukumoto, Yamanaka, & Kato, 2004). Similar experiments in *Drosophila* were associated with defective oogenesis, molting defects or death at larval stage (Oren-Giladi, Krieger, Edgar, Chamovitz, & Segal, 2008; Oron, Mannervik, Rencus, Harari-Steinberg, & Neuman-Silberberg, 2002). The plant *Arabidopsis thaliana* which was the first species in which CSN was knocked down features an altered light response, which gave the COP9 signalosome its name, and lethal seedling (Wei and Chamovitz 1994; Chamovitz et al. 1996). Simple organisms such as fission yeast remain viable despite loss of CSN subunits (Mundt, Liu, and Carr 2002). These findings may suggest that the COP9 signalosome has gained more important and refined control over essential cellular processes throughout evolution.

1.9.1 The COP9 signalosome in neurological disease

Components of the CSN were found to be significant in neurodegenerative and psychiatric disorders. CSN5 was suggested to be involved in the onset of Parkinson´s and Alzheimer´s disease (Oono et al., 2004) and its overexpression was shown to increase amyloid plaque burden and exacerbate learning and memory defects (Wang et al. 2015). CSN3 was associated with Smith-Magenis syndrome, a congenital intellectual disability syndrome (Potocki et al. 2000; Elsea et al. 1999; Potocki, Chen, and Lupski 1999). CSN2 was identified in the neuronal differentiation of embryonal carcinoma (Akinama et al., 2003). In addition to the CSN itself, several proteins that depend on the CSN have been associated with disease. Nedd8, which is cleaved from E3 ubiquitin ligases by the COP9 signalosome, was found accumulated in Lewy bodies of Parkinson´s disease and in glial inclusions of Machado-Joseph disease (F. Mori et al. 2005; Kuazi et al. 2003; Ferro et al. 2007; Y. S. Choo et al. 2012). CUL4B, an ubiquitin E3 ligase scaffold protein, was found altered in an X-linked syndrome involving intellectual disability (Zou et al. 2007; Tarpey et al. 2007). The role of CSN in dendritic morphogenesis was characterized in *Drosophila*. In its larval peripheral nervous system CSN was shown to act as a multilayer regulator of dendritic arborization, both stimulating and repressing dendritic branching via control of different cullins (Djagaeva and Doronkin 2009b; Djagaeva and Doronkin 2009a). The direct and indirect involvement of the COP9 signalosome in various neurological diseases justifies for a more thorough characterization of CSN-associated neuropathology as presented with this work.

2. AIMS OF THE PROJECT

In neuronal biology the ubiquitin-proteasome system (UPS) was found critically involved in axon and dendrite growth, synaptogenesis, presynaptic function, postsynaptic plasticity and postsynaptic receptor trafficking. The COP9 signalosome, by de-neddylating cullin-based E3 ubiquitin ligases, represents an important regulator of the UPS but has remained largely unexplored in neurons. The aim of this work is to provide a characterization of CSN in the brain through knockdown of its catalytic subunit CSN5, via the *Cre-loxP* system. By driving *Cre* expression through neuron-specific promoters we aim to characterize CSN function in a sequence of developmental stages. Loss of CSN5 will be induced in proliferating neuroblasts, early postmitotic and mature neurons. Consequently, analysis will focus on neuronal morphology, embryonic brain development and behavior.

Aim 1: Demonstrating significance of CSN for development of neuronal morphology *in vitro*

As an initial approach, CSN5 will be knocked down in primary neuronal cell cultures to demonstrate significance of CSN for the development of neuronal morphology *in vitro*. Quantitative analysis of the parameters dendritic length and dendritic complexity will be performed. Subsequently *in utero* electroporation will be used to prove requirement of CSN for post-mitotic neurons during development *in vitro* and *in vivo*.

Aim 2: Proving dependency on CSN for embryonic brain development *in vivo*

Knockdown of CSN5 *in vivo* through neuronal subtype-specific *Cre* lines will be performed to sort out dependency on CSN during embryonic brain development. In particular, embryonic lethality of the constitutive knockdown is supposed to be overcome by restricting the *Cre*-mediated loss of CSN5 to proliferating neuroblasts and early postmitotic neurons, by placing it under *Nestin* und *Nex* promotor control, respectively.

Aim 3: Characterizing CSN *in vivo* via establishment of a transgenic mouse line employing the inducible *Cre/LoxP* system

In order to overcome the limitations of embryonic knockdown, we aim to establish a mouse line by which we are able to induce loss of CSN5 in a spatially and temporally restricted manner, making use of the *Cre/LoxP* system, in the adult stage. By employing the *CaMKIIαCreERT2* mouse line, knockdown of CSN5 will be restricted to glutamatergic neurons of the forebrain. These neurons exhibit a characteristic, polarized morphology and are involved in specific cognitive functions. Therefore a comprehensive analysis of neuronal morphology including dendritic length and complexity, spine density and behavioural experiments will be conducted.

3. MATERIALS AND METHODS

3.1 Plasmids, antibodies and reagents

Plasmids

All plasmids used in this thesis are listed in table 1.

Plasmid	Purpose	Source
CAG-ERT2-Cre-ERT2	Tamoxifen inducible *Cre*	Matsuda & Cepko
D151N mutant CSN5	CSN5 mutant form	Thilo Hagen
pcRII-mouse CSN5	In vitro transcription (Sp6, T7 polym.)	cloned
CAG-IRES-GFP	Retroviral expression vector GFP	cloned
CAG-IRES-mRFP	Retroviral expression vector mRFP	cloned

Table 1: Plasmids.

List of all plasmids used in this work, including their purposes and sources.

Primary and secondary antibodies and fluorescence dyes

All antibodies used in this thesis are listed in table 2 and 3.

Antibody	Company, cat. #	Species	Dilution
DAPI	Sigma Aldrich, #D8417, 20mg/ml	-	1:10.000
α-GFP	Abcam, #ab6556	rabbit	1:3000
α-FLAG M2	Sigma Aldrich, #F3165	mouse	1:2000
α-MAP2	Abcam, #ab5392	chicken	1:2000

α-IkBα	Cell Signalling, #9242	rabbit	1:1000
α-p27	Abcam, #32034	rabbit	1:500
α-CSN5	Sigma Aldrich, #J3020	rabbit	1:8000
α-Nedd8	Epitomics, #1571-1	rabbit	1:500 – 1:1000
α-Cullin1	Invitrogen, #322400	mouse	1:750
α-α-Tubulin	Sigma Aldrich, #T5168	mouse	1:100.000 (WB)
α-cyclin D3	Abcam, #28283	mouse	1:1000
α-β-Actin	Cell Signaling Technology, #4967	rabbit	1:3000

Table 2: Primary antibodies and fluorescent dyes

WB, Western Blot

Antibody (Epitope)	Company, cat. #	Dilution
Alexa Fluor 488 goat α-chicken IgG	Invitrogen, #A11039	1:1000
Alexa Fluor 488 goat α-mouse IgG	Invitrogen, #A11029	1:1000
Alexa Fluor 488 goat α-rabbit IgG	Invitrogen, #A11034	1:1000
Alexa Fluor 594 goat α-chicken IgG	Invitrogen, #A11042	1:1000
Alexa Fluor 594 goat α-mouse IgG	Invitrogen, #A11032	1:1000
Alexa Fluor 594 goat α-rabbit IgG	Invitrogen, #A11037	1:1000
α-mouse-IgG HRP	CST, #7076	1:2000
α-rabbit-IgG HRP	CST, #7074	1:2000
α-rat-IgG HRP	CST, #7077	1:2000

Table 3: Secondary antibodies

General Buffers and Solutions

1x PBS

137mM NaCl
2.7mM KCL
20mM Na_2HPO_4
2mM KH_2PO_4
Adjust pH 7.4

20x SSC

3 M NaCl
0.3 M Sodium Citrate
Adjust pH 7.4

6x DNA Loading Buffer (Orange)

1g Orange G
10ml 2 M Tris/HCL, pH 7.5
150ml Glycerol

50x TAE Buffer

2 M Tris-Base
1 M Acetic Acid
100mM EDTA, pH 8.0
Adjust pH 8.1

10x SDS-PAGE Running Buffer

1% (w/v) SDS
250mM Tris
1920mM Glycine
Adjust pH 8.3

1x Transfer Buffer (Western Blot)

0,025 M Tris
0,192 M Glycine
20% Methanol
Adjust pH 8.3

4x Protein Loading Buffer

50% (v/v) Glycerol
125mM Tris-HCL, pH 6.8
4% SDS
0.08% (w/v) Bromophenol blue
5% β-Mercaptoethanol

1x TBS

50mM Tris
150mM NaCl
Adjust pH 7.6

Reagents

Tamoxifen was purchased at Sigma Aldrich. For injections experiments *in vivo* it was first dissolved in absolute Ethanol (100mg/ml) and then diluted 1:10 with sunflower seed oil (Sigma Aldrich) to reach a final concentration of 10mg/ml. Diluted Tamoxifen was stored at 4 °C and used for a maximum of 3 consecutive days. Alternatively, Tamoxifen was administered orally via purchased tamoxifen food pellets (see below).

3.2 Animal experiments

3.2.1 Animals and housing

Animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Government of Bavaria, Germany.

In all experiments mice were housed under standard laboratory conditions (22±1 °C, 55±5% humidity) on a 12h light-dark cycle with food and water ad libitum. At the age of 3 to 4 weeks littermates were separated from parents, numbered by ear-punching, and a small tail biopsy was taken for genotyping. Respective the staging of embryos, noon on the day of the appearance of a vaginal plug was counted as embryonic day 0.5 (E0.5), and the day of birth was considered postnatal day 0 (P0).

If required, a Tamoxifen dosage of 1 mg twice (every 12h) per day for 5 consecutive days was administered by intraperitoneal (i.p.) injections of sunflower seed oil (10mg/ml). Alternatively, in order to avoid injections in some experiments animals were fed with tamoxifen food pellets (LAS CRdiet CreActive TAM400, LASvendi GmbH, Soest, Germany) for 7 consecutive days during postnatal weeks 8-10, and analyses were performed 1-2 weeks later.

3.2.2 Transgenic mouse lines used or generated for this thesis

- *CSN5*$^{lox/lox}$ mice were obtained from Ruggero Pardi (Panattoni et al. 2008)
- *CSN5*$^{CamKII\alpha\ CreERT2}$ mouse line
- *CSN5*^{NestinCre} mouse line
- *CSN5*^{NexCre} mouse line
- *Thy1*^{eGFP} *- CSN5*$^{CamKII\alpha\ CreERT2}$ mouse line

3.3 Genotyping

For genotyping, mouse tail biopsies were taken at the age of 3 to 4 weeks and, by alkaline lysis of the tissue, genomic DNA was prepared. 100µl of 50mM NaOH was added to a mouse tail of approximately 1-2 mm length and heated to 95 °C for 30 min in order to lyse the tissue. After lysis, samples were cooled to 4°C and neutralized by addition of 30µl 1 M Tris-HCl (pH 7.5, containing 4mM EDTA), vortexed at slow intensity and centrifuged at 4°C with maximum speed in a table top centrifuge for 1 min. These samples were used as PCR templates and stored at 4°C. For genotyping, 1µl of the sample of genomic DNA was used in a 25µl-PCR reaction, containing 2.5µl 10x PCR buffer (Abgene), 1.5µl 25mM MgCl2, 0.5µl dNTPs (10mM each, Roche), 0.5µl of each primer (1, 2 and 3) and 0.5µl Taq DNA polymerase (5 units (U)/µl, Abgene).

Routinely a standard PCR program, 95 ºC for 5 min, 35 cycles of 98 ºC for 45 sec, 58-60 ºC for 30 sec, 72 ºC for 20 sec to 1 min, followed by 72 ºC for 10 min, then holding at 8 ºC, was carried out. If required, the annealing temperature and extension time of a PCR program were adjusted to amplify a specific genomic DNA sequence. For readout, small aliquots of the PCR samples (1-5µl) were mixed with 6x Orange loading buffer and PCR products were analyzed by gel electrophoresis in a 1-2% (w/v) agarose (Invitrogen) gel (1x TAE), containing ethidium bromide for visualization of DNA. After electrophoresis, gels were analyzed using a UV transilluminator and a BioDoc II gel documentation system from Biometra.

3.4 Radioactive *in situ* hybridization (ISH)

Single *in situ* hybridization (ISH) procedures were performed as previously described (Lu et al. 2008). Adult mice were sacrificed by an overdose of isoflurane (Forene®, Abbott), brains were carefully dissected out and immediately shock-frozen on dry ice. Frozen brains were cut on a cryostat into 20µm thick sections and mounted on SuperFrost Plus slides (Menzel GmbH). Slides were stored at -20°C until further processing. The riboprobes for CSN5 used in this project were amplified from a plasmid containing the following sequence:

5'-

tcaccactactttaaatactgcaaaatctcagcattggctctactgaaaatggtgatgcatgccaggtcaggaggcaac
ttggaagtgatgggtttgatgctcgggaaagtcgacggcgagaccatgatcatcatggacagtttcgctttgcctgtaga
gggcacagaaactcgagtaaatgctcaagctgctgcgtatgagtatatggctgcatacatagaaaatgccaaacag

3'

Specific riboprobes were generated by PCR applying T7 and T3 or SP6 primers using plasmids containing the above-mentioned cDNA fragments as templates. Radiolabeled sense and antisense cRNA probes were generated from the respective PCR products by in vitro transcription with ^{35}S-UTP (Perkin Elmer) using T7 and T3 or SP6 RNA polymerases (Roche).

Hybridization was performed overnight (o.n.) with a probe concentration of 7 x 106 counts per minute (c.p.m.) ml^{-1} at 57 °C and slides were washed at 64 °C in 0.1 X saline sodium citrate (SSC) and 0.1 M dithiothreitol. Hybridized slides were dipped in autoradiographic emulsion (type NTB2, Eastman Kodak), developed after 2-6 weeks and counterstained with cresyl violet. Dark-field photomicrographs were taken with Zeiss AxioCam MRm and AxioCam MRc5 digital cameras adapted to a Zeiss Axioplan 2 imaging microscope and a stereomicroscope (Leica). Image digitalization was performed with AxioVision 4.5, and afterwards photomicrographs were integrated into plates using Adobe Photoshop CS2 9.0.2 and Adobe Illustrator CS2 12.0.1 image-editing software. Only sharpness, brightness and contrast were adjusted. For an adequate comparative analysis in corresponding mutant and wild-

type sections the same adjustments were undertaken. Brain slices were digitally cut out and set onto an artificial black background.

3.5 Primary neuronal cell cultures and transfection of neurons

All cell culture reagents were purchased from Invitrogen and all cell culture dishes were purchased from Nunc unless stated otherwise.
Primary hippocampal and cortical neurons were prepared from mouse embryos as described (Carlos G. Dotti, Sullivan, and Banker 1988; Goslin and Banker 1989; Kaech and Banker 2006). *In vitro* plated neurons were transfected via a modified calcium phosphate protocol (Jiang and Chen 2006).

3.5.1 Preparation of hippocampal and cortical neuronal cell cultures

Hippocampi and cerebral cortices from embryonic day (E) 17.5 mice were separated from diencephalic structures and digested with 0.25% trypsin containing 1 mM EDTA for 20 min at 37 ºC with gentle shaking. Tissue pieces were then washed three times with DMEM supplemented with 10% FCS and afterwards triturated with a fire-polished Pasteur-pipette in order to obtain dissociated cells. Cells were centrifuged at 90x G for 5 min, cell pellet was carefully resuspended in Neurobasal-A medium supplemented with B27, and cell number and viability was assessed by counting the number of living cells in a Trypan Blue stained cell dilution. Cells were plated at the desired density (5-7x10^4 cells/well in a 24-well plate, 10^5 cells/well in a 12-well plate, 3x10^5 cells/35 mm glass dish (MatTEK Corporation), 4x10^5 cells/24 mm Transwell® with 3µm pore polyester membrane insert (Corning), 5x10^5 cells/well in a 6-well plate, 5-10x10^6 cells/10 cm plate), on Poly-D-Lysine- (0.05mg/ml, 30.000-70.000 MW, Sigma Aldrich) and Laminin- (1 µg/ml, Invitrogen) coated cell culture plates or on 12 mm glass coverslips (ThermoFisher Scientific) and maintained in Neurobasal-A medium supplemented with 2% B27-supplement and 0.5mM GlutaMAXI at 37 ºC and 5% CO2.

3.5.2 Transfection of primary neurons

Transfection of plated cells *in vitro* was performed with expression plasmids at the desired day *in vitro* (DIV) by using a modified calcium phosphate protocol (Jiang and Chen, 2006). 1ml transfection mixtures (5-8µg plasmid DNA, adjusted with Ampuwa H_2O to 50µl, 12.5µl 1 M $CaCl_2$, 50µl 2x BBS (50mM BES, 280mM NaCl, 1.5 mM Na_2HPO_4, pH 7.26) and 900µl Neurobasal-A medium supplemented with 2% B27-supplement and 0.5 mM GlutaMAXI) were prepared in sterile 1.5ml eppendorf tubes. Ampuwa water was mixed with $CaCl_2$ by vortexing at full speed, then plasmid DNA, isolated with MAXI-Preps (Qiagen) and dissolved in Ampuwa water at a minimal concentration of ≥ 0.5 µg/µl, was added and mixed by pipetting up and down for 10 times. 2x BBS buffer was added drop-wise into the H_2O-$CaCl_2$-DNA mixture during slow vortexing. Neurobasal-A medium, preincubated in the cell culture incubator, was added and the complete transfection mix was vortexed at full speed for 10 seconds and incubated 15 min at room temperature (RT). The conditioned medium from the neurons was collected and the transfection mix was applied to the neurons for 2 to 4 h depending on the age and density of the neuronal culture and based on the size and appearance of the precipitate formed by the transfection. Neurons were then washed 8 to 12 times with warm HBSS buffer containing 0.01 M HEPES, and the conditioned medium, filled up with new Neurobasal-A medium, was pipetted back onto the neurons. The time outside of the incubator was minimized during and after transfection in order to avoid damage to the neurons. Expression efficiency, usually between 0.5 and 1% of total neurons, was verified 24 h after transfection and was evaluated by expression of fluorescent proteins.

3.6 Immunocytochemistry

After transfection and/or treatment, neurons on glass coverslips were fixed in 4% (v/v) paraformaldehyde containing 5% (w/v) saccharose for 20 min at RT, washed 3 times with PBS and permeabilized with PBS-TritonX-100 0.1% 3x 5 min.
After blocking in 5% BSA (w/v) in PBS-TritonX-100 0.1% for 1 h at RT, neurons were incubated with primary antibodies diluted at an appropriate concentration in 5% BSA (w/v) in PBS-TritonX-100 0.01% at 4 ºC overnight. After washing 3x 10 min with PBS-TritonX-100 0.01% neurons were incubated with secondary antibodies, Alexa dye-conjugated antibodies (Invitrogen) diluted 1:1000 in PBS-TritonX-100 0.01%, at RT for 2h. Coverslips were then washed 3x 10 min with PBS-TritonX-100 0.01% and mounted with anti-fading VectaShield medium (Vector) (if desired, containing DAPI to stain the nucleus).

3.7 Image acquisition and analysis of neuronal morphology

For analysis of neuronal morphology in culture and brain slices, images of individual neurons were captured randomly in a blind manner using an Olympus IX81 inverted laser scanning confocal microscope and Fluoview 1000 software. Pictures were taken with a 10x UPlanSApo, 0.40 numerical aperture (NA), 20x UPlanSApo, 0.75 NA, 40x PlanApo, 0.9 NA WLSM or 60x UPlanSApo, 1.2 NA WLSM Olympus objective. Labeled neurons were excited at 405nm (DAPI, Alexa-405), 488nm (GFP, Venus, Alexa-488), 559nm (RFP, dsRed, cherry, Alexa-594) and 635nm (Alexa-647), and emission was collected at 425-475nm, 500-545nm, 575-675nm and 680-750nm, respectively. Usually a Z-stack of pictures was collected with 0.4-1.2µm step size and 800x800 to 1024x1024 pixel picture size, depending on the specimen. Confocal pictures were exported to and processed for analysis with open access ImageJ (http://rsbweb.nih.gov/ij/) software and pictures were compiled and

composed using Adobe Photoshop CS2. The morphology of primary neurons was analyzed via manual tracing of neurons followed by length measurements of tracings using the NeuronJ plugin (http://www.imagescience.org/meijering/software/neuronj/) in ImageJ, and the Sholl analysis plugin (http://www.biology.ucsd.edu/labs/ghosh/software/) to measure dendritic arborization. Axons and dendrites of neurons were identified based on morphology. Total dendrite length was determined by summing the lengths of all dendrite processes measured from a single neuron (Meijering et al. 2004). Complexity of dendritic arborization was assessed by Sholl analysis, counting the number of dendrite intersections with concentric circles around the center of the soma of the neuron with increasing radius (10µm step size) (Sholl 1953). For spine analysis dendritic protrusions were counted per 10µm segments on secondary apical dendrites of cortical layer V and hippocampal CA1 pyramidal excitatory neurons. Mean signal intensities were measured with ImageJ in 8-bit gray pictures of single slices of the Z-stacks, in which puncta appeared brightest, substracting background intensity values. For dendrite analysis (total dendrite length and Sholl analysis) on Golgi-stained brain sections, neurons were directly traced on a Zeiss Axioplan microscope equipped with a Zeiss AxioCam camera and analyzed with Neurolucida software (mbf Bioscience).

3.8 *In vivo* experiments

3.8.1 *In utero* intraventricular injection and electroporation of plasmid constructs

Timed to E13.5 pregnant female CD1 mice carrying the *floxed* CSN5 allele ($CSN5^{lox/lox}$, as determined by genotyping) were anesthetized via intraperitoneal (i.p.) injection with a mixture of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight). Eyes were protected from drying during the surgery by a drop of eye cream (Bepanthen eye and nose cream). The abdomen was shaved, cleaned with 70% ethanol and a 3-cm long midline laparotomy was performed. The uterine horns were carefully exposed, placed on sterile gauze and hydrated with saline (0.9% NaCl solution), prewarmed to 37 ºC. 1-2µl of high concentrated expression plasmids (2-4 µg/µl), mixed with fast green dye (for visualization of the injection), were microinjected into the lateral ventricle of mouse embryos using a glass micropipet and plunger (Drummond PCR micropipets, 1-10µl). After DNA injection, electroporations were performed using an Electro Square Porator ECM830 and tweezertrodes (BTX Genetronics). The developing cortex was targeted with 7-mm diameter tweezertrodes (BTX Genetronics) as described in (Nakahira and Yuasa 2005) and five pulses with 40V, 50ms duration and with 950ms intervals were delivered to each embryo. After the embryos were injected and electroporated, the uterine horns were placed back in the abdominal cavity and antibiotic/antimitotic solution (100x stock solution, Invitrogen), diluted 1:100 in saline, was administered to reduce the risk of infection. Surgical sutures (Johnson & Johnson) were used to close the abdominal wall and skin and 7.5% povidone-iodine solution (Braunol) was applied to the abdominal skin around the sutures. For pain management, Metacam (1 mg/kg body weight), diluted in saline, was injected subcutaneously in the neck after surgery and again 18 to 24 h later. The pregnant mouse was allowed to recover from the anesthesia on a heating plate at 30 ºC before it was placed back into a new clean cage (Saito and Nakatsuji 2001; Saito 2010; Saito 2006; Tabata and Nakajima 2001).

3.8.2 Generation of transgenic mice

In this work we induced gene inactivation *in vivo* in a spatially and temporally restricted manner by making use of the *Cre/loxP* system (Hoess et al. 1982; Branda et al. 2004, Nagy 2000). Briefly, *loxP* (locus of crossover [x] of P1) sites are inserted into the gene of interest via homology-based gene targeting. The *Cre* (cyclization recombination, derived from bacteriophage P1) DNA recombinase recognizes and efficiently catalyzes the recombination between two pairs of *loxP* sites (the "*floxed*" fragments) (Argos et al. 1986) leading to excision of the DNA fragment. By driving *Cre* expression through a tissue-specific promoter it is possible to induce gene inactivation in a specific tissue or subpopulation of cells and/or a specific developmental stage (Gaveriaux-Ruff and Kieffer, 2007; Deussing, 2013). Additional temporal control over gene inactivation is obtained upon combination of a *floxed* gene of interest with a *Cre* recombinase that is fused to the ligand-binding domain of the estrogen receptor (*CreERT2*). This *CreERT2* is restricted to the cytoplasm by the heat shock protein 90 (HSP90). Only in presence of 4-hydroxy tamoxifen (4-OH-TAM) it is able to translocate into the nucleus and disrupt the gene of interest (Feil et al. 1996). The advantage of this tamoxifen-inducible *Cre* is that it enables targeting of genes in the adult stage whose disruption during earlier developmental stages would be lethal.

In this work, CSN5-*floxed* ($CSN5^{lox/lox}$) mice were obtained from the research group led by Ruggero Pardi (Panattoni et al. 2008) and bred to the respective *Cre*-driver mouse lines which were available in our animal facility. For selective disruption of CSN5 in forebrain glutamatergic developing neurons, $CSN5^{lox/lox}$ mice were bred to *NestinCre* (Tronche et. al., 1999) and *NexCre* (Goebbels et al., 2006) mouse lines. For tamoxifen-inducible selective disruption in forebrain glutamatergic adult neurons, $CSN5^{lox/lox}$ mice were bred to the *CaMKIIα-CreERT2* mouse line (Erdmann, Schütz, and Berger 2007). For morphological analysis of dendritic spines, $CSN5^{CaMKIIα\text{-}CreERT2}$ mice were bred to *Thy1-eGFP* mice that express GFP in a subset of neurons, including CA1 pyramidal neurons in the hippocampus and pyramidal neurons in the cortex (G. Feng et al. 2000). All mouse lines were maintained in a C57BL/6N genetic background.

Genotyping of *Cre/iCre* transgenic mice

All animals used in this work were genotyped by PCR using the following primers:

i*Cre*-for1: 5'-GGT TCT CCG TTT GCA CTC AGG A-3'

i*Cre*-rev1: 5'-CTG CAT GCA CGG GAC AGC TCT-3'

i*Cre*-rev2: 5'-GCT TGC AGG TAC AGG AGG TAG T-3'

Cre-for: 5'-GAT CGC TGC CAG GAT ATA CG-3'

Cre-rev: 5'-AT CGC CAT CTT CCA GCA G-3'

CSN5-for: 5'-GGT CAG AAA GCT AGG CCT AAG AAG G-3'

CSN5-rev: 5'-GGC ATG CAT CAC TTT CAG TAG-3'

Thy1-for: 5'-TCT GAG TGG CAA AGG ACC TTA GG-3'

Thy1-rev: 5'-CCA CTG GTG AGG TTG AGG-3'

eGFP: 5'-GTC CTC CTT GAA GTC GAT GC-3'

Standard PCR conditions with primers CSN5-for and CSN5-rev resulted in a 397-bp wildtype and a 484-bp *floxed* CSN5 PCR product. In *Cre*-positive mice genotyping with primers Cre-for and Cre-rev resulted in a 574-bp *Cre* product. In the case of the tamoxifen-inducible *CSN5*$^{CamKII\alpha\text{-}CreERT2}$ mouse line genotyping with *iCre* primers resulted in a 290-bp wild-type and a 375-bp *Cre* product. Genotyping of Thy1eGFP expressing mice resulted in a 593-bp eGFP and a 372-bp wild-type PCR product.

3.8.3 Preparation of brain slices

Electroporated and stereotactically injected animals were anesthetized with isoflurane (Forene®, Abbott) and transcardially perfused with a peristaltic pump for 1 min with PBS, 5 min with 4% PFA (w/v) in PBS, pH 7.4, and 1 min with PBS at a flow of 10 ml/min. Brains were removed, post-fixed for 1h in 4% PFA at 4ºC and cryoprotected in 15% (w/v) saccharose in PBS, pH 7.6 overnight (o.n.) at 4 ºC. Brains were washed with PBS and schockfrozen on dry-ice for cryo-sections (MICROM HM 560, ThermoScientific) or embedded in warm 4% (w/v) agarose (Invitrogen) in PBS for vibratome-sections (MICROM HM 650V, ThermoScientific). 20µm thick cryo-sections and 50µm thick vibratome-sections were stored at -20 ºC in cryopreservation solution (25% (v/v) glycerol, 25% (v/v) Ethylenglycol, 50% (v/v) PBS, pH 7.4) until immunohistochemistry, DAPI staining and mounting.

3.8.4 Immunofluorescence stainings on brain sections

Brain sections were washed 3x with PBS and permeabilized with PBS-TritonX-100 0.1% 3x 5 min. After blocking in 5% BSA (w/v) in PBS-TritonX-100 0.1% for 1 h at room temperature, sections were incubated with primary antibodies diluted at an appropriate concentration in 5% BSA (w/v) in PBS-TritonX-100 0.01% o.n. at 4 ºC. After washing 3x 10min with PBS-TritonX-100 0.01%, sections were incubated with secondary antibodies, Alexa dye-conjugated antibodies (Invitrogen), diluted 1:1000 in 5% BSA (w/v) in PBS-TritonX- 100 0.01% for 2h at RT. After that, brain sections were washed 3x 10 min with PBS-TritonX- 100 0.01%, stained with DAPI and mounted with anti-fading fluorescence VectaShield medium (Vector).

3.8.5 Golgi staining on brain sections

Golgi staining was performed using the FD Rapid GolgiStain Kit (FD NeuroTechnologies, Inc.). Animals were deeply anaesthetized using isoflurane (Forene®, Abbott) and subsequently transcardially perfused with a peristaltic pump for 10 min with 0.9% NaCl. Then brains were dissected out, treated and prepared according to the manufacturer's protocol. After Golgi treatment was completed, for the preparation of slices, brains were rinsed 3 times with PBS, embedded in warm 4% (w/v) agarose (Invitrogen) in PBS and cut using a vibratome obtaining 200µm thick slices. The slices were mounted on pre-prepared 2 % gelatine glass slides and dehydrated by employing increasing alcohol concentrations according to the protocol issued by the manufacturer. After dehydration, slides were covered with Eukitt and coverslips and left to dry for 2 weeks in a dark and ventilated area.

3.9 Immunoblotting

Cells and tissue was lysed in RIPA buffer (50mM Tris, pH 8.0, 150mM NaCl, 0,1% SDS, 1.0% NP-40 or Triton X-100 and 0.5% sodium deoxycholate) containing protease inhibitors (complete protease inhibitor tablets, Roche). Protein concentrations were measured using Bradford assay. For immunoblotting, protein samples were separated by 8-14% SDS-PAGE (Laemmli 1970) and transferred to 0.45µm PVDF membranes (Millipore). Membranes were blocked in 5% nonfat milk (Roth) in TBS-Tween 20 0.01% (Sigma Aldrich) for 1 h at RT, followed by incubation with primary antibodies o.n. at 4ºC. Membranes were washed 3x 10 min with TBS-T 0.01% and then incubated with the appropriate secondary horseradish peroxidase-IgG-conjugated antibody for 2 h at RT. After 3x 10 min washing of membranes, signals were revealed by enhanced chemoluminescence (Millipore) and signal was acquired by ChemiDoc XRS+ (Bio-Rad Laboratories, Inc.). The obtained images were analyzed using Image Lab 6.0.1 software, measuring integrated intensities of gray values. All signals were normalized to their respective house-keeping protein control bands.

3.10 Behavioral experiments

All experiments were performed with male *CSN5*^{CaMKIIαCreERT2} mice that were equally old (eight weeks) and were habituated to single housing and test room conditions two weeks before testing. For induction of *Cre* activity, animals were fed with tamoxifen food pellets for one week, and behavioral testing was started after a one-week washout interval. Behavioral testing took place in the first half of the animals' dark period. Urine and feces were removed from the experimental setting after every individual animal to eliminate distraction of and possible cues for the following animal.

Water - cross maze

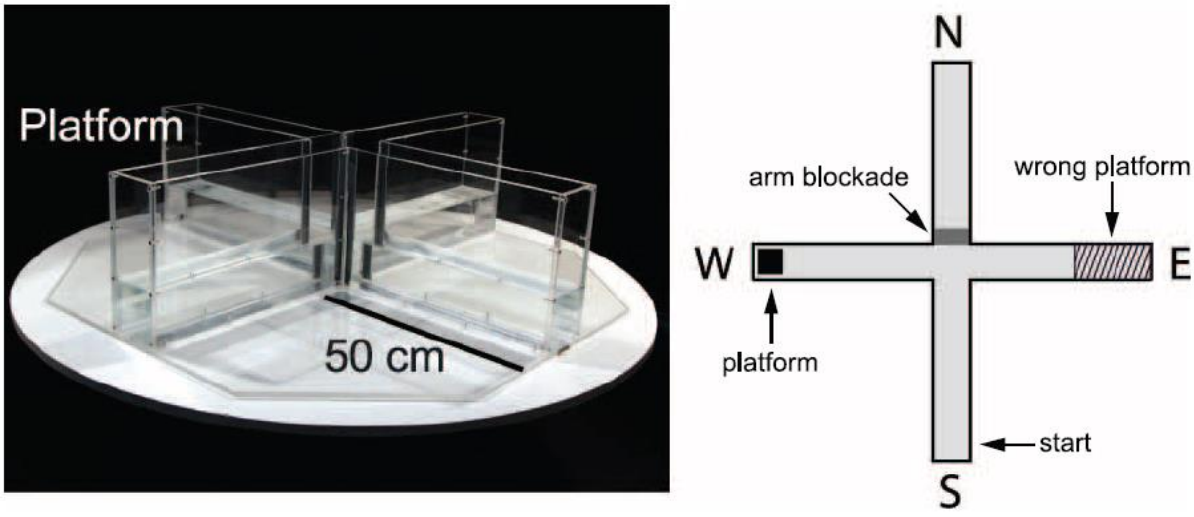


Figure 10: Photograph and schematic representation of the water – cross maze used for experiments (obtained from Kleinknecht et al. 2012)

Assessing hippocampus-dependent spatial learning was performed as previously described (Kleinknecht et al. 2012). We used the water-cross maze (WCM, custom made, Max-Planck-Institute of Psychiatry) which is made from 5 mm thick clear acrylic glass and consists of four arms forming a cross (Figure 10). Each arm is 50 cm long, 10 cm wide and 30 cm high. The arms are labelled North, South, East and West. The acrylic glass enables the mouse to visually orientate itself via distal extra-maze cues in the experimental room, such as a sink, a small gray cabinet or tubes at

the ceiling and on the walls. On every test day the maze was filled with fresh tap water (23°C) up to a height of 11 cm. A platform made from the same acrylic glass was submerged at the end of an arm either left or right to the start arm, 1 cm below the water surface and thus invisible to the mouse. The arm opposite to the start arm was closed with a transparent plastic sheet. The testing room was dimly lit by four lights in every corner of the room emitting indirect regular spectrum light (14 lux at the level of the mouse). Before every trial, each mouse was brought from the adjacent holding room into the testing room, carefully taken out of its cage and placed into the start arm of the maze. Similarly to the classic Morris water maze, the WCM makes use of water-based motivation, but additionally allows for simple assessment of learning strategies. In order to locate the platform, the animal was left to explore the maze. The experimenter remained motionless behind the start arm in order to avoid distraction and cues to the platform´s position.

The task for the mouse was to locate the platform correctly. Each animal performed 6 trials per day, for 5 consecutive days. During this period, the animal was supposed to learn the position of the platform in the maze using external cues, thus decreasing the time spent in the maze and increasing its accuracy for primarily entering into the correct arm. The platform was always located in the same arm, however the starting position of the mouse was changed in a pseudorandom manner, ensuring that it would rely solely on external cues for locating the platform (i.e. place learning) and not on route following based on body turns (i.e. response learning). The water in the maze was renewed every day and cleaned from mouse feces after every individual trial. A trial was considered accurate (i.e., value 1), if the animal directly entered the arm containing the platform and climbed onto it. Other behavior was considered as non-accurate (i.e., value 0). Thus, accuracy reflects the percentage of accurate trials on each day per animal.

Upon finishing the 5 day long learning protocol, mice were allowed 2 days rest and then subjected to a re-learning paradigm for 3 consecutive days, for which the position of the platform was changed.

Radial arm maze

The radial arm maze is an experimental setup for assessment of working memory and was employed as previously described (Kiyota, Miyamoto, and Nagaoka 1991; Davis et al. 1986; Volpe et al. 1984). It consists of a round center with eight arms made from clear plexiglas all equally grouped around it. In our setup the maze itself did not contain any clues, the only clues were the ones in the room (e.g. a sink, a door, light tubes on the ceiling, etc.), easily recognizable through the transparent walls. Each mouse was placed in the center of the maze and then subsequently started exploring the arms around it. Every visit to every arm was noted, and as soon as all arms had been visited, the mouse was taken out of the maze. Ideally, a mouse would visit all arms without entering one that it already had entered before. Thus, the number of visits to arms it had already entered before were counted as mistakes and thus served to quantify working memory performance. Each animal was tested once a day for 6 consecutive days. In order to have a starting value, the average value from the first 3 days was calculated and plotted accordingly.

Open field (OF) test

The open field (OF) test was used to assess explorative behavior and general locomotor activity in a novel environment. Open field boxes (50 x 50 x 60 cm) were made up of grey polyvinyl chloride (PVC) and evenly illuminated (<15 Lux in order to minimize anxiety effects on locomotion). The test duration was 30 minutes. The parameters assessed were total distance travelled and immobility time using the ANY-maze software.

Auditory and contextual fear conditioning

Contextual and auditory fear memory was assessed in conditioning chambers (ENV-307A, MED Associates) as previously described (Kamprath and Wotjak 2004; Refojo et al. 2011). Context-dependent fear memory was assessed in a cubic-shaped conditioning chamber with metal grid floors that had been thoroughly cleaned and sprayed with 70% ethanol before each animal was introduced. Auditory (tone-dependent) fear memory was assessed in a neutral context that was made up of a round plexiglas cylinder filled with bedding (sawdust, same as in home cage) and had been cleaned and sprayed with 1% acetic acid. For foot shock application (on

day 0) mice were placed into the conditioning chamber for 3 min. After a habituation phase of 180 sec, a flashing light and a sine wave tone (80 dB, 9 kHz) was presented for 20 sec (conditioned stimulus), which co-terminated with a 2 sec scrambled electric foot shock (unconditioned stimulus) of 0.7mA. The mice remained in the conditioning chamber for another 60 sec before they were returned to their home cages. In order to measure freezing responses to the conditioned stimulus, mice were placed into the neutral environment (plexiglas cylinder) on the following day (day 1). Three minutes later, a 3-min tone (80 dB, 9 kHz) was presented. The animals were returned to their home cages 60 sec after the end of tone presentation. Contextual (associative) fear was tested on day 2 by re-exposing the animals to the conditioning grid chamber for 3 min. All behavior during the conditioning and testing sessions was recorded on DVD for further off-line analysis. As a measure of fear, we assessed freezing behavior in both setups defined as the absence of all movements except for respiration and the animal's head remaining in a horizontal position. Freezing was scored by a trained observer unaware of the experimental group by means of customized freeware software EVENTLOG.

Tail suspension test (TST)

Mice that are subjected to short term inescapable stress will become immobile. The tail suspension test (TST) was used to measure stress-coping behavior and was performed as previously described (Can et al. 2012; Steru et al. 1985). Each animal was attached to an aluminum rack by its tail with adhesive tape and left for six minutes hanging upside down approximately 10 cm above the ground. During this time the animal struggled, tried to escape and reach for the ground. Each animal was tested individually, only once and out of view from the other animals. Every animal was recorded using a camcorder und later the time struggling as a percentage of the total amount of time was measured. A previously described problem that occurred during this test is tail climbing, especially among C57BL/6 mice (Mayorga and Lucki 2001). In our set of experiments this behavior occurred in 2 out of 24 animals. The recorded data from these animals was excluded from statistical analysis for this test.

3.11 Statistics

Each set of numerical data shown was obtained in two to five independent experiments. Statistical analysis was carried out using GraphPad Prism 7 software. All values are given as mean ± SEM. Statistical significance was assessed using Student's *t* test when appropriate. Comparisons between two variables, e.g. treatment and time were evaluated using two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test. When required, repeated measures (RM) ANOVA was applied. Differences were considered statistically significant at *$p<0.05$, **$p<0.01$, ***$p<0.001$

4. RESULTS

4.1 CSN5 controls dendrite development *in vitro*

It was previously shown that neddylation controls spine development during neuronal maturation and spine stability in mature neurons, and that inhibition of neddylation results in synaptic loss, impaired neurotransmission and severe cognitive deficits (Vogl et al. 2015). A key regulator of neddylation, the COP9 signalosome (CSN), was shown to have multiple regulatory functions in a number of different tissues and diseases (Chamovitz 2009; Cope and Deshaies 2003; Schwechheimer 2004; Richardson and Zundel 2005). However, in the brain, the function of the CSN as a key regulator of neddylation, is still poorly understood. In the *Drosophila* larval peripheral nervous system (PNS) CSN was demonstrated to act as a multilayer regulator of dendritic arborization, both stimulating and repressing dendritic branching via control of different cullins (Djagaeva and Doronkin 2009b; Djagaeva and Doronkin 2009a). Beyond these findings there is no study that characterizes CSN in neuronal development of the rodent brain.

As an initial approach to studying the role of CSN in neuronal development we knocked down its catalytic subunit CSN5 which harbors the deneddylase activity, *in vitro*. Primary neuronal cell cultures from the cortex and hippocampus of $CSN5^{lox/lox}$ mice were prepared and transfected with a *Cre* containing plasmid at DIV 7 in order to induce loss of CSN5 in these neurons. One week later (at DIV 14) the neurons were fixed and subjected to morphological analysis measuring both total dendritic length and complexity of the dendritic network, as quantified by Sholl analysis.

Total dendritic length of hippocampal neurons and dendritic arborization of both hippocampal and cortical neurons as measured by Sholl analysis was found reduced whereas total dendritic length of cortical neurons remained unchanged (Figure 11).

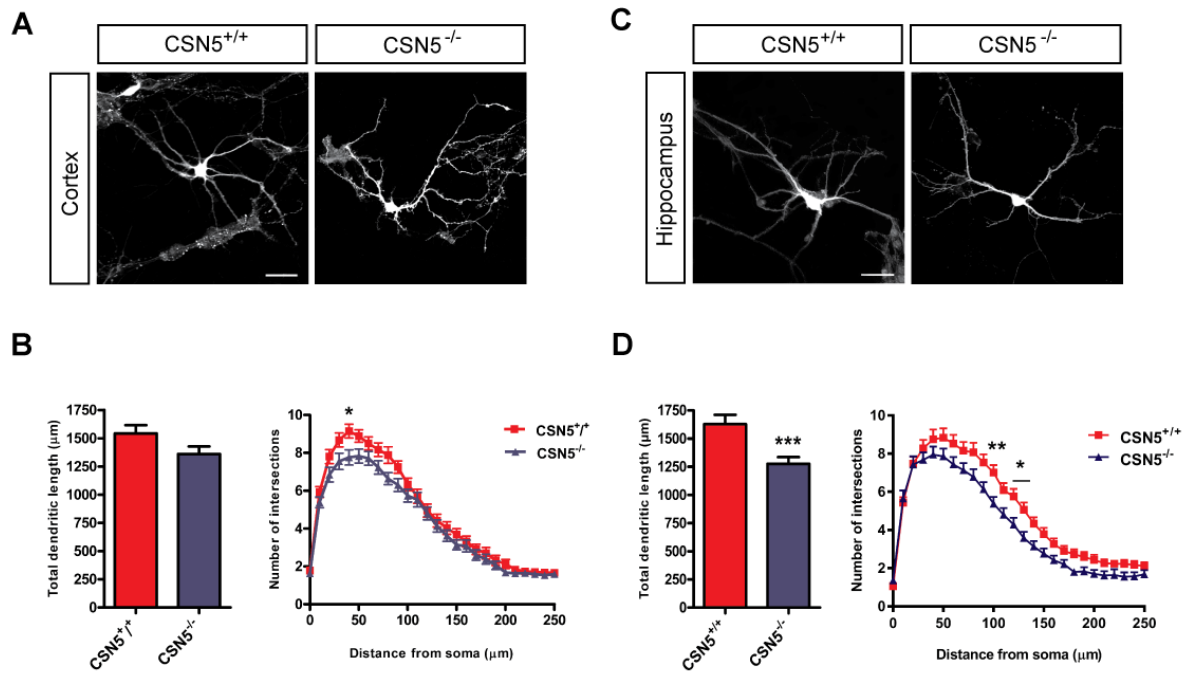


Figure 11: Loss of CSN5 affects dendritic development *in vitro*

Representative pictures of (**A**) primary cortical neurons and (**C**) primary hippocampal neurons transfected at DIV 7 with *Cre* recombinase and fixed at DIV 14. GFP was cotransfected for visualization. Scale bar represents 50 µm. (**B**) Quantification of total dendritic length (for B: $CSN5^{+/+}$ = 1544 ± 72.39 µm vs $CSN5^{-/-}$ = 1361 ± 65.73 µm, p = 0.0647, t = 1.868; for D: $CSN5^{+/+}$ = 1628 ± 82.63 µm vs $CSN^{-/-}$ = 1275 ± 60.2 µm, p = 0.0008, t = 3.452; mean ± SEM, two-tailed unpaired Student´s *t* test) and quantification of dendritic complexity by Sholl analysis (for B: F (29, 2940) = 0.9839, p = 0.4899; number of intersections: 40µm, p =0.0132; for D: F (29, 2940) = 1.276, p = 0.1475, number of intersections: 100µm, p = 0.0093; 120µm, p = 0.0371; 130µm p = 0.0343; mean ± SEM, two-way ANOVA , treatment x radius interaction, Bonferroni post-test) of cortical neurons and (**D**) hippocampal neurons transfected with *Cre* recombinase. (**B**, **D**) *p < 0.05, **p < 0.01, ***p < 0.001, n = 50 neurons per condition

The COP9 signalosome was shown to carry a variety of functions besides acting as a deneddylase on cullin proteins (Chamovitz 2009). We consequently wondered if the effects we saw in our first experiment were mediated by the loss of deneddylating activity of CSN5 or by loss of its other referred functions. In order to answer this question we established an experimental setup in which we would deplete cells of CSN5 *in vitro* and simultaneously transfect either $CSN5^{wt}$ or $CSN5^{D151N}$, a mutated form that lacks the deneddylase activity but retains all the other functions of the CSN (Ambroggio, Rees, and Deshaies 2004; Peth et al. 2007; Y. Y. Choo et al. 2011b). Consequently, primary neurons from $CSN5^{lox/lox}$ mice were transfected at DIV 4 with *Cre* and either $CSN5^{wt}$ or $CSN5^{D151N}$. At DIV10, dendritic morphology was analyzed as before. We observed that rescue of CSN5 function with $CSN5^{wt}$ restored total dendritic length, however results were not significant. No significant difference in total

dendritic length or dendritic complexity was observed between CSN5$^{+/+}$ and CSN5$^{-/-}$ + CSN5^{D151N} neurons (Figure 12).

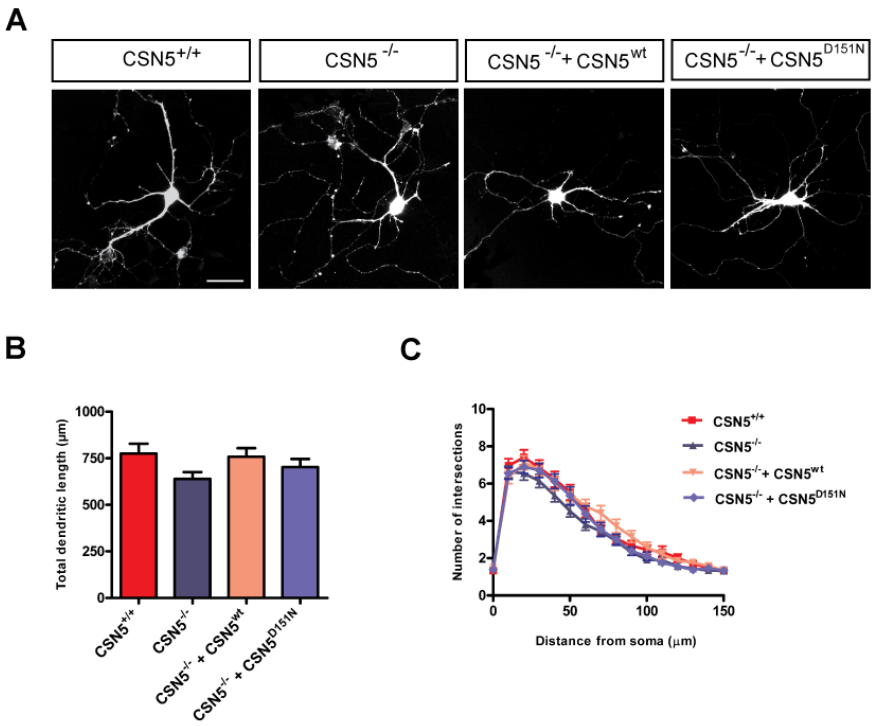


Figure 12: Knockdown and rescue of CSN5 during neuronal development *in vitro*

(**A**) Representative pictures of primary hippocampal neurons transfected at DIV4 with the indicated constructs and fixed at DIV10. GFP was cotransfected for visualization. Scale bar represents 50 µm.
(**B**) Quantification of total dendritic length ($F = 1.846$, $p = 0.1395$, CSN5$^{+/+}$ = 775.4 ± 52.44 µm, CSN5$^{-/-}$ = 639.5 ± 35.95 µm, CSN$^{-/-}$ + CSN5^{wt} = 758.4 ± 46.4 µm, CSN5$^{-/-}$ + CSN5^{D151N} = 703.0 ± 42.9 µm; mean ± SEM, one-way ANOVA and Bonferroni post-test) and (**C**) quantification of dendritic complexity by Sholl analysis of neurons treated as described in (**A**) ($F = 1.504$, $p = 0.2147$, treatment x radius interaction; mean ± SEM, repeated measures (RM) two-way ANOVA and Bonferroni post-test). (**B**,**C**) n ≥ 50 neurons per condition

4.2 CSN5 controls neuronal migration as demonstrated by *in utero* electroporation

The *in utero* electroporation technique allows for manipulating neuronal precursors *in vivo* without disturbing the physiological environment of the developing cortex (Saito and Nakatsuji 2001; Tabata and Nakajima 2001). In order to investigate CSN5 function during cortex formation we performed *in utero* electroporation on $CSN5^{lox/lox}$ mice at E13.5, i.e. injected and electroporated constructs with a *Cre* containing plasmid or empty vector as control, a GFP reporter for *Cre* activity and RFP for visualization. At postnatal day 2 (P2) brains were dissected out and fixed (Figure 13). As apparent from cortical cross-sections, loss of CSN5 leads to a defect in radial migration of pyramidal neurons, evident in an overmigration phenotype. Control neurons migrated to the upper cortical layers and were distributed throughout layer II and III of the cortex, whereas neurons expressing GFP as a reporter of *Cre* activity migrated too far and were lined up at the apical border of layer II, directly adjacent to layer I (the former marginal zone).

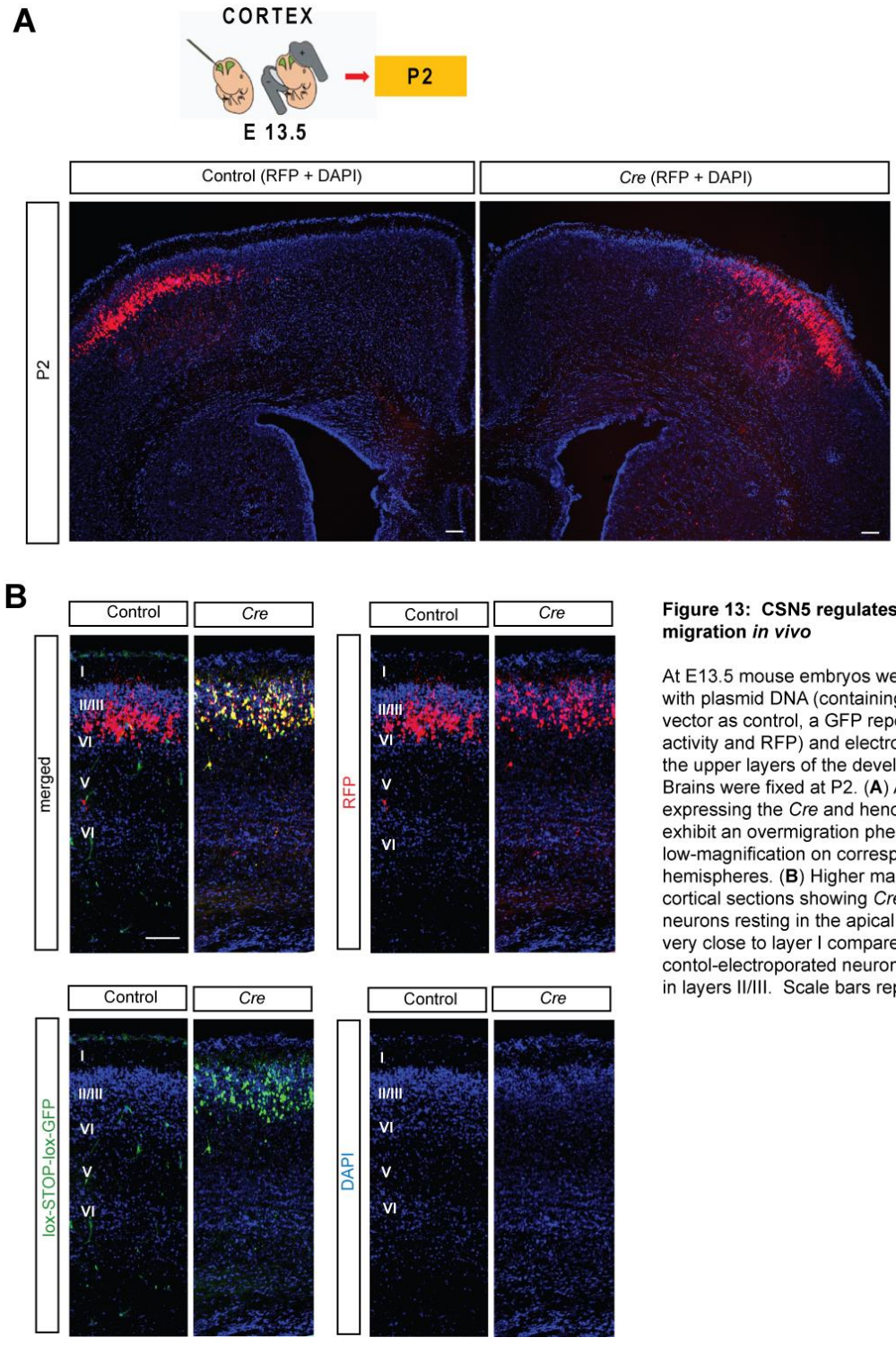


Figure 13: CSN5 regulates neuronal migration *in vivo*

At E13.5 mouse embryos were microinjected with plasmid DNA (containing *Cre* or empty vector as control, a GFP reporter for *Cre* activity and RFP) and electroporated to target the upper layers of the developing neocortex. Brains were fixed at P2. (**A**) At P2 neurons expressing the *Cre* and hence lacking CSN5 exhibit an overmigration phenotype shown in low-magnification on corresponding brain hemispheres. (**B**) Higher magnfication of cortical sections showing *Cre*-electroporated neurons resting in the apical part of the cortex very close to layer I compared to contol-electroporated neurons residing deeper in layers II/III. Scale bars represent 100µm.

4.3 Loss of CSN5 *in vitro* has more pronounced effects on neuronal morphology if induced early

In the previous experiments, loss of CSN5 *in vitro* was induced at E17.5 +DIV4 or +DIV7, respectively, and resulted in minor or no reduction of total dendritic length and neuronal complexity. Knockdown of CSN5 by *in utero* electroporation was performed at E13.5 and resulted in an apparent overmigration phenotype. This observation raises the question if knockdown of CSN5 earlier in embryonic development affects only neuronal migration or changes neuronal morphology, too, if these cells were left to develop *in vitro*. To answer this question we carried out *in utero* electroporation at E13.5 as before but harvested the electroporated cells four days later, at E17.5, from the embryonic brain and left them to develop *in vitro*. At DIV 14 morphological analysis revealed a more pronounced reduction of both total dendritic length and dendritic arborization in the *Cre* - transfected neurons (Figure 14).

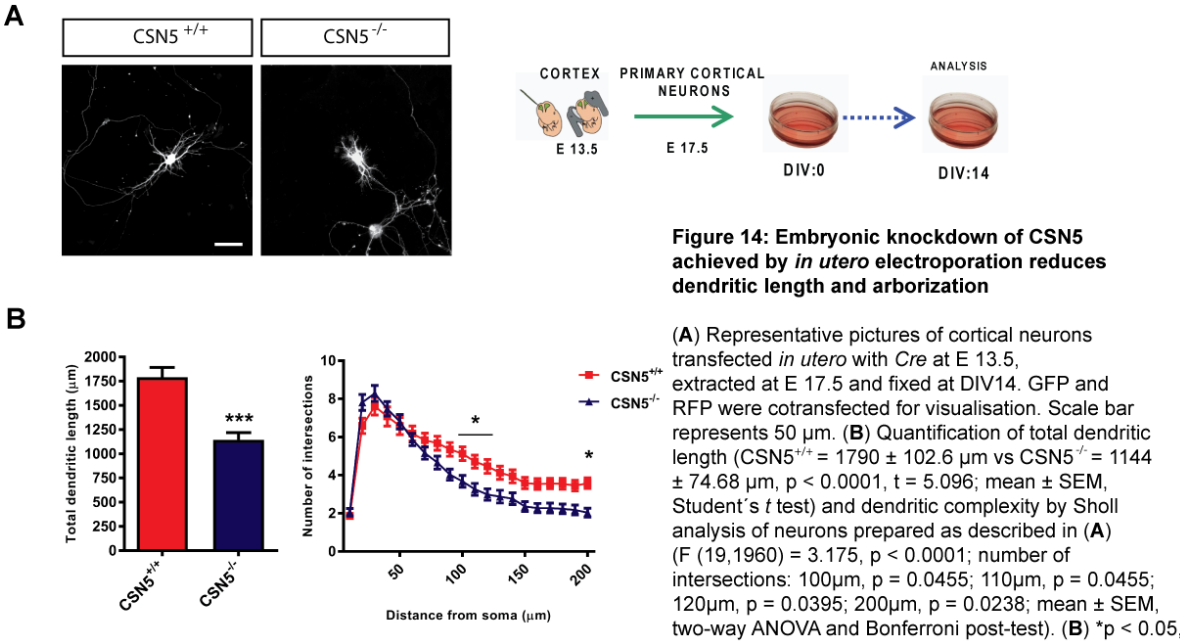


Figure 14: Embryonic knockdown of CSN5 achieved by *in utero* electroporation reduces dendritic length and arborization

(**A**) Representative pictures of cortical neurons transfected *in utero* with *Cre* at E 13.5, extracted at E 17.5 and fixed at DIV14. GFP and RFP were cotransfected for visualisation. Scale bar represents 50 µm. (**B**) Quantification of total dendritic length ($CSN5^{+/+}$ = 1790 ± 102.6 µm vs $CSN5^{-/-}$ = 1144 ± 74.68 µm, $p < 0.0001$, $t = 5.096$; mean ± SEM, Student´s *t* test) and dendritic complexity by Sholl analysis of neurons prepared as described in (**A**) ($F(19,1960) = 3.175$, $p < 0.0001$; number of intersections: 100µm, $p = 0.0455$; 110µm, $p = 0.0455$; 120µm, $p = 0.0395$; 200µm, $p = 0.0238$; mean ± SEM, two-way ANOVA and Bonferroni post-test). (**B**) *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n = 50 neurons per condition

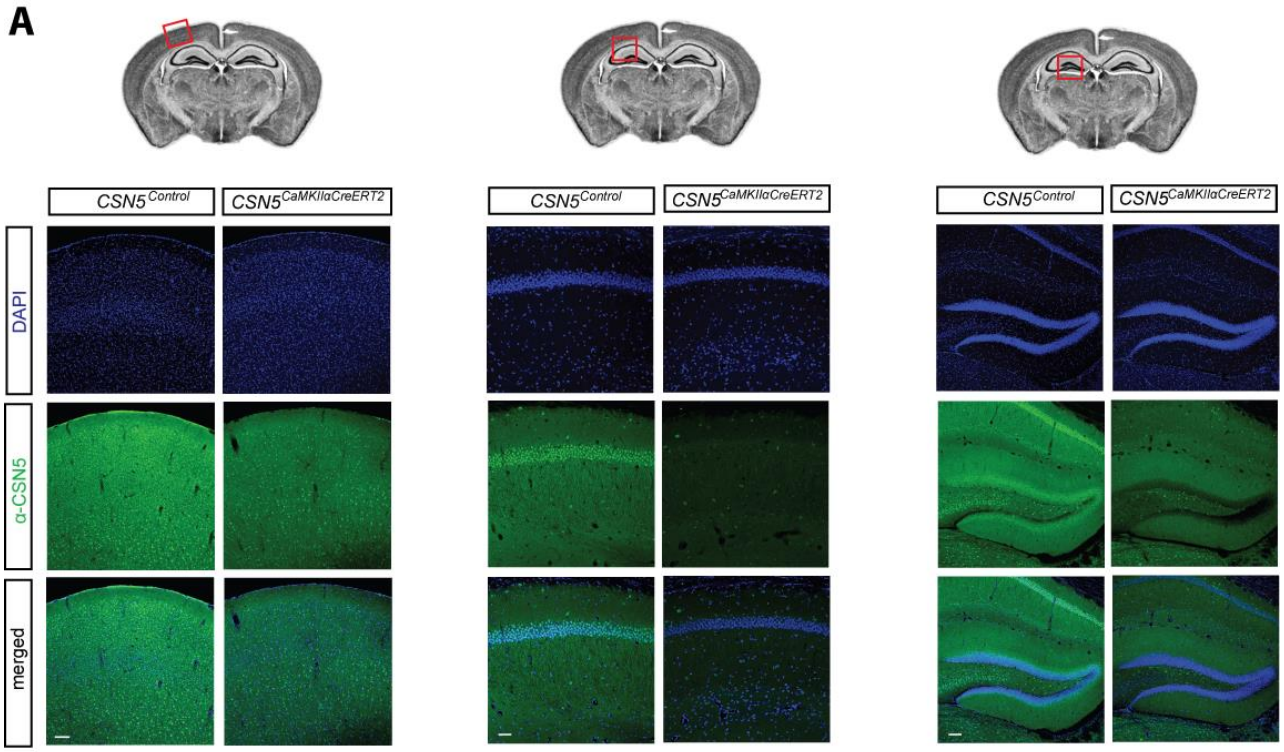
4.4 Studying COP9 function *in vivo* employing the *Cre/LoxP* System

Conditional *CSN5*$^{CaMKII\alpha CreERT2}$ KO mouse

The effects we observed *in vitro* raise the question whether loss of CSN5 would lead to similar results *in vivo*.

As previously described, constitutive knockout of CSN5 is embryonic lethal (Tomoda et al. 2004). To overcome this limitation we made use of the *Cre-LoxP* system. Ca^{2+}/calmodulin-dependent protein kinase II alpha (CaMKIIα) belongs to the serine/threonine protein kinases family (Wayman et al. 2008), is crucial for calcium signaling and involved in several aspects of plasticity at glutamatergic synapses such as hippocampal long-term potentiation (LTP) and spatial learning (Lamsa et al. 2007). By breeding CSN5$^{lox/lox}$ animals to a *CaMKIIα-CreERT2* mouse strain we established a tamoxifen-inducible knockdown of CSN5 confined to glutamatergic neurons of the forebrain, i.e. particularly the cortex and hippocampus.

At the age of eight weeks, after transitioning normally through embryonic and postnatal stage, animals were injected with 1mg tamoxifen twice per day for 5 consecutive days in order to induce *Cre* activity and subsequent loss of CSN5 in the forebrain. As control littermates we used CSN5$^{lox/lox}$ mice that were equally administered tamoxifen but lacked the inducible *CreERT2* recombinase as confirmed by genotyping. After a washout of 1-2 weeks, effectiveness of the knockout was proven by immohistochemistry, *in situ* hybridization and immunoblotting (Figure 15). In *CSN5*$^{CaMKII\alpha CreERT2}$ mice, CSN5 as detected by immunofluorescence was reduced in the cortex and, more pronounced due to a higher density of neurons and less glial cells, in the CA1 region and the dentate gyrus of the hippocampus (Figure 15.A). Similar results were obtained on the mRNA level, as shown by *in situ* hybridization (Figure 15.B). As an additional readout, western blot on protein extracts from cortex and hippocampus showed a strong reduction of CSN5, an increase of neddylation of cullin 1 and accumulation of NEDD8 in the KO (Figure 15.C). Downstream targets of NEDD8 (p27, IkB and cyclin D3) are enriched in the KO to a varying extent (Figure 15.D).



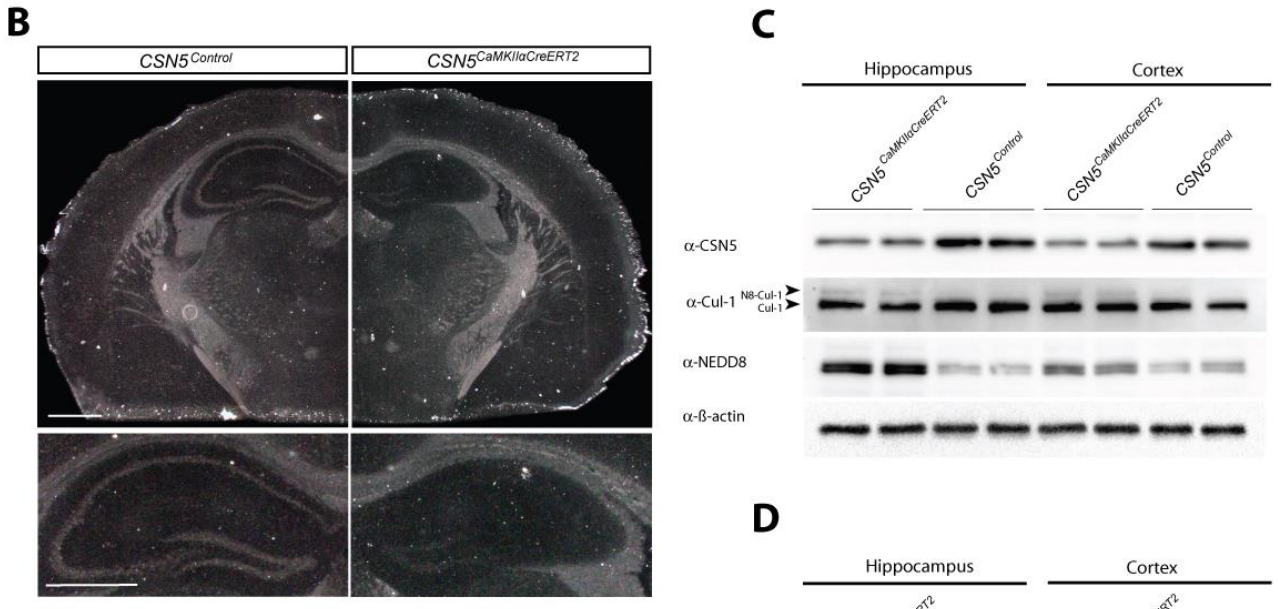


Figure 15: Brain-region specific knockdown of CSN5 mediated by *CaMKIIαCreERT2*

Eight-week-old *CaMKIIαCreERT2*-positive animals and wild-type littermates were injected with 1mg tamoxifen, twice per day for 5 consecutive days.
One week later *CaMKIIαCreERT2*-mediated knockdown of CSN5 was verified by (**A**) immunohistochemistry showing details from the cortex, CA1 region and dentate gyrus. Scale bar represents 100 µm. (**B**) Whole brain and hippocampus coronar sections show reduced CSN5 mRNA expression as detected by *in situ* hypridization. Scale bar represents 1mm. Both demonstrate a strong signal loss in the hippocampus and weaker signal loss in the cortex due to lower neuron density and higher portion of glial cells. (**C**) As an additional readout, CSN5 protein content was determined by western blot. Protein levels of CSN5 in the cortex and hippocampus are reduced in the KO compared to control. An increase in neddylation of cullin 1 is reflected in a faint upper band which is missing in the control. Nedd8 is accumulated in the KO and detected as bound to cullin proteins. (**D**) Downstream targets of NEDD8 are enriched in the KO to a varying extent.

4.5 CSN5 controls dendrite development *in vivo*

Golgi stain is a reliable method to visualize neuronal morphology *in situ* and has previously been used in knockout studies of genes controlling dendritic development (Cubelos et al. 2010). In order analyze dendritic morphology *in situ* we performed Golgi stain on whole brain sections obtained from our $CSN5^{CaMKII\alpha CreERT2}$ mice (Figure 16). *In situ* tracings reveal that total dendritic length in both cortical and hippocampal neurons and dendritic complexity in cortical neurons is significantly reduced in $CSN5^{CaMKII\alpha CreERT2}$ mice versus $CSN5^{Control}$.

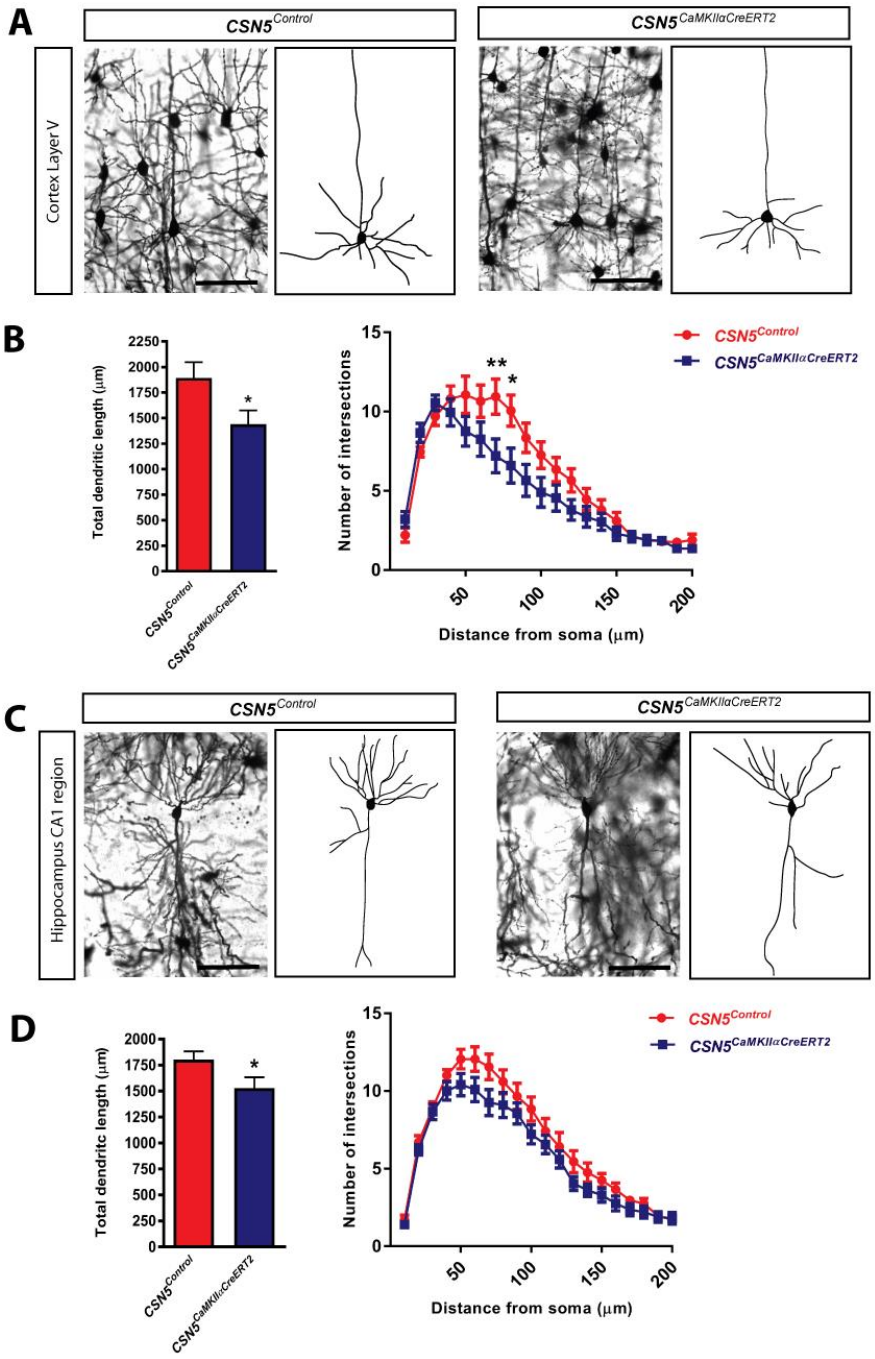


Figure 16: Knockdown of CSN5 decreases dendritic length and complexity in adult *CSN5*^{CaMKIIαCreERT2} mice

Representative pictures of pyramidal neurons in the (**A**) cortex (layer V) and the (**C**) hippocampus (CA1 region) and their corresponding tracings. Scale bar represents 100 µm.
Quantification of total dendritic length (for **B** cortical neurons: *CSN5*^{CaMKIIαCreERT2} = 1893 ± 153.7 µm vs *CSN5*^{Control} = 1440 ± 134.6 µm, p = 0.0327, t = 2.216; for **D** hippocampal neurons: *CSN5*^{CaMKIIαCreERT2} = 1804 ± 78.27 µm vs *CSN5*^{Control} = 1530 ± 102.3 µm, p = 0.0398, t = 2.129; mean ± SEM, two-tailed unpaired Student´s *t* test).
Quantification of dendritic complexity by Sholl analysis (for **B** cortical neurons: F (19, 760) = 1.988, p = 0.0074; number of intersections: 70µm, p = 0.0049; 80µm, p = 0.0146; for **D** hippocampal neurons F (19, 760) = 0.5975, p = 0.9099; mean ± SEM, two-way ANOVA , treatment x radius interaction, Bonferroni post-test).
*p < 0.05, **p<0.01.
n = 20 neurons per condition recruited from 3 mice per genotype.

4.6 Spine density is not affected by loss of CSN5 *in vivo*

It was shown previously that inhibition of neddylation affects spine development (Vogl et al. 2015). Conversely, consequences of inhibition of CSN5 for spine maintenance *in vivo* have been untested. By breeding *CSN5*$^{CaMKII\alpha CreERT2}$ animals to a *Thy1-eGFP* mouse strain (G. Feng et al. 2000) we obtained mice that express GFP in the same subset of neurons that lack CSN5, including CA1 pyramidal neurons in the hippocampus and pyramidal neurons in the cortex. This allowed for accurate analysis of dendritic spines using confocal microscopy. Spine number on secondary apical dendrites of both cortical and hippocampal pyramidal neurons was analyzed and found unchanged between KO and control (Figure 17).

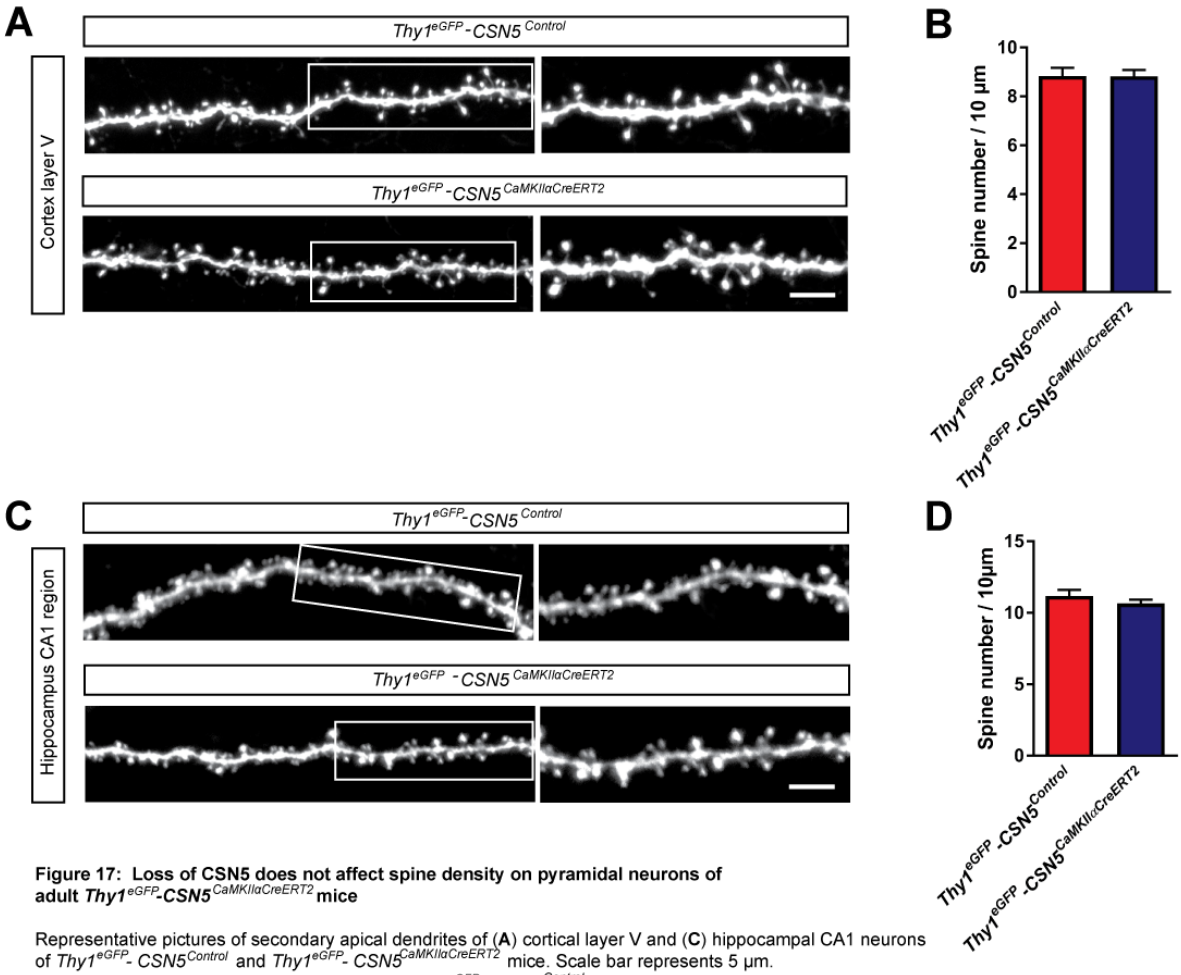


Figure 17: Loss of CSN5 does not affect spine density on pyramidal neurons of adult *Thy1*^{eGFP}-*CSN5*$^{CaMKII\alpha CreERT2}$ mice

Representative pictures of secondary apical dendrites of (**A**) cortical layer V and (**C**) hippocampal CA1 neurons of *Thy1*^{eGFP}- *CSN5*^{Control} and *Thy1*^{eGFP}- *CSN5*$^{CaMKII\alpha CreERT2}$ mice. Scale bar represents 5 µm. Quantification of spine density on (**B**) cortical (*Thy1*^{eGFP} - *CSN5*^{Control} = 8.841 ± 0.3293 vs *Tyh1*^{eGFP} - *CSN5*$^{CaMKII\alpha CreERT2}$ = 8.827 ± 0.2582, p = 0.9719, t = 0.03541) and (**D**) hippocampal neurons (*Thy1*^{eGFP} - *CSN5*^{Control} = 11.19 ± 0.407 vs *Thy1*^{eGFP} - *CSN5*$^{CaMKII\alpha CreERT2}$ = 10.66 ± 0.2594, p = 0.2561, t = 1.147). Values represent mean ± SEM, two-tailed unpaired Student´s *t* test, n ≥ 25 neurons recruited from ≥ 5 mice per genotype.

4.7 Behavioral assessment of *CSN5*$^{CaMKII\alpha CreERT2}$ mice

As changes of dendritic morphology were shown to be associated with cognitive deterioration (Kaufmann and Moser 2000) we subjected *CSN5*^{Control} and *CSN5*$^{CaMKII\alpha CreERT2}$ mice to a comprehensive battery of behavioral tests.

In the open field test (Figure 18.A), *CSN5*$^{CaMKII\alpha CreERT2}$ mice exhibited increased immobility time (*CSN5*^{Control} = 723 ± 20.65s vs *CSN5*$^{CaMKII\alpha CreERT2}$ = 850.7 ± 46.92s; t = 2.316, p = 0.0302, mean ± SEM, two-tailed unpaired Student´s *t* test), reduced distance travelled (*CSN5*^{Control} = 6598 ± 209.9cm vs *CSN5*$^{CaMKII\alpha CreERT2}$ = 5456 ± 416.1cm; t = 2.431, p = 0.0237, mean ± SEM, two-tailed unpaired Student´s *t* test) and hypolocomotion throughout the entire 30 min test duration (two-way RM-ANOVA, genotype x time interaction F(5, 110) = 0.822, p = 0.5366, genotype F(1,22) = 5.91, p = 0.0237, time F(5, 110) = 15.64, p < 0.0001; Bonferroni post hoc test: 1-5 min p = 0.6842, 6-10 min p = 0.1636, 11-15 min p = 0.2126, 16-20 min p = 0.8688, 21-25 min p = 0.2244, 26-30 min p = 0.0247).

Spatial, hippocampus-dependent memory performance was assessed with the water cross maze (WCM) and did not differ between the two groups (two-way RM-ANOVA, accuracy: time × genotype interaction, p = 0.2995, F (4, 72) = 1.245; genotype, p = 0.9483, F (1, 18) = 0.004317; time, p < 0.0001, F (4, 72) = 18.57; Bonferroni post hoc test, accuracy: day 1, p = 0.8303; day 2-5, p > 0.9999). No difference was observed during the re-learning paradigm (two-way RM-ANOVA, accuracy: time × genotype interaction, p = 0.9195, F (2, 36) = 0.08411; genotype, p = 0.3501, F (1, 18) = 0.9201; time, p < 0.0001, F (2, 36) = 82.01; Bonferroni post hoc test, accuracy: day 1-3, p > 0.9999) (Figure 18.B).

Short term memory performance was tested with the radial arm maze and not found compromised (two-way RM-ANOVA, accuracy: time × genotype interaction, p = 0.7970, F (3, 42) = 0.3393; genotype, p = 0.5040, F (1, 14) = 0.4704; time, p = 0.6321, F (3, 42) = 0.5789; Bonferroni post hoc test, accuracy: avg day 1-3 and day 4-6, p > 0.9999) (Figure 18.C).

The fear conditioning experiment in which animals were re-exposed to the tone 24 h after conditioning and to the context 48h after conditioning revealed no deficits in both cue- and context-dependent fear memories, as expressed by the percentage of time that was spent freezing (contextual fear memory: $CSN5^{Control}$ = 19.33 ± 3.932% vs $CSN5^{CaMKII\alpha CreERT2}$ = 30.4 ± 5.603%; t = 1.617, p = 0.1282; auditory fear memory: $CSN5^{Control}$ = 25.96 ± 2.647% vs $CSN5^{CaMKII\alpha CreERT2}$ = 32.7 ± 4.369%; t = 1.359, p = 0.1974; mean ± SEM, two-tailed unpaired Student´s *t* test) (Figure 18.D). Stress coping behavior was evaluated in the tail suspension test and found equal between the two groups, as expressed by the percentage of time that the animals spent struggling ($CSN5^{Control}$ = 32.33 ± 1.504% vs $CSN5^{CaMKII\alpha CreERT2}$ = 32.04 ± 1.173%; t = 0.1537, p = 0.8794; mean ± SEM, two-tailed unpaired Student´s *t* test) (Figure 18.E). Body weight of $CSN5^{CaMKII\alpha CreERT2}$ animals was found increased compared to $CSN5^{Control}$ before, during and after the behavioral testing (two-way RM-ANOVA, accuracy: time × genotype interaction, p = 0.0017, F (2, 28) = 8.076; genotype, p = 0.0010, F (1, 14) = 17.02; time, p < 0.0001, F (2, 28) = 17.16; Bonferroni post hoc test, accuracy: day 1, p = 0.0281; day 6, p = 0.0241, day 28, p < 0.0001) (Figure 18.F).

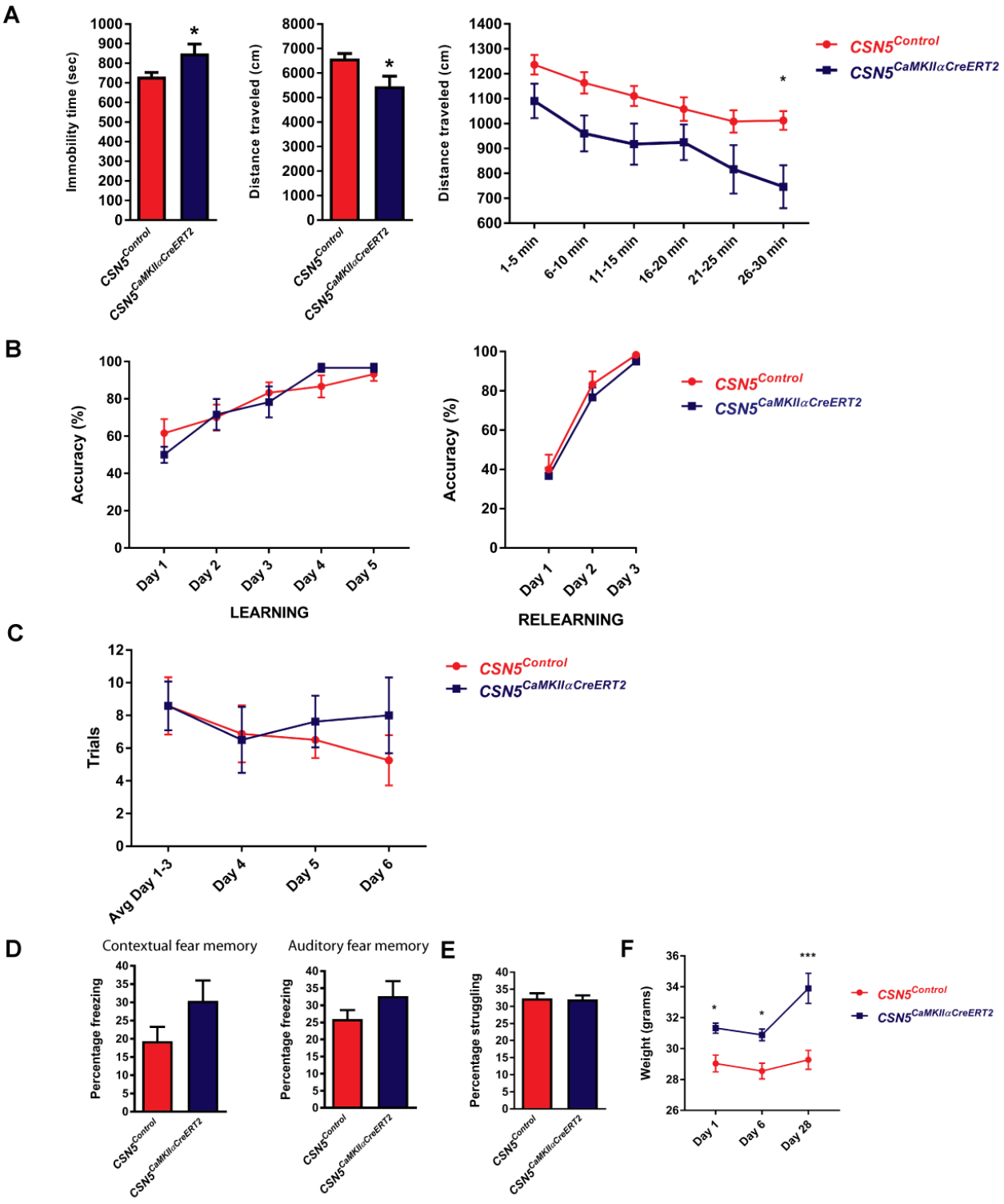


Figure 18: Behavioral testing of *CSN5*$^{CaMKII\alpha CreERT2}$ mice

(**A**) Locomotor activity measured as immobility time and distance travelled (both total and per time interval) was reduced in *CSN5*$^{CaMKII\alpha CreERT2}$ mice compared to control littermates.
(**B**) Spatial memory performance evaluated in the water-cross maze did not differ between the two groups.
(**C**) Short term memory assessed in the radial arm maze was found equal among the two groups.
(**D**) Contextual and auditory fear conditioning was not affected within the two groups.
(**E**) Stress coping behavior evaluated in the tail suspension test was found equal in the two groups.
(**F**) Body weight of *CSN5*$^{CaMKII\alpha CreERT2}$ mice was found higher at the beginning, during and after the behavioral testing phase.
Values represent mean + SEM, *p<0.05, **p<0.01, ***p<0.001. Student´s *t* test for the distance travelled and immobility time in (**A**) and for (**D**) and (**E**).
Two-way RM-ANOVA and Bonferroni post hoc test for (**A,** time intervals), (**B**), (**C**) and (**F**).
n = 12 mice per genotype in (**A**), (**B**) and (**E**).
n = 8 mice per genotype in (**C**), (**D**) and (**F**).

4.8 Embryonic knockdown of CSN5 confined to the central nervous system *in vivo* is associated with embryonic lethality

Based on the observations from our *in utero* electroporation experiments performed at E13.5 we aimed for knockdown of CSN5 *in vivo* in the early embryonic stages of brain development. In order to do so we bred $CSN5^{lox/lox}$ animals to a *NestinCre* and *NexCre* mouse line, respectively. The Nestin promoter and neural enhancer drives *Cre* expression in neuronal and glial precursors, i.e. proliferating cells, as early as embryonic day 10.5 (Tronche et al., 1999; Dubois et al., 2006). The helix-loop-helix transcription factor Nex (also known as NeuroD6/Math2) is a marker of embryonic cortical neuronal precursors and is expressed starting at E11.5 (Wu et al., 2005). In the adult brain Nex is expressed in mature glutamatergic cortical neurons. Accordingly, *NexCre*-mediated recombination is essentially restricted to postmitotic glutamatergic neurons of the telencephalon: the olfactory bulb, projection neurons of the cortex, pyramidal neurons of the CA1 and CA3 regions of the cornus ammoni, granule cells of dentate gyrus, glutamatergic mossy cells of the hilus of the dentate gyrus and the cortical-related amygdaloid nuclei (Goebbels et al., 2006).

Thus, the observations from this experiment result either from early, i.e. in proliferating precursors, or later, i.e. in post-mitotic cells, embryonic knockdown of CSN5 *in vivo.*

Interestingly, we were not able to obtain $CSN5^{NestinCre}$ –positive animals or embryos despite repeated breedings, which leads us to believe that the *NestinCre* mediated loss of CSN5 in these animals is early embryonic lethal.

In contrast, we were able to obtain $CSN5^{NexCre}$ embryos but not viable newborn animals. Analysis of morphology on the embryos showed no alterations in size, shape or symmetry (Figure 19). However, we found that some animals from this cohort died early at postnatal day 1 (P1), i.e. within hours after birth, without significant reason. This leads us to believe that *NexCre* mediated loss of CSN5 starting around E11.5 in post-mitotic glutamatergic neurons may cause functional alteration of electrophysiological properties or disruption of brain circuitry, which ultimately results in late embryonic or early postnatal death.

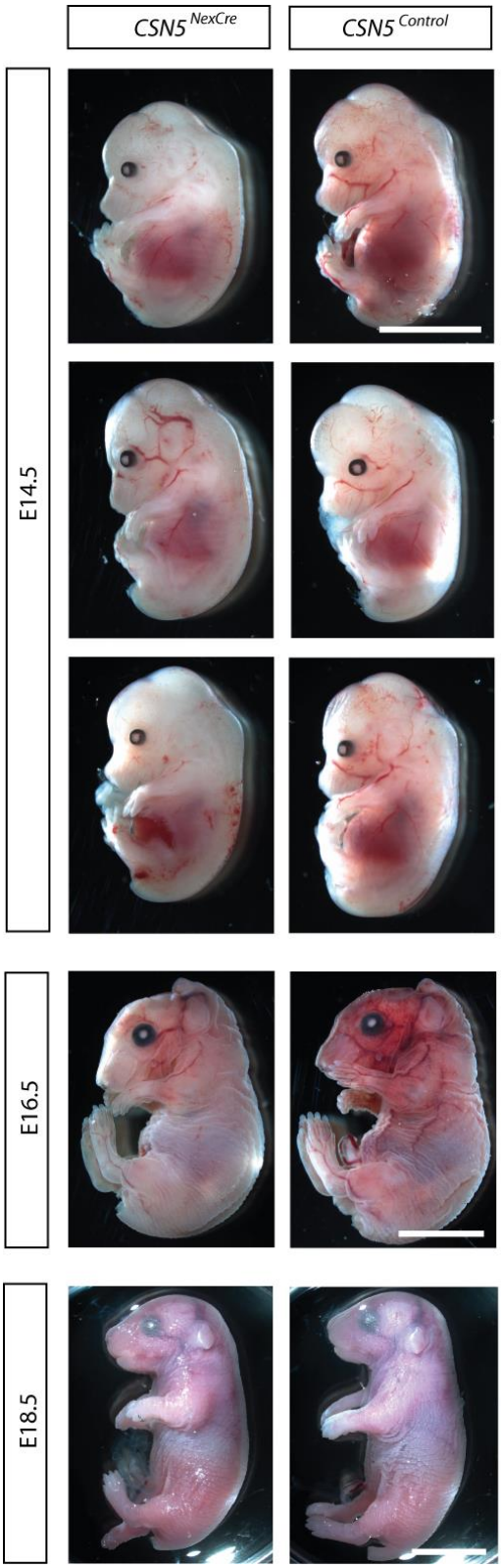


Figure 19: Aspect of *CSN5*^{NexCre} embryos during development *in utero*

Binocular microscope images of CSN5^{NexCre} embryos and *CSN5*^{Control} embryos taken at E14.5, E16.5 and E18.5. There are no conspicuous changes in size, symmetry or shape of the body. As demonstrated, *CSN5*^{NexCre} embryos can remain viable at least until E18.5. Scale bar represents 5mm.

5. DISCUSSION

The purpose of the present work was to characterize the role of the COP9 signalosome in the mouse brain with focus on neuronal morphology, embryonic brain development, cognitive function and stress-coping behavior by disrupting its catalytic subunit CSN5. The COP9 signalosome has been studied extensively in various tissues and cell lines and was found involved in diverse developmental and physiological processes including, but not limited to embryonic development, immune responses, cell cycle control, and cardiovascular function (Lykke-Andersen et al. 2003; Su et al. 2011; Tomoda et al. 2004; Panattoni et al. 2008; Lei et al. 2011). In the brain, it has been implicated in a number of neurological diseases (Wang et al. 2015; Oono et.al. 2004; Akanima et. al. 2003; Potocki et al. 2000; Potocki et al. 1999; Elsea et al. 1999). CSN5 was shown to co-localize with Nedd8 during murine embryogenesis (Carrabino et. al 2004) and, in the brain, Nedd8 was shown to be expressed throughout embryonic development and critically involved in maintenance and maturation of spines via the synaptic protein PSD95 (Vogl et al. 2015). Being a critical regulator of Nedd8, the role of CSN in the mouse brain for the most part has remained unexplored.

5.1 Dendritic morphology is controlled by CSN *in vitro* and *in vivo*

It was shown previously in *Drosophila* that the COP9 signalosome regulates dendritic development in larval peripheral nervous system (PNS) neurons by either stimulating or repressing dendritic branching via control of different cullins (Djagaeva and Doronkin 2009b; Djagaeva and Doronkin 2009a). In this work we show that knockdown of CSN5, the catalytic center of CSN, is associated with reduced dendritic length and complexity in mouse pyramidal neurons *in vitro* and in *vivo*. As opposed to the observations in *Drosophila* we did not notice excessive branching.

Interestingly, the reduction of dendritic length and dendritic complexity was greater when loss of CSN5 was induced *in utero*. This observation hints at a stronger regulatory function during the embryonic phase of brain development than in the postnatal state – a conclusion which is supported by studies that found CSN5 involved in neuronal differentiation. Mechanistically, it was shown recently that CSN5 interacts with Copine1 (CPNE1), a calcium-dependent phospholipid-binding protein that plays a role in calcium-mediated intracellular processes (Yoo et al. 2018). Its loss in primary neural stem cells derived from mouse embryonic hippocampus was associated with decreased proliferation and multi-lineage differentiation potential, and downregulated mTOR signaling (T. H. Kim et al. 2018). In the mouse brain, CPNE1 was found higher expressed in earlier embryonic stages (i.e. E11 and E15) compared to postnatal and adult stages. As we performed *in utero* electroporation at E 13.5 and observed a stronger reduction of dendritic morphology than in later transfected primary neurons and the *CSN5*$^{CamKII\alpha CreERT2}$ mouse line it may be argued that CSN5 exerts its regulatory function during embryonic brain development via CPNE-1. Complementary to our experiments in which knockdown of CSN5 reduced dendritic morphology, overexpression of both CPNE1 and CSN5 in Nestin-positive HiB5 cells effectively increases neurite outgrowth, suggesting that CSN5 positively regulates the neuronal differentiation ability of CPNE1 (Yoo et al. 2018).

The anaphase-promoting complex (APC) is a large multi-subunit E3 ubiquitin ligase that contains the cullin homologue apc2 (Yamano 2019; Z. Zhou et al. 2016; Alfieri, Zhang, and Barford 2017). In addition to cullin1 and cullin3, which mediate the effects of CSN on dendritic morphogenesis in *Drosophila* (Djagaeva and Doronkin 2009b; Djagaeva and Doronkin 2009a), anaphase promoting complex 2 (apc2) may constitute another target of CSN. Although apc2 lacks the carboxyl-terminal Nedd8 consensus sequence and is therefore functionally distinct from the other cullins (Brown and Jackson 2015), it was shown that the COP9 signalosome interacts with APC independently of Nedd8 (Kob et al. 2009). In cerebellar granule neurons, shRNA mediated knockdown of CDC20-APC decreases total dendrite length *in vitro* and *in vivo* (A. H. Kim et al. 2009), which is consistent with our results from the knockdown of CSN in pyramidal neurons. Further evidence is needed to

elucidate the extent of CSN interaction with APC and its implications for the development of neuronal morphology.

5.2 Assessment of spine density and cognitive performance upon disruption of CSN in glutamatergic pyramidal neurons

It was reported previously from our laboratory that inhibition of neddylation in the adult mouse brain via knockdown of NAE1 by an inducible *CaMKIIαCreERT2* results in severe reduction of spine density and size in both cortical and hippocampal pyramidal neurons and is associated with specific cognitive deterioration (Vogl et al. 2015). In the present work we identified that inhibition of deneddylation via knockdown of CSN5 by an inducible *CaMKIIαCreERT2* does not affect spine density on cortical or hippocampal pyramidal neurons. A comprehensive battery of behavioral tests revealed reduced locomotor activity and increased body weight in the KO but no further specific cognitive deterioration, especially no compromise of spatial or working memory, as would have been expected given the localization of these functions in the cortex and hippocampus. This finding hints at a rather marginal role of the COP9 signalosome for spine maintenance in the adult brain or, alternatively, at the presence of sufficient compensatory mechanisms. Respective the moderately increased body weight of our KO group compared to control, it cannot be answered if this increase is attributable i) to the expression of the *Cre*, ii) to the deletion of the transgene or iii) to an increase in sedentary behavior as suggested by the open field test independent of the expression of the *Cre* or the deletion of the transgene. Various effects on metabolism and physiology induced by the *Cre*-based technology have been reported (Harno, Cottrell, and White 2013). Although reduced body weight was found a general feature in the *Nestin-Cre* mouse line and can be attributed to a mild hypopituitarism (Giusti et al. 2014; Galichet, Lovell-Badge, and Rizzoti 2010), the finding of increased body weight in *CaMKIIα-CreERT2* mice is new and was not described in the initial report by Erdmann et al. (2007).

5.3 CSN exhibits a developmental stage dependency

We show that knockdown of CSN5 by *in utero* electroporation, apart from reducing dendritic arborization, disturbs cortical layering, apparent in an overmigration phenotype. Perturbations in cortical layering result from defective neuronal migration during the formation of the cortical plate. Migration is coordinated through rearrangement of the cellular cytoskeleton and the cell membrane proteins (which involve cell surface receptors, cell adhesion proteins and intracellular signalling cascades) (Hippenmeyer 2014; Walsh and Goffinet 2000; Lui, Hansen, and Kriegstein 2011). The extracellular protein reelin which is secreted by Cajal–Retzius cells in the marginal zone of the developing cortex is significant for neuronal migration during cortical development. Disruption of reelin function causes disintegration of the layered cortical cytoarchitecture (Tissir and Goffinet 2003; Förster et al. 2006; Lambert de Rouvroit and Goffinet 2001). Reelin itself was found to be controlled through ubiquitin-dependent degradation (Arnaud, Ballif, and Cooper 2003). Specifically, it was found that cullin 5 which serves as core scaffold for ubiquitin ligases (Okumura et al. 2016) and is modified by Nedd8 (Hori et al. 1999) regulates neuron positioning via Disabled-1 (Dab1) (L. Feng et al. 2007; Simó, Jossin, and Cooper 2010). Cullin-based ubiquitin ligases are regulated by the COP9 signalosome (Y. Y. Choo et al. 2011b; Merlet et al. 2009; Cavadini et al. 2016; Z. Zhu et al. 2019; Lamsoul et al. 2016). Our finding of defective neuronal migration in consequence of disruption of the COP9 signalosome lets us hypothesize that impaired deneddylation may affect the functioning of cullin-RING ligases and thus perturb downstream targets such as Dab1 or brain-specific kinase 2 (BRSK2). BRSK2 is a human serine/threonine-proteine kinase, highly expressed in the mammalian forebrain and was associated with neurodevelopmental disorders (Hiatt et al. 2019). It was shown that CSN5 interacts with BRSK2 and promotes its ubiquitin-dependent degradation (J. Zhou et al. 2012). Interestingly, BRSK2 shares high sequence homology with two mice SAD (synapses of amphids defective) kinases. SAD kinases have emerged as key regulators of neuronal polarization, axon

arborization and synapse maturation (Kishi et. al. 2005; Xing et al. 2013; Lilley et al. 2014; Barnes et al. 2007; Wu et al. 2015). Knockdown of both SAD kinases in mice causes defects in neuronal polarity and death within 2 hours after birth (Kishi et. al. 2005). It was shown that CSN5 co-localizes with BRSK2 in the perinuclear region, promotes its ubiquitination and proteasome-dependent degradation. CSN5 also promotes also cell cycle progression in a BRSK-dependent manner (J. Zhou et al. 2012). These findings are well in line with our observation of newborn *NexCre* animals dying at P1 and may suggest impaired circuitry formation as a possible cause.

Brain-1 and -2 (Brn-1/-2) are transcriptions factors that are expressed in the developing neocortex (He et. al 1989). Disruption of both Brn-1 and Brn-1 leads to hypoplastic neocortex with inversion, and selective knockdown of Brn-1 causes renal malformation and is lethal within 3 days after birth (McEvilly et. al. 2002). Homozygous deletion of Brn-2 does not impair neuroblast proliferation, generation of postmitotic neurons or lateral migration to correct loci but is lethal within 10 days after birth due to failure of differentiation of migratory precursor cells into mature neurosecretory neurons in the paraventricular nuclei (PVN) and the supraoptic nuclei (SO) of the hypothalamus and the posterior pituitary gland. The subsequent death of these neurons results from inability to activate genes encoding regulatory neuropeptides or to make correct axonal projections (Nakai et al. 1995; Schonemann et al. 1995). In fact, Brn-2 protein is found exclusively in postmitotic cells, indicating that, in the developing neocortex, Brn-2 may be involved in the maturation process of immature neuronal cells (Hagino-Yamagishi et. al. 1997). As CSN5 was shown to bind Brn-2 (Huang et al. 2005) it may be argued that the effects we observed after knockdown of CSN5 in the embryonic brain in part result from impaired interaction of Brn-2 with CSN5.

5.4 Need for further investigation beyond deneddylation of cullins

The COP9 signalosome has been described primarily as a deneddylator of cullins, whereas DEN1 (NEDP1) is thought of as a deneddylator of non-cullin proteins (Chan et al. 2008; Gan-Erdene et al. 2003; Cope and Deshaies 2003; Wolf, Zhou, and Wee 2003). Apart from cullins, only p53, p73, EGFR, PINK1, parkin and PSD95 were verified as substrates of the Nedd8 pathway (Harper 2004; Oberst et. al. 2005; Oved et al. 2006; Um et al. 2012; Choo et al. 2012; Vogl et al. 2015). Bearing in mind that CSN function is not limited to protease activity (Chamovitz 2009; Wei, Serino, and Deng 2008), the mechanisms of regulation of neuronal development by the COP9 signalosome remain to be elucidated. Mechanistically, it may be that i) executing proteins constitute downstream targets of E3 ubiquitin-RING ligases, ii) additional proteins (other than cullins) are deneddylated by CSN and/or iii) CSN exerts its function in neuronal development as a transcriptional regulator or corepressor.

6. APPENDIX

6.1 Index of Figures

6.2 Index of Tables

7. BIBLIOGRAPHY

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