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Multiple functions of neddylation in neuronal development

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SUMMARY/ZUSAMMENFASSUNG

Multiple functions of neddylation in neuronal development

Among the ubiquitin family members, ubiquitin and SUMO have been implicated in the regulation of various neurobiological processes such as growth, synaptic plasticity and neurotransmission. Accordingly, dysregulation of the ubiquitin-proteasome- and SUMO-system is associated with several neurological disorders in humans, such as Alzheimer's or Parkinson's disease. In contrast the function of other ubiquitin-like proteins in the brain, such as Nedd8, remains elusive. The aim of this work was to identify and characterize the functions of neddylation in neuronal development and in the adult mammalian brain as well as to uncover cellular targets and molecular mechanisms underlying Nedd8 function.

Analysis of the expression pattern of the different components of the neddylation pathway revealed a strong and ubiquitous expression in the central nervous system during embryonic development as well as in the adult mouse brain, and an enrichment of the components of the neddylation pathway in neurons.

To address the role of neddylation during neuronal development we employed dissociated primary neuronal cells as a well characterized model of neuronal growth. Blocking neddylation by genetic tools and via application of a pharmacological inhibitor during early developmental stages in hippocampal and cortical neurons resulted in strongly reduced growth and branching of the axon as well as decreased length and complexity of dendritic trees. In later stages blockade of neddylation impaired spine formation and synaptogenesis and resulted in increased generation of filopodia. Morphological defects elicited by blockade of the Nedd8 pathway led to a reduction in synaptic neurotransmission, measured in electrophysiological recordings of miniature excitatory post-synaptic potentials (mEPSPs). Further, we applied animal models to study cortical and hippocampal neuronal development, including *in utero* microinjection and electroporation of mouse embryos and injection of retroviruses into the dentate gyrus to label newborn neurons in the adult brain. This enabled us to further characterize the role of the Nedd8 pathway *in vivo* during axon and dendritic growth as well as spine formation and synaptogenesis, confirming the results obtained in primary neurons.

Beyond regulation of neuronal development in embryos and young postnatal stages, neddylation is also active in the mature synapse of adult animals. There it targets different pre- and post-synaptic proteins and controls spine maintenance and stability as shown by long-term live cell imaging studies of mature primary neurons and with inducible genetic tools, based on the Cre-ERT2/loxP system *in vivo*.

Moreover, functional and biochemical experiments identified PSD-95, a major scaffold protein of the post-synaptic density of spines, as a direct target of Nedd8 conjugation, mediating the effects of Nedd8 on spine formation during development and synapse stability in mature neurons. We showed that neddylation critically regulates oligo-/multimerization of PSD-95 thus controlling synaptic anchoring and function of PSD-95. Site-directed mutagenesis of specific lysine residues of PSD-95, that were identified to be conjugated to Nedd8 via mass spectrometry analysis, led to impaired spine formation and increased filopodia formation in primary neurons, establishing a crucial role for neddylation of PSD-95 in neuron development and functioning.

To further dissect the functions of neddylation *in vivo* during brain development and in the postnatal brain we developed conditional transgenic mouse models based on the Cre/loxP system. By breeding these to specific Cre mouse lines, expressing Cre-recombinase in defined and restricted cell populations in the brain and in specific neurotransmitter circuits, we are able to block the neddylation pathway in a spatio and temporal controlled manner. In the scope of this project a Ubc12 (Nedd8-conjugating enzyme) – LacZ reporter mouse line, a conditional knockout mouse of Appbp1 (one subunit of the Nedd8-activating enzyme (Nae1)) and a knockin mouse line, conditionally overexpressing a dominant-negative version of Ubc12 in the *ROSA26* locus, were generated using gene targeting via homologous recombination in embryonic stem (ES) cells.

In summary, we describe neddylation as a new post-translational protein modification in the mammalian brain, targeting multiple substrates and exerting specific functions during neuronal development as well as in the mature synapse.

Multiple Funktionen der Neddylierung in der neuronalen Entwicklung

Ubiquitin und SUMO gehören zur Familie der Ubiquitin-Proteine, die als post-translationale Proteinmodifikation viele wichtige neurobiologische Prozesse steuern und regulieren, wie z.B. das neuronale Wachstum, die synaptische Plastizität und die Signalübertragung an Synapsen. Die Fehlregulationen des Ubiquitin-26S Proteasom-Systems (UPS), sowie des Sumoylierungsweges werden als wichtige Faktoren bei der Entstehung neurologischer Erkrankungen, wie z.B. Alzheimer oder Parkinson, angesehen. Dahingegen ist die Rolle von Nedd8, eines verwandten Ubiquitin-ähnlichen Proteins, nahezu unbekannt und unerforscht.

Das Ziel dieser Arbeit war die Identifizierung und Charakterisierung der Funktionen der Neddylierung während der Entwicklung des zentralen Nervensystems und im adulten Säugetiergehirn. Im Verlauf der Arbeit konnten die zugrunde liegenden zellulären und molekularen Mechanismen aufgedeckt werden. Mittels biochemischer Methoden entdeckten wir mit Nedd8 modifizierte Nervenzell-spezifische Zielproteine und untersuchten die Funktion der neddylierten Proteine in der Nervenzellentwicklung.

Die Expressionsanalyse zeigte, dass die einzelnen Komponenten des Neddylierungssystems während der embryonalen Entwicklung des zentralen Nervensystems, wie auch im adulten Gehirn der Maus stark und weit verbreitet exprimiert sind. Des Weiteren konnte gezeigt werden, dass der Neddylierungsweg besonders in Nervenzellen aktiv ist.

In Zellkulturexperimenten mit dissoziierten primären Nervenzellen untersuchten wir die Rolle der Neddylierung im Nervenzellwachstum und bei der Synapsenentwicklung. Sowohl die pharmakologische, als auch die genetische Inhibiton des Neddylierungsweges, während der frühen Entwicklung hippokampaler und kortikaler Neurone, reduzierte das Wachstum und die Verzweigung der Axone und führte zu einer stark verringerten Länge und Komplexität der Dendriten. Die Blockade der Nedd8-Kaskade in späteren Entwicklungsstadien der Nervenzellen beeinträchtigte die Bildung der Synapsen und resultierte in einer vermehrten Ausbildung sogenannter dendritischer Filopodien. Als Folge der morphologischen Defekte, wurde mittels elektrophysiologischer Messungen von spontanen postsynaptischen Miniaturströmen (mEPSCs), eine verringerte synaptische Signalübertragung festgestellt.

Versuche in Tiermodellen bestätigten die Ergebnisse aus den Zellkulturexperimenten und erweiterten die Rolle des Nedd8 Weges während der Gehirnentwicklung. Mittels *in utero* Elektroporation von Mausembryonen und Applikation retroviraler

Expressionsvektoren zur Markierung neugeborener Neurone im Gyrus dentatus, wurde gezeigt, dass der Nedd8 Weg das Axon- und Dendritenwachstum, die Ausbildung dendritischer Spines und die Synapsenentwicklung hippokampaler und kortikaler Neurone *in vivo* steuert.

Darüber hinaus ist die Neddylierung auch in Synapsen ausgereifter Neurone im adulten Gehirn aktiv. Sie bewirkt dort die Modifizierung verschiedener prä- und postsynaptischer Proteine mit Nedd8 und kontrolliert so die Stabilität und Erhaltung dendritischer Spines. Dies konnte in langzeit 'Live-Cell Imaging' Studien in primären Neuronen und *in vivo* mittels induzierbarer Plasmide, basierend auf dem Cre-ERT2/loxP System, gezeigt werden.

Durch biochemische und funktionelle Experimente identifizierten wir PSD-95 als ein direktes neuronales Zielprotein, das mit Nedd8 modifiziert wird. PSD-95 ist ein wichtiges strukturgebendes Protein im komplexen Proteinnetzwerk in der postsynaptischen Membran, der sogenannten postsynaptischen Verdichtung ('postsynaptic density' (PSD)), das die Effekte von Nedd8 auf die Synapsenbildung und –erhaltung vermittelt. Die Modifizierung mit Nedd8 beeinflusst die Oligo-/Multimerisierung von PSD-95 Proteinen und kontrolliert somit die synaptische Verankerung von PSD-95 und dessen Funktion in der Postsynapse. Mittels Massenspektrometrie wurden spezifische Lysinreste im PSD-95 Protein identifiziert, die kovalent an Nedd8 konjugiert werden. Die gezielte Mutagenese dieser Lysinreste, führte zu einer verringerten Spine-Bildung und vermehrten Filopodienentwicklung in primären Neuronen, was auf eine grundlegende Funktion von neddyliertem PSD-95 in der Nervenzellentwicklung, insbesondere in Synaptogeneseprozessen, schließen lässt.

Zur detaillierteren Analyse der Funktionen der Neddylierung in der Gehirnentwicklung und im adulten postnatalen Gehirn *in vivo*, entwickelten wir konditionale transgene Mausmodelle des Nedd8 Weges auf der Grundlage des Cre/loxP Systems. Dabei ermöglicht das Cre/loxP System die örtlich und zeitlich begrenzte Ausschaltung der Neddylierungskaskade, z.B. in definierten Zellpopulationen oder Neurotransmittersystemen im Gehirn. In dieser Arbeit wurden folgende Mausmodelle mittels spezifischer genetischer Manipulation ('Gene Targeting'), über homologe Rekombination in embryonalen Stammzellen (ES Zellen), hergestellt und charakterisiert. Eine Ubc12 (Nedd8 Konjugationsenzym)-LacZ Reportermauslinie, eine konditionale Appbp1 (eine Untereinheit des Nedd8 Aktivierungsenzyms) 'Knockout' Maus und eine

’Knockin’ Mauslinie, in der die dominant-negative Form des Nedd8 Konjuagtionsenzyms Ubc12 im *ROSA26* Genlokus konditional überexprimiert wird.

Zusammengefasst konnten wir zeigen, dass die Neddylierung eine wichtige posttranslationale Proteinmodifikation im Säugetiergehirn darstellt, die die neuronale Entwicklung und die Funktion ausgereifter Synapsen steuert.

DECLARATION

All experiments shown in this PhD Thesis were performed by me, except otherwise stated. For details please see the table of contributions on pages 273-275. In addition, collaborations are also stated in Materials and Methods, Results and in the figure legends. These results are included in the thesis because they are part of a bigger experiment and are necessary to fully describe our findings. Furthermore, I used the term “we” and not “I” throughout this work to write it similar to a publication.

Annette Vogl

1. INTRODUCTION

1.1. General principles of neuronal development and function in the central nervous system

A central goal in neuroscience is to disentangle the molecular and cellular principles organizing neuronal development, synaptic communication and complex circuits finally governing behavior. Primate central nervous systems contain billions of neurons and even up to 10 times greater numbers of glial cells. In the human central nervous system the estimated 100 billion (10^{11}) neurons are interconnected via a predicted 1000x higher number (10^{14}) of synapses, which establish a highly organized but at the same time extremely plastic network (Kandel et al., 2000; Ullian et al., 2001; Noctor et al., 2007; Azevedo et al., 2009). During development, neurons acquire a highly polarized morphology, and this polarization of axon and dendrites underlies the ability of neurons to integrate and propagate information within the brain. Neurons communicate among each other and transmit information, encoded in electrical currents, via synapses, complex junctions established between pre-synaptic axon terminals and post-synaptic dendritic sites (or dendritic protrusions, called spines). The formation, maintenance and plastic remodelling of synapses are processes thought to underlie information storage as well as learning and memory. Dysregulation of processes that orchestrate neuronal migration, axon and dendrite formation, synaptogenesis, synapse maintenance and synaptic plasticity leads to severe neurodevelopmental, neuropsychiatric and neurodegenerative diseases (Fiala et al., 2002; Penzes et al., 2011; Emoto, 2011; Liu, 2011). The development and functions of neurons are studied using different *in vitro* cell culture systems and *in vivo* animal models, which will be summarized in the following sections.

1.1.1. Modeling neuronal development *in vitro* – characteristics of dissociated primary neurons in culture

A detailed understanding of the molecular mechanisms involved in specifying different steps of neuronal development that ultimately lead to the high complexity of neuronal networks requires an accurate dissection of the different developmental steps. Cultures of embryonic neural tissues such as organotypic cultures, re-aggregate cultures, and dissociated primary cell cultures recapitulate many aspects of neuronal development *in vivo*, including polarization (Dotti et al., 1988; Delima et al., 1997), maturation (Mains and Patterson, 1973; Seeds and Haffke, 1978; Trapp et al., 1979; Bartlett and Banker, 1984a) and synaptogenesis (Bornstein and Model, 1972; Trenkner and Sidman, 1977; Yavin and Yavin, 1977; Bartlett and Banker, 1984b). Neurons in cultures often follow a similar time course of development as their *in vivo* counterparts with regards to morphological as well as functional properties (Mains and Patterson, 1973; Spitzer and Lamborghini, 1976; Yavin and Yavin, 1977; Kozak, 1977; Huettner and Baughman, 1986; Ogura et al., 1987; Habets et al., 1987; Dotti et al., 1988; Murphy et al., 1992; Kuroda et al., 1992; Ichikawa et al., 1993; Powell et al., 1997). Moreover, primary neurons are readily accessible for manipulations, as e.g. transfection and viral-mediated gene delivery to alter gene expression and patch-clamp or extracellular recordings to characterize their electrophysiological properties (Segal, 1983; Maeda et al., 1995; Maeda et al., 1998; Gross and Kowalski, 1999; Jimbo et al., 1999; Segev et al., 2001; Ailles and Naldini, 2002; Beggs and Plenz, 2003; Beggs and Plenz, 2004; van Pelt et al., 2004; Jiang and Chen, 2006; Janas et al., 2006; Zhang et al., 2006; Zeitelhofer et al., 2007; Anliker et al., 2010).

Dissociated hippocampal and cortical neurons are among the best-characterized model systems to study different apects of neuronal development (Dotti et al., 1988). Since the first description of primary neuronal cell cultures by Banker and Cowan (Banker and Cowan, 1977), considerable progress has been made in refinement of the culture conditions and possibilities to manipulate gene expression in individual neurons (Goslin and Banker, 1991; de Hoop et al., 1997; Goslin et al., 1998; Kaech and Banker, 2006). Careful analysis of dissociated hippocampal and cortical neurons that grow and mature under controlled growth conditions, mainly free of unspecified external cues, led to the observation that these neurons can adopt spatially and functionally distinct dendritic and

axonal domains via a transition through several well-defined stages (Dotti et al., 1988; Delima et al., 1997; Bradke and Dotti, 2000b; Barnes and Polleux, 2009; Polleux and Snider, 2010) (Figure 1). At stage 1 (days in vitro (DIV) 0), freshly plated, unpolarized cells display intense lamellipodial and filopodial protrusive activity, which results in the emergence of stage 2 (DIV 1-2) neurons bearing multiple immature neurites. At stage 3 (DIV 2-4), a single neurite grows fastly to become the future axon, while the remaining neurites acquire dendritic identity. This first morphological sign of axon formation is a crucial hallmark of neuronal polarization, as it marks the initial break in symmetry during neuronal development (Goslin and Banker, 1989; Craig and Banker, 1994). Stage 4 (DIV 4-15) is characterized by extensive axon growth and dendritic growth and branching. By stage 5 (> DIV 15) neurons are terminally differentiated and reach functional polarization. Mature neurons form functional synaptic contacts among each other and generate electric currents (Segal, 1983; Bartlett and Banker, 1984b; Fletcher et al., 1994).

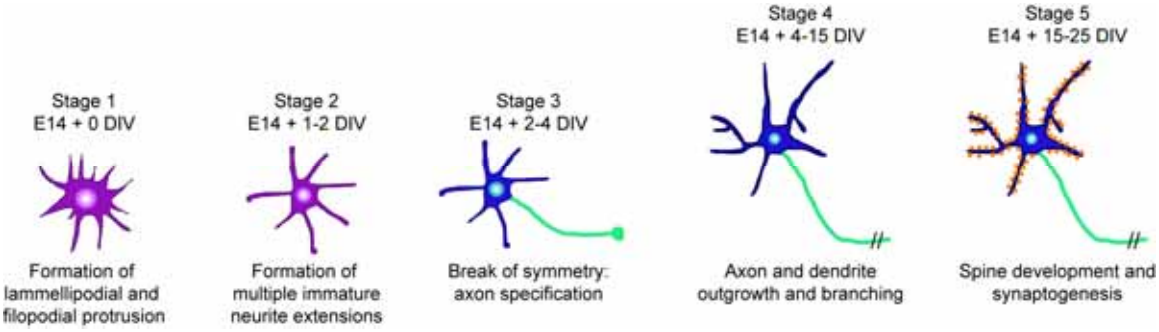


Figure 1: Development and polarization of primary neurons *in vitro*.

In dissociated neuronal cultures, postmitotic neurons isolated from the embryonic rat or mouse hippocampus and cortex transition through several defined stages, 1 to 5, to finally adopt their characteristic mature polarized neuronal morphology. Stage 1 (DIV 0): unpolarized, symmetric neurons freshly after plating, isolated from the embryonic hippocampus or cortex, are surrounded by lamellipodia and filopodia that display high protrusive dynamics. Stage 2 (DIV 1-2): neurons develop several minor immature neurites (purple). Stage 3 (DIV 2-4): neuronal symmetry breaks and the axon (green) is specified and grows faster than the remaining neurites that adopt dendritic identity (blue). Stage 4 (DIV 4-15): rapid and extensive axon and dendrite outgrowth. Stage 5 (> DIV 15): neurons are terminally differentiated into mature pyramidal neurons possessing functional synapses formed between dendritic spines and axonal boutons (orange spots on dendrites). (Adapted from Dotti et al., 1988; Barnes and Polleux, 2009.)

The cell-type composition and accessibility, as well as the applicable manipulations in hippocampal and cortical tissue account for the common and widespread use of dissociated hippocampal and cortical neurons as model systems of neuronal development. The population of nerve cells in the hippocampus is relatively homogeneous, with excitatory glutamatergic pyramidal cells accounting for approximately 90% of the neurons and GABA-ergic interneurons making up the remaining 10% of neurons (Bayer, 1980a; Bayer, 1980b; Benson et al., 1994; Soriano et al., 1994). In the cerebral (neo-) cortex, two morphologically distinguishable principal neuronal cell types can be found, excitatory glutamatergic pyramidal neurons and stellate (granule) cells, representing the GABA-ergic interneurons (Bayer et al., 1991; Marin-Padilla, 1992; Mcconnell, 1995; Super and Uylings, 2001). Since hippocampal and cortical cultures are routinely prepared from late-stage rodent embryos, mouse and rat E17.5-E18.5 embryos, the tissue contains relatively few glial cells, is easy to dissociate and the isolated neurons have a high survival rate. Neurons grown in culture are readily accessible for a variety of manipulations using sophisticated pharmacological, genetic or electrophysiological methods and protocols. On the one side, pharmacological inhibitors and activators of specific cellular pathways can be applied to a whole cell population that allows subsequent assessment and read-outs of the treatment. On the other side, gene expression can be altered in individual neurons at different developmental stages using calcium- or electroporation-based transfection methods as well as viral-mediated gene transfer. (Kaech and Banker, 2006; Jiang and Chen, 2006; Janas et al., 2006; Zeitelhofer et al., 2007; Zeitelhofer et al., 2009). By this means it is possible to overexpress particular proteins or to knock-down the expression of specific genes to study their function in the context of neuronal development (Zeringue and Constantine-Paton, 2004; Miller et al., 2005). Fluorescently tagged proteins are commonly employed to study the intracellular localization and cellular trafficking of proteins as well as for live cell imaging studies to analyse dynamic processes inside living neurons or as reporters for intracellular processes (Marshall et al., 1995; Stepanova et al., 2003; Huttelmaier et al., 2005; Jacobson et al., 2006; Heim et al., 2007; Bains and Heidenreich, 2009). Neuronal cultures prepared from mutant mice e.g. transgenic or knockout mice, allow the analysis of effects of specific gene alterations. Furthermore, neurons grown in culture enable the investigation of postnatal aspects of neural development of knockout (KO) animals that die at or shortly after birth from systemic defects (Okabe et al., 1997; Esteban et al., 2001). Since neurons establish functional networks in culture it is

possible to record electrophysiological changes, such as miniature excitatory/inhibitory post-synaptic currents (mE/IPSCs) or membrane properties, from neurons previously subjected to gene manipulation or pharmacological treatment (O'Brien et al., 1998; Bolton et al., 2000a; Bolton et al., 2000b; Copi et al., 2005; Szczot et al., 2010).

Although cell culture techniques have been successfully employed to address specific cell biological or functional questions and are thus invaluable to systematically study and dissect the complex processes building up mammalian nervous systems, they have clear limitations and drawbacks compared to *in vivo* models. Several developmental processes only occur in the intact living brain, e.g. neuronal migration to form the complex architecture of brain regions such as the cortex, hippocampus or cerebellum. Many neurodevelopmental processes, like cellular differentiation into specific cell types, neuronal and glial lineages and axon guidance, are controlled by external cues from the brain's microenvironment. Moreover, higher brain functions, such as learning and memory, emotional behavior or motor behaviour and the underlying synaptic circuits can only be analyzed in living organisms. Hence, animal models are indispensable to elucidate molecular mechanisms controlling brain development and neuronal functions as well as functional adaptations and plasticity of neuronal circuits *in vivo*. The following section describes the most important animal models and techniques that were used in the scope of this project.

1.1.2. Three approaches to study neuronal development and function *in vivo* – *in utero* electroporation of mouse embryos, retroviral injections to label adult newborn neurons and the generation of transgenic and knockout mice

Conclusions from studies of invertebrate and lower vertebrate nervous systems provide the basis for the analysis of complex mammalian brains. Mouse and rat are commonly used as model organisms to study neuronal development, neuronal structure, synaptic function and ultimately behaviour. They are small, relatively cheap in housing, easy to breed and many aspects of human neurobiological disorders can be modeled in mice and rats. In addition the upsurge of mouse genetic tools, that allowed the development of transgenic, knockout (KO) and knockin (KI) mouse lines, have revolutionized the way to study the influence of single genes in the context of whole organisms. In the present section different approaches will be described, that are employed to dissect and analyze gene function *in vivo* in the developing and adult mouse brain, including their advantages and disadvantages.

1.1.2.1. *In utero* electroporation technique

The *in utero* electroporation technique of mouse embryos is a fast, efficient and powerful tool to study the function of specific genes during development *in vivo*. The method was adapted from the *in ovo* electroporation system originally developed for gene transfer in chick embryos (Muramatsu et al., 1997; Funahashi et al., 1999; Itasaki et al., 1999; Momose et al., 1999; Tabata and Nakajima, 2001; Saito and Nakatsuji, 2001; Fukuchi-Shimogori and Grove, 2001; Oberg et al., 2002; Tabata and Nakajima, 2002; Takahashi et al., 2002; Saito, 2006; Langevin et al., 2007; Tabata and Nakajima, 2008; Saito, 2010). The protocol of *in utero* electroporation of mouse embryos was first developed to study the development of the cerebral cortex and was later adapted to also target additional brain and CNS regions, such as the hippocampus, cerebellum, striatum, neural retina, or the hypothalamus as well as the spinal cord (Saito and Nakatsuji, 2001; Saba et al., 2003; Ding et al., 2004; Hasegawa et al., 2004; Miyagi et al., 2004; Nakahira

and Yuasa, 2005; Saba et al., 2005; Molyneaux et al., 2005; Mizutani and Saito, 2005; Borrell et al., 2005; Nguyen et al., 2006; Kawauchi et al., 2006a; Matsuda and Cepko, 2007; Garcia-Frigola et al., 2007; Kataoka and Shimogori, 2008; Vue et al., 2009; Tsuchiya et al., 2009; Matsui et al., 2011).

During *in utero* electroporation, circular plasmid DNA is microinjected into the brain ventricle of E12.5-E15.5 mouse embryos *in utero* and square-wave electric pulses are delivered with forceps-type electrodes (Saito, 2006) (Figure 2).

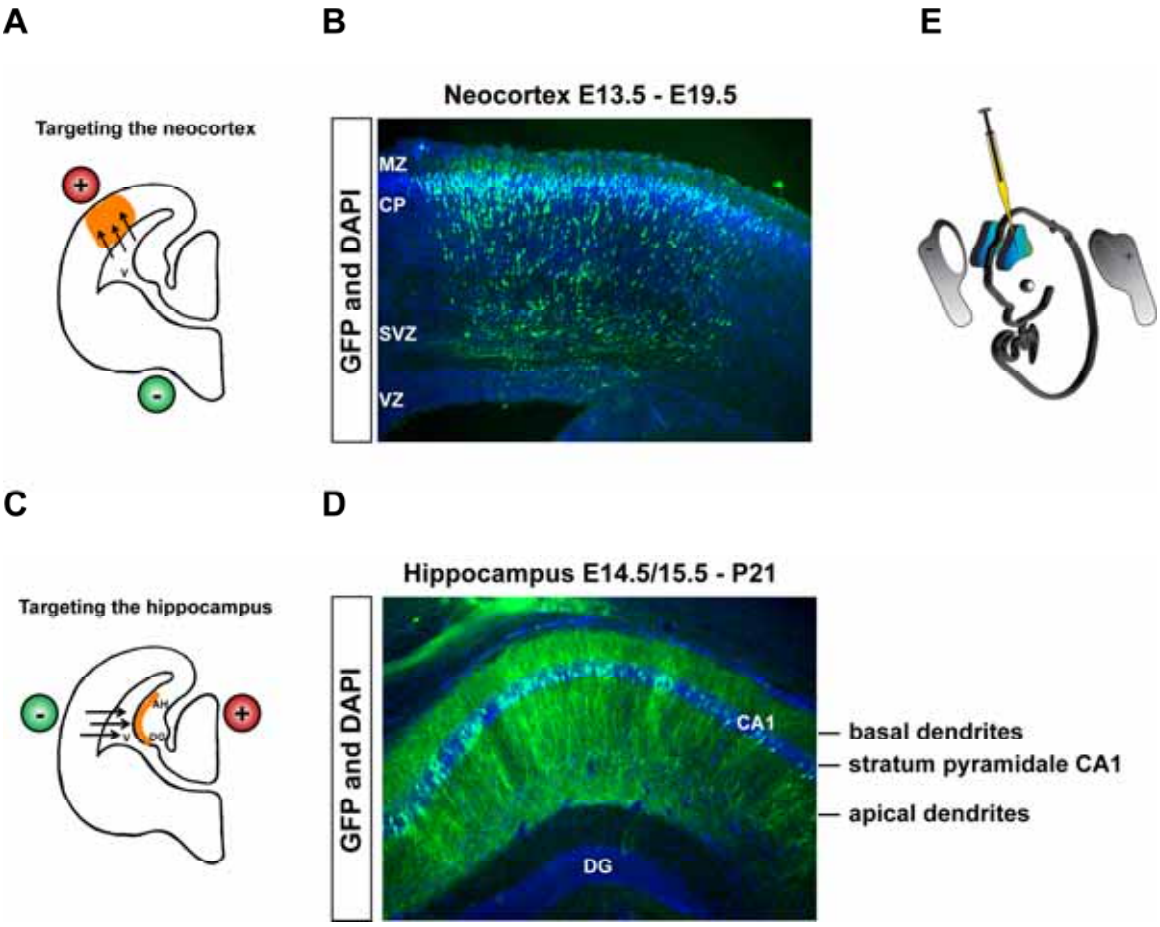


Figure 2: Targeting embryonic mouse central nervous system cells by *in utero* electroporation.

A) and **C)** Schematic overviews of the relationship between the region of vector transfection by *in utero* electroporation and the position of the electrodes (plus and minus pole); arrows indicate the direction of the electric current. Plasmid DNA, injected into the ventricle, is targeted to the restricted neuroepithelium shown in orange. The neocortical neuroepithelium is labeled at E13.5 in the ventral-to-dorsal direction. Whereas, the Ammonic neuroepithelium (hippocampus) is

targeted at E14.5-E15.5 in the lateral-to-medial direction. Photomicrographs show coronal sections, top is dorsal and left is lateral. AH, Ammon's horn, DG, Dentate gyrus, V, Ventricle. (Adapted from Nakahira and Yuasa, 2005.) **B)** and **D)** Green fluorescent protein (GFP) expression after electroporation. **B)** E13.5 mouse embryos were subjected to *in utero* electroporation targeting the neocortex and brains were fixed at E19.5; coronal sections were cut and nuclei were stained with DAPI (blue). GFP-expressing neuroblast cells are migrating from the VZ/SVZ (ventricular zone/subventricular zone) radially to form the cortical layers and differentiate into pyramidal neurons in the CP (cortical plate), below the MZ (marginal zone). **D)** E14.5-E15.5 mouse embryos were subjected to *in utero* electroporation targeting the neuroepithelium corresponding to Ammon's horn and brains were analyzed at P21. Coronal brain sections containing the hippocampus were cut and stained with DAPI (blue) to visualize cell nuclei. Cell bodies of GFP-expressing pyramidal neurons are found in CA1 stratum pyramidale of the hippocampus. Labeled neurons develop basal and apical dendritic trees, form synapses and integrate into the synaptic circuit in the hippocampus. **E)** Plasmid DNA is microinjected with glass capillaries into the lateral ventricles of E13.5 – E15.5 mouse embryos through the uterine wall. Then electric pulses are applied to the brain from outside the uterine wall with tweezer electrodes.

Electroporated embryos survive and develop normally in the uterus, they are later born and reared by their mother. Many of the transfected cells, that are located adjacent to the ventricle, are neuronal progenitors and their descendant neurons will express the transfected genes. Loss of function and gain of function experiments can be readily performed, by expressing shRNA constructs to knock down gene expression or dominant-negative versions of proteins and by overexpression of specific genes, respectively (Saito, 2006). Furthermore, combining *in utero* electroporation with the versatile Cre-ERT2/loxP system allows the functional analysis of particular genes in a spatio-temporal-controlled manner (Matsuda and Cepko, 2007; Kolk et al., 2011). Co-expression of fluorescent proteins allows the identification of transfected cells in the brain.

1.1.2.2. Retroviral injection experiments

To analyze the function of specific genes in mature postmitotic neurons of the adult mouse brain, delivery of genes into various brain regions can be achieved by viral-mediated gene transfer via stereotactic injections. Adeno-associated viruses (AAVs) and lentiviruses (LVs) are commonly used as vectors to infect adult neurons *in vivo*, to induce long-term gain of function as well as loss of function of specific genes, or even as therapeutic vectors (Kaplitt et al., 1994; Xiao et al., 1997; Bartlett et al., 1998; Trono, 2000; Peel and Klein, 2000; Watson et al., 2002; Baekelandt et al., 2002; Passini et al., 2005; Mandel et al., 2006; Wong et al., 2006; Lingor and Bahr, 2007; Husain et al., 2009; Kanninen et al., 2009; Di Benedetto et al., 2009). Transduction efficencies,

subtypes of neurons that are infected, cell toxicity and immune responses triggered by the viral capsule can greatly vary between the different viral serotypes available (Kajiwara et al., 1997; Baekelandt et al., 2003; Burger et al., 2004; Alba et al., 2005; Gao et al., 2005; Chen et al., 2005a; Schmidt et al., 2006).

In contrast to AAVs and LVs, retroviruses (RVs) can only infect actively dividing cells, e.g. neuronal progenitors in the adult brain, because their replication cycle relies on the stable integration into the host-genome. Hence, in the field of neuroscience, retroviruses are intensively used as vectors for gene delivery to study the process of adult neurogenesis in the rodent brain (Price et al., 1987; Lewis and Emerman, 1994; Carleton et al., 2003) (see later 1.1.4).

1.1.2.3. Generation of mouse mutants

The more laborious and time consuming generation of conventional or conditional knockout (KO) as well as transgenic mice allows the stable transmission of the modified DNA to the next generation. Conventional and conditional knockout mice harbor disruptions of specific genes whereas transgenic mice can also be engineered to overexpress the wild-type, mutant, or dominant-negative versions of a specific protein, to express shRNA constructs to knock-down specific genes or to express a reporter gene, such as a fluorescent protein, eGFP, or β-Galactosidase under the control of a cell-type specific promoter. Conventional KO mice, usually generated by the insertion of a selectable marker into the gene of interest (Smith et al., 1988; Capecchi, 1989; Robertson, 1991; Robbins, 1993), often die at or shortly after birth due to systemic defects, precluding analysis of the function of the specific gene deletion in the postnatal brain (Zimmer, 1992). To overcome this limitation a refined knockout strategy termed conditional mutagenesis was developed, by which conditional KO mice are generated using the flexible and versatile Cre/loxP system that allows the deletion of a gene in specific parts of the body or cells with specific identities, e.g. in specific cell populations of the brain (Kilby et al., 1993; Gu et al., 1994; Rajewsky et al., 1996; Kühn and Wurst, 2008; Friedel et al., 2011) (illustrated in Figure 3.B). In this approach, conditional gene inactivation is achieved by the cell type- or tissue-specific expression of the DNA recombinase Cre, while a critical part of the gene of interest is modified by insertion of recombinase recognition (loxP) sites (floxed mouse) (Sauer and Henderson, 1988; Sauer and Henderson, 1989; Orban et al., 1992; Kühn et al., 1995; Sauer, 1998; Ray et

al., 2000; Lewandoski, 2001; Friedel et al., 2011). In addition to a large collection of Cre transgenic mouse lines, that confer spatial control of the gene KO, temporal control can be accomplished by the use of Cre-ERT2 transgenic mice that allow inducible inactivation of floxed alleles in adult mice by administration of the drug Tamoxifen (Feil et al., 1996).

Gene targeting is generally carried out in mouse ES cells via homologous recombination (Smithies et al., 1984; Smithies et al., 1985; Capecchi, 1989; Robertson, 1991; Capecchi, 2001; Tessarollo et al., 2008). Correctly targeted ES cells are injected into blastocysts of wild-type mice to produce chimeric animals that can transmit the modified DNA allele to their offspring (Bradley et al., 1984) (Figure 3.A).

Large scale mutagenesis projects put great effort into the high throughput generation of conditional gene trap and conditional gene-targeted mice to achieve genome-wide systematic mutational and functional analysis of all mouse genes (Capecchi, 2005; Schnutgen et al., 2005; Stanford et al., 2006; Collins et al., 2007; Gondo et al., 2009; Guan et al., 2010; Wurst et al., 2010). Among them the European Conditional Mouse Mutagenesis Programme (EUCOMM), the US-based Knockout Mouse Project (KOMP) and the North American Conditional Mouse Mutagenesis Project (NORCOMM) plan for genome-wide mutagenesis in ES cells attempting to build a functional map of the mammalian genome.

A recently developed technique using recombinant engineered Zinc Finger nucleases to mediate site specific integration of modified DNA via homologous recombination in pronuclear injections or fertilized zygotes allows the single-step modification of the mouse germ line (Meyer et al., 2010; Urnov et al., 2010; Carbery et al., 2010; Goldberg et al., 2010; Cui et al., 2011; Li et al., 2011). This technological attempt holds great potential to achieve conditional mutagenesis and transgenesis in additional mammalian species such as rats, rabbits, pigs and cattle as well as in other important verterbrate model organisms, e.g. zebrafish (Doyon et al., 2008; Meng et al., 2008; Geurts et al., 2009; Jacob et al., 2010; Geurts and Moreno, 2010; Mashimo et al., 2010; Watanabe et al., 2010; Yu et al., 2011; Moreno et al., 2011; Cui et al., 2011; Flisikowska et al., 2011).

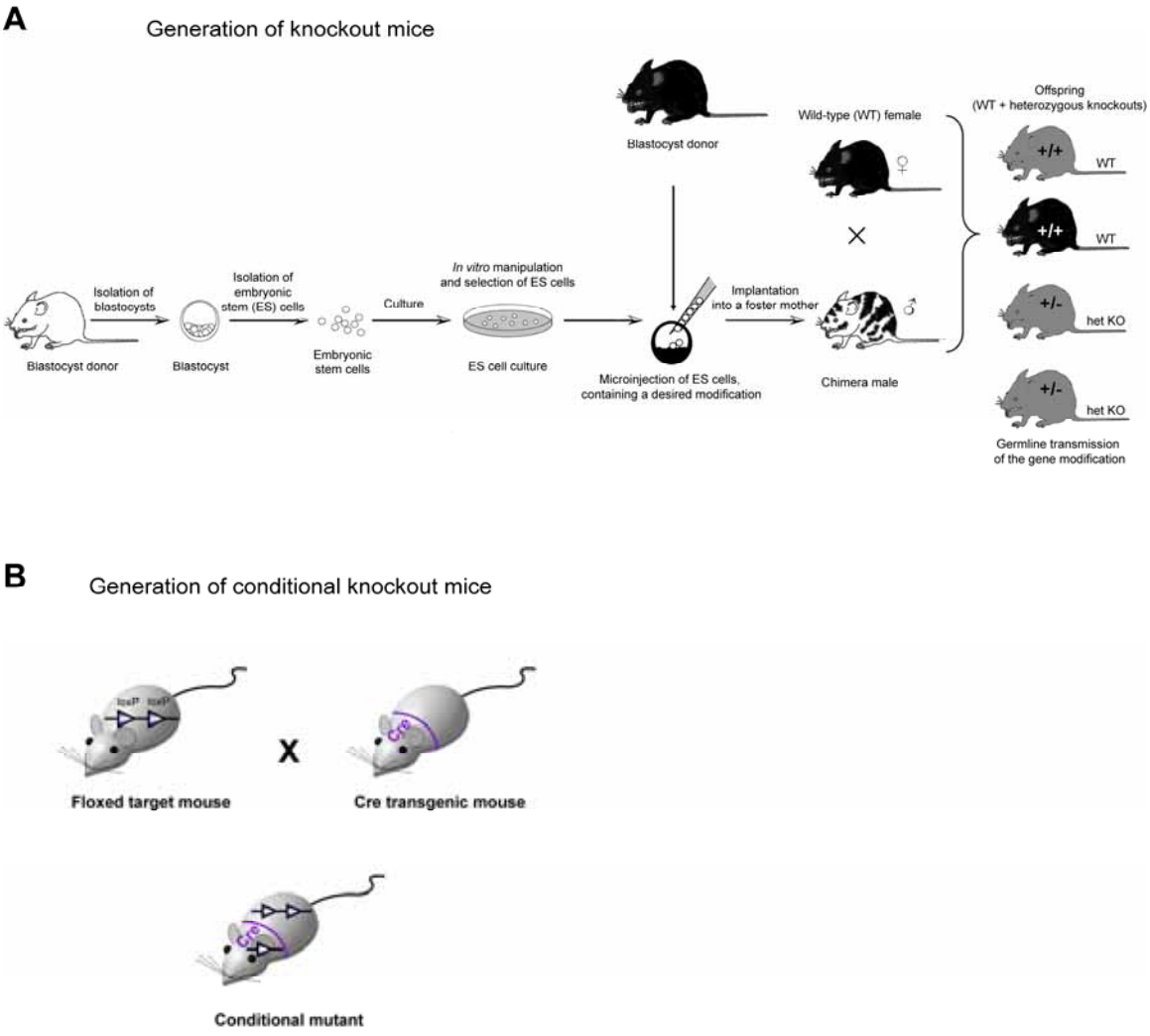


Figure 3: Generation of conditional knockout mice.

A) Generation of mouse germline chimeras from ES cells carrying a targeted mutation. The first step involves the isolation of a clonal ES cell line containing the desired mutation. This can be achieved by homologous recombination in ES cells. After electroporation of the targeting vector, containing homology sequences of the desired locus, positive and negative selction is used to enrich for ES cells containing the modified allele. The second step is to use those ES cells to generate chimeric mice, that are able to transmit the mutant allele to their offspring, by injection of the ES cells into blastocysts of donor females. To facilitate the identification of positive progeny, the ES cells and recipient blastocysts are derived from mice with distinguishable coat color alleles (e.g. ES cells from agouti brown mice and blastocysts from black mice). This allows the evaluation of the percentage of chimerism by coat color and the identification of ES cell contribution to the formation of germ line by the coat color of the progeny of the cimeric animals. **B)** Generation of conditional mouse mutants. To generate conditional mutants, one mouse strain harboring a loxP-flanked gene segment (floxed allele, upper left mouse) is crossed to mice expressing Cre recombinase in specific cell types, tissues or organs (upper right mouse). In the double transgenic offspring, conditional gene inactivation occurs via Cre-mediated excision of the floxed gene segment restricted to Cre-expressing cells (lower middle mouse).

Making use of transgenic and conditional KO mice, the role of specific genes in different aspects of neurogenesis and neuronal function has been elucidated, including guidance molecules, cell adhesion molecules, extracellular matrix proteins, signalling molecules, cytoskeleton-associated proteins, receptors, transcription factors and scaffold proteins (Gaveriaux-Ruff and Kieffer, 2007).

In the following sections a detailed analysis of the main features and open questions in key aspects of developmental neurobiology, including regulation of neuronal migration, polarization, axon and dendrite formation, spinogenesis and synaptogenesis, will be presented and discussed.

1.1.3. Principles and mechansims of neuronal development and migration in the developing cerebral cortex

The basic principles and molecular mechanisms of neuronal development and migration *in vivo* are best described in the developing neocortex. Mutations in key genes controlling neuronal migration and cortical layer formation result in severe neurodevelopmental diseases characterized by abnormalities and malformations of the human cortex including lissencephaly, pachygyria, polymicrogyria, heterotopia, and focal cortical dysplasia. For instance mutations in the genes *lissencaphaly 1* (*LIS1)* and *double cortin* (*DCX)* result in lissencephaly, a cortical dysplasia which means literally “smooth brain” and is characterized by the absence of the typical gyri and sulci that shape the human cortex, abnormal cortical layering, enlarged ventricles and neuronal mislocations (Barkovich et al., 1991; Dobyns and Truwit, 1995; Dobyns et al., 1996; Leventer et al., 2000; Kato and Dobyns, 2003; Liu, 2011).

Neurogenesis in the cortex during embryonic development starts with the generation of neurons from precursor cells near the ventricle and their coordinated migration to form the six distinct cortical layers. Pyramidal neurons, accounting for the most frequent neuronal cell type in the cortex, originate from precursors in the ventricular zone in the dorsal forebrain and migrate radially guided by the processes of radial-glial cells (RGCs) to reach their final positions in the cortical layers. On the other hand, interneurons are

born in the medial ganglionic eminence and lateral ganglionic eminence in the ventral forebrain and follow tangential migratory routes to the cortex (Walsh and Cepko, 1988; Bayer et al., 1991; Price et al., 1992; Tan et al., 1998; Walsh and Goffinet, 2000; Parnavelas, 2000; Maricich et al., 2001; Marin and Rubenstein, 2001; Gilmore and Herrup, 2001; Corbin et al., 2001; Nadarajah et al., 2001; Gupta et al., 2002; Nadarajah and Parnavelas, 2002; Anderson et al., 2002; Nadarajah et al., 2003; Marin and Rubenstein, 2003; Kriegstein and Noctor, 2004; Kriegstein, 2005; Ayala et al., 2007; Marin et al., 2010; Corbin and Butt, 2011) (for details see Figure 4.A).

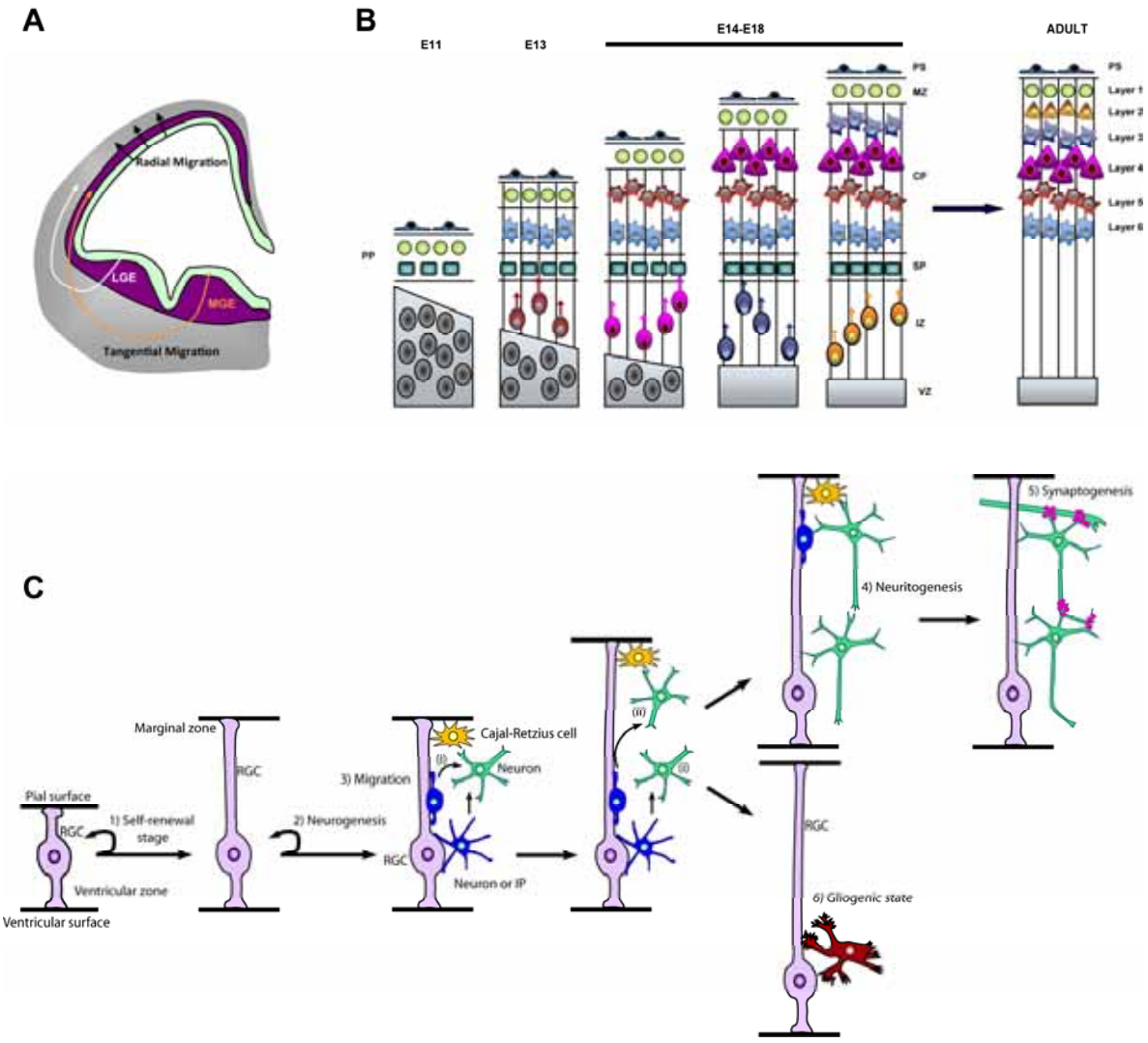


Figure 4: Cortical development.

A) Diagram of a coronal brain section of the developing cerebral cortex illustrating the migratory pathways of both excitatory neurons (radial) and inhibitory interneurons (tangential). Excitatory neurons and inhibitory neurons arise from the dorsal and ventral regions adjacent to the lateral ventricles, respectively. (Adapted from Liu, 2011.) **B)** The organization of the adult neocortex into distinct neuronal layers. Key developmental stages of the radial component of neocortical-layer formation are shown, which occurs mainly by migration of neuroblasts along radial glia (shown as vertical bars). At E11, the preplate (PP) is established by a postmitotic wave of neurons that migrate from the ventricular zone (VZ) to the pial surface (PS). By E13, a second postmitotic neuronal wave migrates 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. (Adapted from Gupta et al., 2002.) **C)** Key steps of neuronal differentiation in the mammalian brain. After neural tube closure, neuroepithelial cells proliferate and differentiate into radial glial cells (RGCs; also known as neural progenitor cells), which retain the potential to proliferate by symmetric cell division (**1**). Premature neurons or intermediate progenitors (IPs) are generated upon asymmetric division of RGCs (**2**). Cajal–Retzius cells are generated in the very early phase of neurogenesis and migrate towards the marginal zone. RGCs sustain the potential to differentiate into premature neurons, IPs and glial progenitors (oligodendrocytes and astrocytes). New neurons migrate along RGC processes (**3**) until they receive a signal from Cajal–Retzius cells, after which they distribute horizontally in the cortical plate (**i**). Later-migrating neurons go further towards the marginal zone and neurons begin to differentiate (**ii**), generating two major processes — the future axon and the future main dendrite shaft. Subsequently, the neurons further extend their processes (**4**) and generate ordered networks by regulated synaptogenesis (shown by red stars) and synapse elimination (**5**). Soon after the neurogenesis stage, RGCs start to generate glial progenitors (**6**). (Adapted from Kawabe and Brose, 2011.)

Notably, as revealed by studies that date cell birth, the layering of the cortical plate occurs in an ‘inside-out’ fashion, meaning that early-born neurons make up the deep layers of the cortical plate, whereas, later-born neurons passes their predecessors and occupy the more superficial layers (Berry and Rogers, 1965; Rakic, 1974; Goffinet and Lyon, 1979; Goffinet, 1984; Parnavelas, 2000; Gupta et al., 2002; Marin and Rubenstein, 2003; Kriegstein, 2005; Cooper, 2008).

Neuronal-layer formation by radial migration occurs in the mouse between E11 and E18. At E11, the neocortex consists of many proliferative precursor cells arranged in a layer known as the ventricular zone. A wave of postmitotic neurons then exits the ventricular zone, and these neurons move in a radial direction towards the pial surface of the brain, establishing a neuronal layer known as the preplate. At ~E13, a second wave of postmitotic neurons migrates radially away from the ventricular zone and splits the preplate into a superficial marginal zone and a deeper subplate. In this way, the second neuronal wave creates a layer between the marginal zone and the subplate, known as the cortical plate. Between E14 and E18, waves of postmitotic neurons continue to exit

the ventricular zone in distinct phases, and migrate radially to traverse the subplate and form the sequential layers of the cortical plate. Once the cortical plate is fully established, the subplate degenerates and leaves behind a six-layered neocortex that persists throughout adulthood (Figure 4.B).
On the cellular level neurogenesis and cortical layer formation follow a defined sequence of sequential steps depicted in Figure 4.C. During early neurogenesis neuroepithelial cells in the ventricular zone proliferate by symmetric cell division and subsequently generate neurons by asymmetric division. Neuroepithelial cells first generate RGCs, which expand by symmetric cell division and undergo asymmetric division at the ventricular zone, thereby producing intermediate progenitor cells and neurons in midgestation, and glial progenitors in late gestation (Mione et al., 1997; Qian et al., 2000; Anthony et al., 2004; Gotz and Huttner, 2005). Each intermediate progenitor cell divides only once to generate two neurons in the subventricular zone. Newly generated neurons migrate along RGC processes, until they receive a signal from Cajal–Retzius cells, after which they stop migration, distribute horizontally in the cortical plate and begin to differentiate into mature neurons (Rakic, 1972; Rakic, 1990; Misson et al., 1991; Orourke et al., 1992; Marin-Padilla, 1998; Frotscher, 1998; Kriegstein and Noctor, 2004; Gotz and Barde, 2005; Sild and Ruthazer, 2011).

The characterization of the naturally occurring mouse mutants reeler carrying mutations in the Reelin gene (Reln), scrambler and yotari carrying mutations in Disabled homolog 1 (Dab1) as well as engineered mice that are double homozygous null mutations for the genes Vldlr (very low density lipoprotein receptor) and Lrp8 (apolipoproetin E receptor 2 (ApoER2)) has provided the first insights into the process of laminar organization and the molecular signalling mechanisms which instruct cells when to stop migration (Falconer, 1951; Goffinet, 1984; Darcangelo et al., 1995; Sweet et al., 1996; Sheldon et al., 1997; Ware et al., 1997; Yoneshima et al., 1997; Trommsdorff et al., 1999; D'Arcangelo et al., 1999). All of these mouse mutants show similar developmental defects in the cortex, an inverted layering (Caviness and Sidman, 1973; Caviness, 1975; Goffinet, 1979; Caviness, 1982). Reln is secreted by distinct cells of the marginal zone, called Cajal–Retzius cells, which results in a high concentration of Reln in the marginal zone or in its immediate vicinity. In contrast to Reln, its receptors Vldlr and ApoER2 as wells as their downstream effector molecule Dab1 are expressed primarily in the neurons that migrate radially towards the pial surface. Binding of reelin to Vldlr or

ApoER2 results in tyrosine phosphorylation of Dab1, which is necessary for the activation of downstream signalling cascades that ultimately mediate the cellular effects of reelin and stop migrating neurons (Howell et al., 1997; Rice et al., 1998; Howell et al., 1999; Hiesberger et al., 1999; Howell et al., 2000; Kerjan and Gleeson, 2007).

Furthermore, p35-Cdk5 signaling was shown to control several aspects of neuronal developemt in the cortex, including directing neurons to their appropriate location, regulation of motility of migrating neurons and regulation of neuronal-glial interactions. This ability is based on the functional property of this signalling complex to transduce guidance signals from the cell membrane to the cytoskeleton thereby regulating and remodelling the microtubular and actin cytoskeleton (Aboitiz, 1999; Ohshima et al., 2001; Keshvara et al., 2002; Ohshima and Mikoshiba, 2002; Xie et al., 2003; Hammond et al., 2004; Kawauchi et al., 2006b).

A network of transcription factors was identified that controls cortical development. Studies identified the proneural genes Neurogenin 1 and 2 (Ngn1 and Ngn2) as well as the homeodomain paired box gene 6 (Pax6) to play an important role in the specification and differentiation of cortical projection neurons. The generation of neurons of the lower and upper cortical layers appears to be a specific output of Ngn1–Ngn2- and Pax6-controlled genetic pathways, respectively (Guillemot, 1995; Kageyama and Nakanishi, 1997; Lee, 1997; Gotz et al., 1998; Bertrand et al., 2002; Heins et al., 2002; Ross et al., 2003; Scardigli et al., 2003; Schuurmans et al., 2004; Mattar et al., 2004; Guillemot, 2005; Guillemot et al., 2006; Cobos et al., 2006; Merot et al., 2009; Huang, 2009; Asami et al., 2011).

In the course of their journey pyramidal neurons begin to differentiate, first generating two major processes, the leading and the tailing process, that subsequently develop into the arborized dendrite and the axon projecting to cortical and subcortical brain regions. Later during postnatal development pyramidal cortical neurons further extend their processes and generate highly ordered neural networks by thightly regulated processes of synaptogenesis and synapse elimination (Figure 4.C).

In the following sections we will describe the basic principles and molecular mechanisms governing the terminal differentiation and maturation of neurons *in vitro* and *in vivo.* But first, we will present a summary of the main developmental and functional characteristics of adult neurogenesis, a process of development that persists in the adult brain, that shares many features of embryonic neurogenesis and that offers unique opportunities to better understand the basic mechanisms of neuronal terminal differentiation.

1.1.4. Adult neurogenesis in the dentate gyrus – generation of new neurons in the adult brain

Adult neurogenesis refers to the persistent production of new functional neurons from precursor cells present in an adult brain. Neurogenesis occurring beyond the embryonic and perinatal development was observed and reported for the first time by Altman and Das in 1962 in the adult rat hippocampus and replicated in different species and conditions afterwards (Altman, 1962; Altman, 1963; Altman and Chorover, 1963; Altman and Das, 1965; Altman and Das, 1966; Altman, 1966) (for review see Ming and Song, 2005). Adult neurogenesis is a prominent example of cellular neuroplasticity, that occurs in most vertebrate species including humans (Eriksson et al., 1998; Gross, 2000; Curtis et al., 2003; Sanai et al., 2004). The two neurogenic niches in the adult rodent brain are the subgranular zone (SGZ) of the dentate gyrus (DG) and the subventricular zone (SVZ) of the lateral ventricle, from where newborn neurons migrate to the olfactory bulb (OB) via the rostral migratory stream (RMS) (Kempermann and Gage, 2000; varez-Buylla and Garcia-Verdugo, 2002; van Praag et al., 2002; Carleton et al., 2003). In the hippocampal dentate gyrus of young adult rodents, thousands of new neurons are generated everyday and this process is dynamically regulated by various internal and environmental factors (Cameron and Mc Kay, 2001; Ming and Song, 2005; Balu and Lucki, 2009). Adult neurogenesis in the DG proceeds through five characteristic developmental stages (Esposito et al., 2005; Zhao et al., 2006). During the first step quiescent radial glia-like precursor cells in the SGZ of the DG are activated and give rise to transient amplifying cells (Seri et al., 2001; Denise et al., 2004). Proliferation of non-radial precursors and intermediate progenitor cells ultimately generates neuroblasts that

differentiate into immature neurons. Immature neurons migrate into the granule cell layer of the DG, where they eventually integrate into the tri-synaptic hippocampal circuit and mature morphologically and functionally into dentate granule neurons, thereby receiving inputs onto their dendrites from the entorhinal cortex and extending axonal projections towards CA3 pyramidal neurons (Lie et al., 2004; Ming and Song, 2005; Zhao et al., 2006; Overstreet-Wadiche et al., 2006; Faulkner et al., 2008; Zhao et al., 2008a) (Figure 5) (see also 1.1.6). Two critical aspects of adult neurogenesis are the selection and survival processes, which are closely coupled. While large numbers of new neurons are born in the OB and DG, only a fraction of these cells survive. In the DG, approximately half of the newborn neurons die by apoptosis within two weeks after birth, though this number might be altered due to the influence of various intrinsic and extrinsic factors that regulate maturation and survival (Kempermann and Gage, 2002; Lie et al., 2004; Zhao et al., 2008a; Ming and Song, 2011).

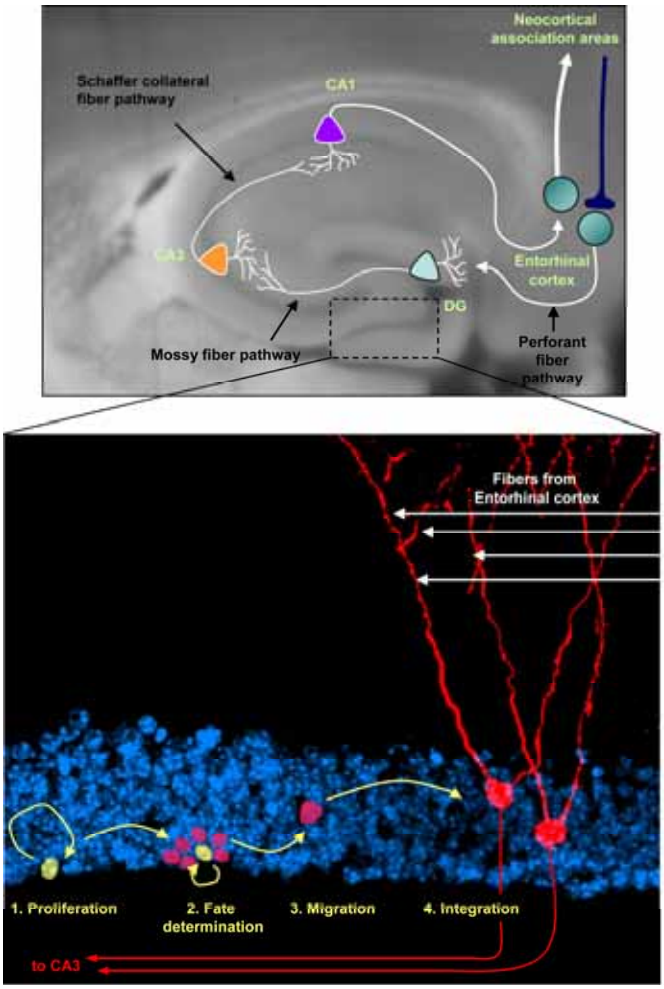


Figure 5: Adult neurogenesis in the dentate gyrus.

Overview of the hippocampus showing the tri-synaptic hippocampal circuit. Granule neurons in the dentate gyrus (DG) receive synaptic input from the entorhinal cortex and project via the mossy fiber pathway to CA3 pyramidal neurons. CA3 neurons in turn form projections, the Schaffer collateral fibers, that connect them to CA1 pyramidal neurons. In the subgranular zone of the dentate gyrus, one neurogenic niche in the adult brain of mammals, new neurons are generated throughout the life. The inset highlights the sequential steps during adult neurogenesis in the dentate gyrus. 1. and 2. Proliferation and fate determination: Stem cells (yellow) in the subgranular zone of the dentate gyrus give rise to transit amplifying cells that differentiate into immature neurons (pink). 3. Migration: Immature neurons migrate into the granule cell layer of the dentate gyrus. 4. Integration: Immature neurons mature into new granule neurons, receive inputs from the entorhinal cortex, and extend projections to CA3. (Adapted from Lie et al., 2004.)

Experiments using different techniques, such as BrdU (Kuhn et al., 1996) or retroviral injections (van Praag et al., 2002; Tashiro et al., 2006) to label newborn neurons and to trace the clonal lineage of dividing precursors, combined with functional measurements identified various molecular players regulating different aspects of adult neurogenesis, and provided insight into the time course of development and functional integration (Duan et al., 2008). Both intrinsic and extrinsic signaling mechanisms, including niche factors and receptors, cytoplasmic complexes, transcriptional factors and epigenetic regulators, control adult neurogenesis (Ninkovic and Gotz, 2007; Ma et al., 2010; Mu et al., 2010; Sun et al., 2011; Ming and Song, 2011). Besides genetic factors that regulate the generation of new neurons, adult neurogenesis was shown to be highly sensitive to physiological and pathological environmental stimuli at almost every stage (Ming and Song, 2005; Zhao et al., 2008a). Physical exercise and an enriched environment were shown to increase cell proliferation and to promote neuron survival during adult neurogenesis, respectively (Kempermann et al., 1997; van Praag et al., 1999a; van Praag et al., 1999b; van Praag et al., 2000). In contrast aging (Drapeau et al., 2003; Bizon and Gallagher, 2003; Merrill et al., 2003; Driscoll et al., 2006; Drapeau et al., 2007; Klempin and Kempermann, 2007) as well as different forms of stress, such as social (Ohl et al., 2000; Czeh et al., 2001), social and unexpected chronic mild stress (Mineur et al., 2007) as well as stress hormones (Gould et al., 1992) reduce cell proliferation and adult neurogenesis in the SVZ and SGZ (Mirescu and Gould, 2006; Klempin and Kempermann, 2007; Rossi et al., 2008). Given that stress is thought to precipitate and exacerbate mood disorders and glucocorticoid levels are often found to be elevated in patients suffering from depression, the potential role and implication of adult neurogenesis in mood disorders has been extensively studied (Kempermann, 2002; Eisch, 2002; Kempermann and Kronenberg, 2003; Sapolsky, 2004; Thome and

Eisch, 2005; Mirescu and Gould, 2006; Sahay et al., 2007; Balu and Lucki, 2009). Interestingly, in contrast to stress, it was found that antidepressant treatments increase proliferation and neuronal survival in the dentate gyrus (Duman et al., 2001; Santarelli et al., 2003; Malberg, 2004; Warner-Schmidt and Duman, 2006; Dranovsky and Hen, 2006). However, controversial data exists regarding the requirement of adult neurogenesis in the behavioral effects of antidepressants (Santarelli et al., 2003; Saxe et al., 2006; David et al., 2009). Furthermore neurogenesis and depressive-like behavior are not well correlated in two rodent models of depression, learned helplessness and chronic mild stress and ablation of adult neurogenesis does not alter anxiety-like, depressive-like or environmental enrichment-related behavior (Vollmayr et al., 2003; Meshi et al., 2006; reviewed in Warner-Schmidt and Duman, 2006). Despite the fact that many depressive patients show hippocampal atrophy, the contribution and involvement of adult SGZ neurogenesis has not been directly demonstrated. Moreover, many studies, combining correlative evidence, ablation experiments and computational modeling, are being performed to gain insight into the putative function of SGZ adult neurogenesis in hippocampus-dependent learning and memory processes (Snyder et al., 2005; Deng et al., 2010). The Morris water maze, a gold standard test for analyzing spatial memory has shown inconsistent results regarding the role of adult neurogenesis in spatial memory. Some researchers described changes in short-term retention, others in long-term memory and some didn't see any discernable effects at all. However, recently a more clear experimental evidence, concerning the role of adult neurogenesis in different cognitive tasks, like spatial pattern separation (Clelland et al., 2009; Sahay et al., 2011) or memory consolidation (Kitamura et al., 2009), has been reported.
Neurogenesis in other adult CNS regions is discussed controversially and is generally believed to be very limited under normal physiological conditions but could be induced after injury (Gould, 2007). This raises the exciting possibility that stimulation of adult neurogenesis could be thought of as a novel potential therapeutic strategy for neurodegenerative diseases in reparative medicine (Lie et al., 2004).

The processes of embryonic and adult neurogenesis share many common features and mechanisms such as maintenance of neuroprogenitor niches and stem cells, proliferation, early differentiation, migration, and late differentiation, including neuronal polarization, axonal growth, dendritic arborization and synaptogenesis. In the next

sections state of the art research on the cellular molecules and the genetic basis of axonal, dendritic and synaptic differentiation will be discussed.

1.1.5. Cytoskeleton-dependent regulation of neuronal polarization and axon growth

Mature neurons are highly polarized cells, typically having one thin and long axon to transmit information encoded in action potentials and several shorter arborized dendrites to receive a myriad of synaptic inputs. This unique and characteristic morphology enables neurons to form the cellular basis of neural networks. Newly generated neurons gradually develop their polar structure by a defined sequence of events, starting with the axon formation (Dotti et al., 1988). In primary hippocampal neuron cultures one of the several neurites of an unpolarized neuron grows faster than all other neurites and adopts axonal identity, whereas the remaining neurites develop into dendrites. Axon formation marks the first break in symmetry during neuronal development and depends on tightly regulated cytoskeletal rearrangements. Different extracellular cues and intracellular signalling pathways regulating neuronal polarity and axon formation were identified that converge on the actin and microtubular cytoskeleton. Phosphoinositide 3-kinase (PI3K), Akt and Glycogen synthase kinase-3β (GSK3β) signalling (Shi et al., 2003; Menager et al., 2004; Jiang et al., 2005; Yoshimura et al., 2005; Kim et al., 2006b), Rho GTPases, such as ras homolog gene family member A (RhoA), cell division cycle 42 (Cdc42) and ras-related C3 botulinum toxin substrate (Rac) 1 (Bradke and Dotti, 1999; Bito et al., 2000; Kunda et al., 2001; Schwamborn and Puschel, 2004; Govek et al., 2005; Nishimura et al., 2005; Yoshimura et al., 2006b; Garvalov et al., 2007; Tahirovic et al., 2010), as well as CRMP-2 (Inagaki et al., 2001; Fukata et al., 2002a) have been described as cell intrinsic key molecules in neuronal polarization and axon formation (reviewed in Fukata et al., 2002b; Arimura et al., 2004; Arimura and Kaibuchi, 2005; Yoshimura et al., 2006a; Arimura and Kaibuchi, 2007; Barnes and Polleux, 2009; Tahirovic and Bradke, 2009; Polleux and Snider, 2010).

Furthermore it has been demonstrated that dynamic rearrangements of the neuronal cytoskeleton are ultimately responsible for axon formation, and are characterized by

local dynamic instability of actin (Bradke and Dotti, 1999) and concomitant stabilization of microtubules (Witte et al., 2008) in one of the neurites, that will become the future axon (Witte and Bradke, 2008; Tahirovic and Bradke, 2009; Hoogenraad and Bradke, 2009; Stiess and Bradke, 2011; Neukirchen and Bradke, 2011b).

1.1.5.1. The neuronal cytoskeleton

The neuronal cytoskeleton is build up by actin filaments and microtubules. Actin microfilaments consist of two helically twisted strands of filamentous actin (F-actin) and with a diameter of approximately 7 nm they are the thinnest fibers of the cytoskeleton. F-actin is composed of polymerized subunits of globular actin (G-actin). G-actin subunits are arranged in a head-to-tail fashion in the actin filament which creates two ends with distinct dynamic properties. At the so-called plus- or 'barbed' end polymerization, the addition of G-actin subunits, prevails, making this the fast growing end of the actin filament. At the minus- or 'pointed' end depolymerisation, the removal of subunits, predominates, letting it grow slowly. Polymerizing G-actin is associated with adenosine triphosphate (ATP), which is then hydrolyzed to adenosine diphosphate (ADP) upon ageing in the actin filament. Regulators of the actin cytoskeleton modulate various processes, such as 'de novo' nucleation of filaments, crosslinking/bundling, severing, depolymerization and ADP/ATP exchange to fine-tune the dynamic properties of the actin cytoskeleton (reviewed in Pollard and Borisy, 2003; Cingolani and Goda, 2008).

Microtubules are polymers of α- and β-tubulin dimers that form a hollow cylinder with a diameter of 25 nm. As actin filaments, microtubules also have two distinct ends, a fast-growing 'plus' end and a slow growing 'minus' end. The minus ends are usually anchored to the centrosome, which is responsible for nucleating microtubules, whereas plus-ends grow outwards towards the cell periphery. Microtubule ends alternate between prolonged phases of polymerization and depolymerization, a phenomenon called 'dynamic instability' (Mitchison and Kirschner, 1984). The transition from polymerization to depolymerization is referred to as 'catastrophe', whereas the opposite transition from depolymerization to polymerization is referred to as 'rescue' (Walker et al., 1991).

While neuronal microtubules are structurally similar to those in non-neuronal cells, the organization of microtubules in neurons is different and strikingly more diverse. Brain microtubules contain tubulin subunits of many different isotypes, with many different post-translational modifications and neuronal microtubules are bound to a variety of

microtubule-associated proteins (MAPs), which regulate the assembly and dynamics as well as the organization of microtubules into dense bundles (reviewed in Cassimeris and Spittle, 2001; Westermann and Weber, 2003; Dehmelt and Halpain, 2005; Hammond et al., 2008; Conde and Caceres, 2009; Janke and Kneussel, 2010). Specific MAPs stabilize microtubules, for instance by stimulating the addition of tubulin subunits to the microtubule polymer, by blocking catastrophes, or by promoting rescue events. MAP2 and Tau act as microtubule stabilizing proteins and down-regulation of MAP2 inhibits neurite formation while down-regulation of Tau inhibits axon formation (Caceres and Kosik, 1990; Caceres et al., 1992). Plus-end tracking proteins (+TIPs) accumulate at growing microtubule plus ends and regulate microtubule dynamics (Galjart, 2005). The end binding (EB) proteins 1 and 3 are highly enriched in growth cones and promote persistent microtubule growth and stability by preventing microtubule catastrophe (Tirnauer and Bierer, 2000; Geraldo et al., 2008; Komarova et al., 2009). Additionally, EBs interact with many other +TIPs thereby recruiting them to growing microtubule ends (Komarova et al., 2005; Akhmanova and Hoogenraad, 2005; Akhmanova and Steinmetz, 2008). Moreover, EB3, bound to microtubule tips, was shown to bind the F-actin-binding protein drebrin in neuronal growth cones thereby coordinating actin-microtubule interactions. Accordingly, inhibition of EB3 function in cortical neurons leads to strong impairments in neuritogenesis (Geraldo et al., 2008). Microtubule stabilization is also achieved by cross-linking protofilaments (Chapin and Bulinski, 1992; Gustke et al., 1994) or bundling of microtubules (Kanai et al., 1992; Masson and Kreis, 1993; Preuss et al., 1997). On the other hand, some MAPs, e.g. Op18/Stathmin, destabilize microtubules by promoting catastrophes and inhibiting rescues, by severing microtubules, sequestering tubulin subunits and weakening lateral interactions between protofilaments (Cassimeris and Spittle, 2001; Morii et al., 2006; Watabe-Uchida et al., 2006). Moreover, the stability of microtubules is correlated also with their post-translational modifications (PTMs) (Westermann and Weber, 2003; Hammond et al., 2008; Janke and Kneussel, 2010). Mainly the carboxy-terminal tails of α- and β-tubulin heterodimers are subjected to various reversible PTMs including acetylation and tyrosination. Acetylation is found in stable long-lived microtubules, whereas tyrosinated tubulin is confined to dynamic microtubules with a high turnover.

Axonal microtubules can be more than 100 mm long and have a uniform polarity, with all plus-ends distal to the cell body. In contrast dendritic microtubules are typically shorter and often exhibit mixed polarity (Baas et al., 1988; Baas et al., 1989; Baas and Black,

1990). This different microtubule polarity and organization gives rise to differential protein sorting and trafficking involved in maintaining neuronal polarity (Conde and Caceres, 2009; Kapitein et al., 2010; Kapitein and Hoogenraad, 2011).
In general, most microtubules in proliferating and developing cells are very dynamic, whereas, in differentiated neurons, most microtubules are stabilized owing to their interactions with structural MAPs.

1.1.5.2. Neuronal polarization and axon growth

Of central importance for axon formation and neuronal polarity is the specialized, highly motile cellular compartment at the tips of growing axons, termed growth cone (reviewed in Tahirovic and Bradke, 2009). This structure supports growth by sensing environmental cues (Dickson, 2002) and transducing those signals to the cytoskeleton. The axonal growth cone is composed of a central region filled with organelles and microtubules and a peripheral, highly dynamic, actin-rich region containing lamellipodia and filopodia (Figure 6.C) (Forscher and Smith, 1988; Gordon-Weeks, 2004; Dehmelt and Halpain, 2005; Stiess and Bradke, 2011). Lamellipodia are broad veil-like cellular protrusions that contain branched actin filaments. Filopodia are thin protrusions made out of unbranched and parallel F-actin bundles. Barbed (fast-growing) ends of actin filaments are oriented towards the rim and pointed (slow-growing) ends towards the base of the growth cone (Figure 6.C). G-actin subunits continuously incorporate into the barbed end while they dissociate from the pointed end, resulting in a “treadmilling” of F-actin and regulation of growth cone dynamics (Katoh et al., 1999; Mallavarapu and Mitchison, 1999). This process is further modulated by myosin motors and intracellular trafficking as well as by additional actin regulators (Ishikawa and Kohama, 2007; Hirokawa et al., 2010).

Rearrangements of the actin cytoskeleton and microtubules are crucial for the initial establishment of polarity (Figure 6.A). Before morphological polarization occurs, the future axon shows enhanced growth cone dynamics and, thereby, actin turnover (Bradke and Dotti, 1999; Witte et al., 2008). In contrast, future dendrites, which are not growing at that stage, have a static growth cone with a rigid actin cytoskeleton (Figure 6.B). Pharmacological depolymerization of the actin cytoskeleton, e.g. by Cytochalasin D treatment, transforms nongrowing dendrites into growing axons (Bradke and Dotti, 1999; Bradke and Dotti, 2000a; Kunda et al., 2001; Schwamborn and Puschel, 2004). This

suggests, that the actin filaments of future dendritic growth cones form a barrier for the protrusion of microtubules, whereas the axonal growth cone contains an actin structure permissive for microtubule protrusion (Forscher and Smith, 1988). Microtubules are also actively involved in neuronal polarization (Witte et al., 2008). Axonal microtubules are more stable as shown by the high abundance of acetylated tubulin in axons compared to minor neurites and as demonstrated by the fact that pharmacological stabilization of microtubules, with low concentrations of Taxol, is sufficient to induce axon formation (Witte et al., 2008). Moreover +TIPs, such as CLIP-115, CLIP-170 and EB3, promote microtubule growth and stabilization and coordinate interactions of microtubules with actin filaments. Thus, microtubules are able to protrude through the destabilized actin network of the axonal growth cone, and thereby sustaining axonal growth (Geraldo et al., 2008; Neukirchen and Bradke, 2011a).

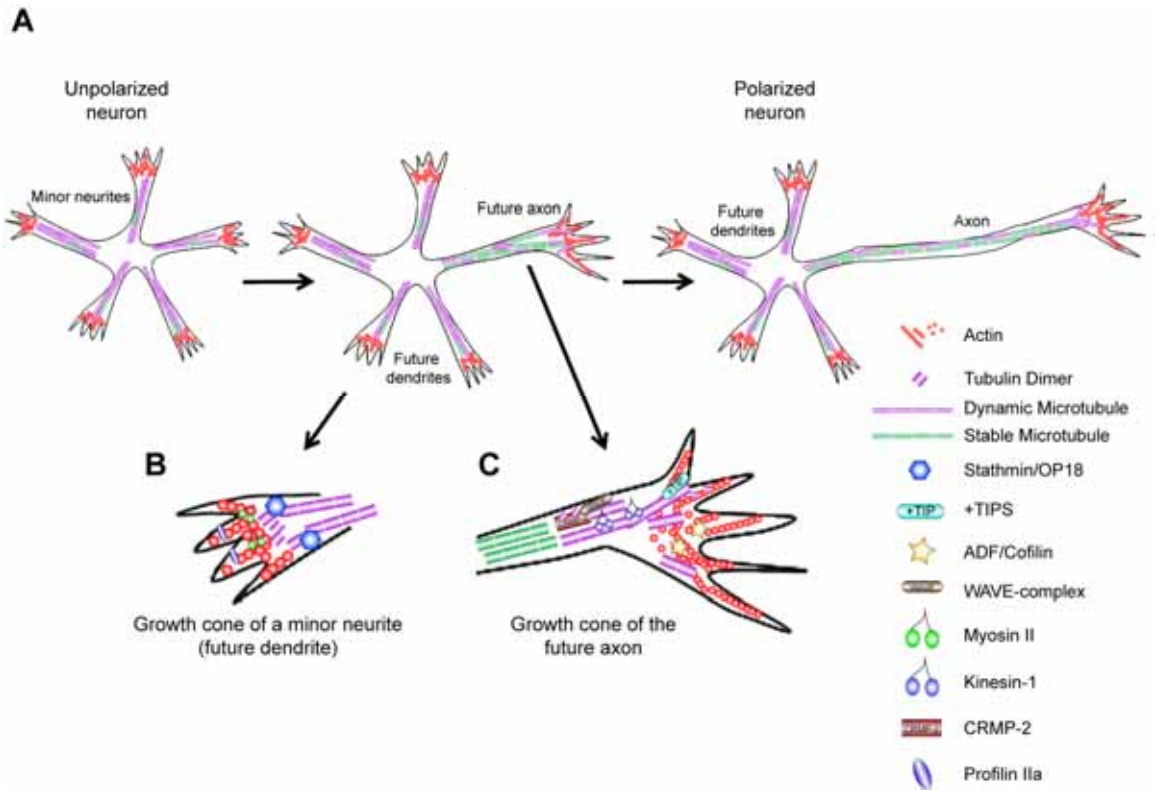


Figure 6: Neuronal polarization and axon growth.

A) Coordinated rearrangements of the actin and microtubular cytoskeleton promote neuronal polarization and axon growth. During polarization of neurons, one of the minor neurites elongates rapidly to become the future axon. Microtubules become more stabilized in the shaft of the future axon. Moreover, the growth cone of the future axon enlarges and becomes more dynamic

compared to the growth cones of the remaining neurites. Additionally, the actin cytoskeleton becomes destabilized and allows dynamic microtubules to explore the growth cone. In contrast, the actin cytoskeleton in growth cones of minor neurites forms a rigid barrier for exploring microtubules that are less stabilized in minor neurites. Therefore, these neurites do not elongate and will develop into dendrites only later. **B)** The growth cone of the minor neurite is smaller and less dynamic than the axonal growth cone. The motor protein myosin II contracts the actin cytoskeleton and profilin IIa increases actin polymerization leading to a dense and rigid actin barrier, which microtubules cannot pervade. Moreover, stathmin destabilizes microtubules in the minor neurite. **C)** In contrast, the growth cone of the future axon is larger and more dynamic. The actin network becomes destabilized by ADF/Cofilin. Microtubule plus-end tracking proteins (+TIPs) promote microtubule assembly and thus, microtubules are able to protrude through the destabilized actin network. Furthermore, growth promoting factors like tubulin dimers and the WAVE-complex are selectively targeted into the future axon via a CRMP-2/Kinesin-1-based transport. Together, these events lead to enhanced growth of the future axon. (Adapted from Stiess and Bradke, 2011.)

In contrast to primary neurons in culture, in which axon specification depends largely on cell autonomous signaling cascades, *in vivo* axon formation, growth and branching is controlled and influenced additionally by extracellular guidance cues.

Netrins, slits, semaphorins and ephrins provide attractive and repulsive guidance signals to stimulate and orient the asymmetrical growth of neurites, direct axon pathfinding and thereby facilitating the development of neuronal circuits.

Wingless-proteins (Wnts) and their receptors frizzled as well as neurotrophic factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT-3) through their tyrosine receptor kinases (Trks) modulate intracellular signaling cascades thereby modifying axon growth (Hedgecock et al., 1990; Serafini et al., 1994; Cohencory and Fraser, 1995; Wadsworth et al., 1996; Tessierlavigne and Goodman, 1996; Hong et al., 1999; Cohen-Cory, 1999; Tessier-Lavigne, 2000; Huang and Reichardt, 2003; Dent et al., 2004; Tang and Kalil, 2005; Adler et al., 2006; Prasad and Clark, 2006; Hilliard and Bargmann, 2006; Arimura and Kaibuchi, 2007). Moreover, many extacellular matrix and cell adhesion proteins, e.g. laminin and NgCAM, were shown to enhance and accelerate neurite outgrowth and axon elongation (Stoeckli and Landmesser, 1995; GarciaAlonso et al., 1996; Fitzli et al., 2000). Addtionally, *in vivo*, different neuronal cell populations, e.g. projection neurons and local interneurons in the neocortex, show diverse modes of axonal growth and different signalling mechanisms regulate various forms of axonal branching in different types of neurons in the brain as well as in the spinal cord (Portera-Cailliau et al., 2005; Schmidt and Rathjen, 2010). These processes finally establish the well-ordered topographic relationships in the nervous system.

As a consequence of the remarkable differences in axon formation between *in vivo* and *in vitro* models, it is recommendable to validate and re-evaluate effects observed in cell culture systems in model organisms *in vivo*.

1.1.6. Dendritic growth and branching of pyramidal neurons in the forebrain

The proper formation and morphogenesis of dendrites and their particular shapes are fundamental to neural circuit assembly in the brain and provide the basis for neurons to receive and propagate information. The morphology of a dendritic arbor determines the processing and integration capabilities of incoming electrical signals (London and Hausser, 2005). In this regard, the extend and branching pattern of dendrites critically controls the number and type of synaptic contacts that a neuron establishes (Vetter et al., 2001; Parrish et al., 2007; van Elburg and van Ooyen, 2010). Accordingly, different neuronal subclasses show a wide variety of dendritic morphologies that serve their specific functions (Hausser et al., 2000). However, the mechanisms that specify the development of subclass-specific dendritic arbors in vertebrates remain to be elucidated (Whitford et al., 2002; Parrish et al., 2007).

Misregulation of dendrite development as well as abnormal dendrite remodeling is associated with several severe neurodevelopmental as well as neurological brain diseases, including mental retardation, fragile X syndrome, Down's syndrome, Rett syndrome and schizophrenia (reviewed in Emoto, 2011). Moreover dendrite and dendritic spine vulnerability were observed in mouse models of neurodegenerative diseases such as Alzheimer disease (AD) (Duyckaerts et al., 2008; Luebke et al., 2010). Furthermore it has been shown that hippocampal and neocortical neurons can undergo extensive reversible dendritic remodeling in response to external or environmental stimuli as well as pathological conditions, such as chronic stress, hibernation, physical activity, enriched environment, sensorimotor learning tasks, chronic exposure to drugs, epilepsy, ischemia and brain injury (Popov and Bocharova, 1992; Popov et al., 1992;

Spigelman et al., 1998; Lambert et al., 1998; Ruan et al., 2006; Magarinos et al., 2006; Hickmott and Ethell, 2006; Emoto, 2011).

Dendritic arbor development is characterized by a sequence of complex and interrelated steps, which include neurite outgrowth and branching, guidance and synaptic targeting, limitation of growth, stabilization and remodeling (Scott and Luo, 2001). Many of the molecules and cellular mechanisms controlling dendritic growth and arborization in various neuronal cell types, species and experimental model systems have been revealed and will be discussed in the following section (Whitford et al., 2002; Parrish et al., 2007; Urbanska et al., 2008; Corty et al., 2009).

Genetic programs that regulate the size and complexity of dendritic fields are orchestrated and tightly controlled by transcription factors. Studies mainly in *Drosophila* identified a large number of transcription factors that are required for dendrite development of different neuronal types. In this context, two major scenarios may occur during dendrite development and examples supporting both models have been described.

In the first one, different types of neurons express a specific set of transcription factors that determines their particular dendritic fate. Thus, the choice between external sensory (es) and multiple dendrite (md) neurons in the peripheral nervous system of *Drosophila* depends on the transcription factor Hamlet. Hamlet specifically inhibits md neuron morphology and promotes es neuron fate in postmitotic neurons (Moore et al., 2002). In the second model, dendritic development depends on the combined action of multiple transcription factors, as decribed for the specification of different classes of md neurons in *Drosophila*. More than 70 transcription factors were identified that regulate the morphogenesis of sensory neurons in *Drosophila* (Parrish et al., 2006). Among them combinatorial expression levels of the transcription factors Cut, Abrupt and Spineless specifiy the development of class I, II, III and IV *Drosophila* dendritic arborized (da) neurons, a class of md neurons (Grueber et al., 2003; Li et al., 2004; Sugimura et al., 2004; Kim et al., 2006a).

In mammalian brains, the identification of intrinsic cues that regulate dendrite development is more difficult, because migration of neurons and the acquisition of their pyramidal dendritic morphology are coordinated developmental processes, as discussed above (Kriegstein and Noctor, 2004). Therefore, the interpretation of results is often difficult and obscured, because processes that affect migration influence dendritic

growth and vice versa. Despite these difficulties, expression analyses demonstrated neuron-type specific expression of mammalian transcription factors (Gray et al., 2004) and Ngn2, a basic helix-loop-helix (bHLH) transcription factor, was identified to specify pyramidal dendrite morphology (Hand et al., 2005). Many transcription factors, including Ngn2 also function as proneural genes. Nevertheless, the functions of Ngn2 in migration and dendrite development depend on phosphorylation of a single tyrosine residue at position 241 and could be separated from the proneural activity of Ngn2. Another example are the homeobox transcription factors Cux1 and Cux2, which are the vertebrate homologs of *Drosophila* homeobox Cut, which function as regulators of dendrite branching, spine development and synapse formation in layer II and II pyramidaII neurons of the cerebral cortex (Cubelos et al., 2010).

Furthermore, several transcription factors were identified that regulate activity-dependent dendritic growth, including NeuroD, calcium-responsive transactivator (CREST) and cAMP response element binding protein (CREB) (Shieh et al., 1998; Redmond et al., 2002; Aizawa et al., 2004; Gaudilliere et al., 2004; Redmond and Ghosh, 2005; Wayman et al., 2006). One of the main roles of transcription factors involved in dendritic growth is to regulate the expression of neurotrophins, guidance cues, cell adhesion and other extracellular proteins that critically control spatial and temporal dimensions of dendritic field development. For example CREB was shown to regulate BDNF and Wnt2 expression, secreted neurotrophic factors which promote dendritic outgrowth (Shieh et al., 1998; Tao et al., 1998; Wayman et al., 2006). In vertebrates, BDNF, neurotrophin 3 (NT-3), neurotrophin 4 (NT-4) and NGF mediate neurotrophin signaling through members of the Trk receptor family. The neuron's response to secreted neurotrophic factors is determined by the combination of Trks expressed in the membrane. Hence, BDNF stimulates dendritic growth in layer IV cortical neurons, but inhibits dendritic outgrowth in layer VI neurons (McAllister et al., 1997). Extrinsic signals likely regulate dendritic growth by modulating the cytoskeleton, as it was shown for the non-canonical Wnt pathway, which regulates dendritic growth by targeting the cytoskeleton via Disheveled (Dvl), Rac and c-Jun N-terminal protein kinase (JNK) signaling cascades (Rosso et al., 2005; Fradkin et al., 2005; Srahna et al., 2006)

In contrast to its function as a repulsive cue for axons, semaphorin 3A (Sema3A) acts as an attractive cue for apical dendrites in the cortex near the marginal zone (Polleux et al., 2000). Sema3A acts through plexin and neuropilin receptors (Takahashi et al., 1999),

and the asymmetric localization of soluble guanyl cyclase (SGC) towards the pial surface at the site of apical dendrite growth mediates the chemoattractive response of pyramidal dendrites to Sema3A (Polleux et al., 2000).

Ephrins and Eph receptors, mainly EphB1, B2 and B3, were shown to regulate dendritic complexity. The PDZ-domain scaffold molecule glutamate receptor interacting protein 1 (GRIP1) interacts with EphB2 and the microtubule motor kinesin KIF-5, and thereby regulates intracellular trafficking and cell surface expression of EphB2 (Hoogenraad et al., 2005).

Furthermore the boundaries and territories of dendritic fields are established mainly by repulsive dendro-dendritic interactions. To cover and innervate neuronal receptive fields completely but without redundancy, neurons of certain functional groups exhibit 'tiling' of their dendrites via dendritic repulsion. Two evolutionarily conserved proteins, Tricornered (Trc) and Furry (Fry), are essential for tiling and and self-avoidance control of *Drosophila* sensory neuron dendrites (Grueber et al., 2002; Emoto et al., 2004; Gallegos and Bargmann, 2004). Moreover, cell surface molecules were shown to mediate dendro-dendritic repulsion. For instance, in cortical neurons, Notch receptor activation is triggered by interneuronal contacts and is sufficient to block dendritic growth (Sestan et al., 1999; Redmond et al., 2000). In contrast, N-cadherins were found to mediate dendro-dendritic contacts (Zhu and Luo, 2004).

During development dendrites have to recognize their correct synaptic partners and establish axo-dendritic contacts to ensure proper wiring in the CNS. Many adhesive molecules were shown to regulate synaptic partner choice, including the heterophilic interaction of Nectin-1, an immunoglobulin domain adhesive protein, preferentially expressed in axons, with Nectin-3, which is expressed not only in axons but also in dendrites (Togashi et al., 2006). Similarly, pre-synaptic neurexins and post-synaptic neuroligins mediate synaptic adhesion via heterophilic interactions (Boucard et al., 2005; Lise and El-Husseini, 2006).

Dendritic fields have to be maintained during the lifetime of an organism. Interestingly, Down syndrome is characterized by a progressive reduction in dendritic arborization of cortical neurons that starts postnatally (Becker et al., 1986; Schulz and Scholz, 1992; Kaufmann and Moser, 2000).

Dendritic stability is closely related to synaptic stability (as it will be discussed later). Synaptic activity, as well as adhesive contact between pre- and post-synaptic compartments and trophic signaling between the two compartments, was shown to enhance dendritic spine stability and this synaptic support maintains the integrity of dendritic arborization. The loss of this support results in destabilization of dendritic spines followed by dendritic simplification (Jones and Thomas, 1962; Matthews and Powell, 1962; Coleman and Riesen, 1968; Wu and Cline, 1998; Rajan et al., 1999; Niell et al., 2004; Sfakianos et al., 2007; Lin and Koleske, 2010).
BDNF signaling through TrkB was also shown to be required for maintenance of cortical dendrites, because late onset knockout of *BDNF* or *TrkB* results in a reduction of dendritic complexity (Xu et al., 2000; Gorski et al., 2003). Interactions with the extracellular matrix, e.g. with integrins, stabilize dendrites (Marrs et al., 2001), and cadherins as well as protocadherins were shown to play diverse roles in shaping and maintaining dendritic arbors (Formstone and Little, 2001; Shima et al., 2002; Tissir et al., 2002; Shima et al., 2004; Ye and Jan, 2005).

In addition to stabilization and maintenance over long time periods, dendritic arbors can be refined and remodeled in response to environmental stimuli and neuronal activity. The effects of sensory neuronal activity are largely mediated by N-methyl-D-aspartate (NMDA) receptors and downstream signaling molecules, such as Calcium/calmodulin-dependent protein kinases (CamKs) and mitogen-activated kinase (MAPK) (Wu and Cline, 1998; Rajan and Cline, 1998; Wu et al., 2001; Redmond et al., 2002; Datwani et al., 2002; Sin et al., 2002; Vaillant et al., 2002). Activity-dependent stimulation of CamKII and MAPK pathways enhances the interaction between MAP2 and microtubules, thereby stabilizing microtubules in dendrites (Vaillant et al., 2002). Additionally. activity-dependent dendritic growth requires activation of Rac1 via Tiam1 as well as Cdc42 (Nakayama et al., 2000; Sin et al., 2002; Tolias et al., 2005). Tiam1 is activated by phosphorylation in response to NMDA receptor stimulation. Tiam1 then activates Rac1, leading to rearrangements of the actin cytoskeleton and furthermore Rac1 activates JNK1, which controls microtubule dynamics (Chang et al., 2003; Tolias et al., 2005). Furthermore, astrocytes release soluble factors, such as ATP and adenosine, in response to neural activity, which might play a role in activity-dependent dendritic remodeling (Volterra and Meldolesi, 2005; Wang et al., 2006b).

Pyramidal neurons in the mammalian forebrain

Pyramidal cells are the main projection neurons of the neocortex and allocortex in the mammalian brain. Assisted by their long and complex apical and basal dendritic trees, principal neurons of the cortex, hippocampus and amygdala integrate enormous quanta of information conveyed by multiple intracortical, thalamic and subcortical afferents and trigger electrical responses as part of more complex engrams of associative, motor or coginitive programs (Spruston, 2008). The essence of the integrative capability of dendritic arborizations stems on the fact that dendrites are decorated with a myriad of heteromorphous protrusions, called spines, that represent post-synaptic platforms of reception of the excitatory synaptic inputs that release neurotransmitters, mainly glutamate. Due to its highly ordered cytoarchitecture, its defined neuronal populations (pyramidal CA1 and CA3 neurons, granule neurons of the dentate gyrus, and a relatively small number of interneurons) (Bayer, 1980a; Bayer, 1980b; Benson et al., 1994; Soriano et al., 1994), its defined tri-synaptic circuit (Amaral and Witter, 1989; Amaral and Witter, 1995) (see Figure 7.A for details), and its involvement in memory storage and spatial navigation (RempelClower et al., 1996; Reed and Squire, 1997; Milani et al., 1998; Neves et al., 2008), the hippocampus represents an ideal brain structure to analyze changes in neuronal morphology and synaptic physiology of pyramidal neurons in unison.

The basic tri-synaptic hippocampal circuit is depicted in Figure 7.A. Neurons from the entorhinal cortex innervate granule neurons in the dentate gyrus via the perforant path. The granule neurons of the dentate gyrus project to CA3 pyramidal neurons via the mossy fiber pathway. CA3 cells then in turn project axons to innervate CA1 pyramidal cells through the Schaffer collateral or the commissural pathway. CA1 cells project outside the hippocampus, through the subiculum and entorhinal cortex to several cortical and subcortical areas (Amaral and Witter, 1989).

Pyramidal neurons in the hippocampus consist of a typical pyramidal-shaped soma, an axon and two distinct highly branched apical and basal dendritic trees. The basal and proximal apical dendrites of CA1 pyramidal neurons extending into stratum oriens and radiatum, respectively, mainly receive input from CA3 neurons via the Schaffer collateral pathway, whereas the distal apical dendrites, so-called apical tufts, mainly receive inputs from the entorhinal cortex via the perforant path as well as from thalamic nuclei (Andersen et al., 1966a; Andersen et al., 1966b; Spruston, 2008) (Figure 7.B). Presynaptic terminals of pyramidal neurons release the neurotransmitter glutamate and

their excitatory glutamatergic post-synaptic inputs are localized at dendritic spines, mushroom-shaped specializations that oppose the pre-synaptic terminals. Pyramidal neurons also receive inhibitory GABA-ergic synaptic input from interneurons directly onto their dendritic shafts.

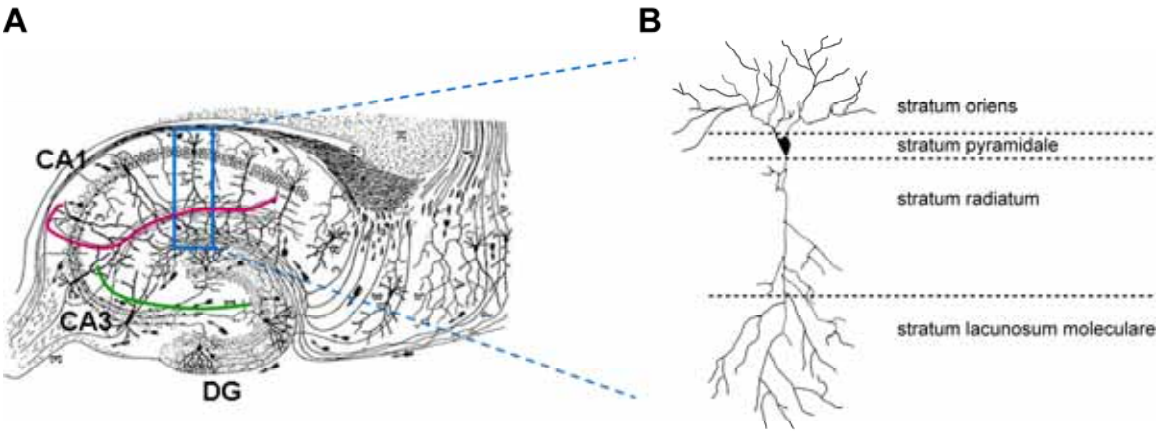


Figure 7: Pyramidal neurons in the hippocampus.

A) Illustration of the hippocampus showing the basic tri-synaptic hippocampal circuit. The hippocampus consists of two major pyramidal neuron populations in the CA1 and CA3 regions, whereas the dentate gyrus DG, a neighboring region, consists of granule neurons. Most external inputs are conveyed to the hippocampus through superficial layers of the entorhinal cortex. Neurons of the entorhinal cortex project to the DG and the CA3 through the perforant path. The granule neurons of the dentate gyrus relay input to CA3 pyramidal neurons via the mossy fiber pathway (marked in green). CA3 neurons in turn form projections, the Schaffer collaterals (marked in pink), that connect them to CA1 pyramidal neurons (blue box). CA1 neurons project outside the hippocampus to the deep layers of the entorhinal cortex (layer V). (Adapted and modified from Ramón y Cajal, 1911.). **B)** Drawing of a CA1 pyramidal neuron. The triangular-shaped cell bodies of CA1 pyramidal neurons lie in stratum pyramidale and their apical and basal dendrites extend into stratum radiatum/stratum lacunosum moleculare and stratum oriens, respectively.

The following chapter describes the generation of spines and mechanisms of synapse formation during brain development.

1.1.7. Regulation of spinogenesis and synaptogenesis in the brain

During brain development neurons establish contacts among each other via formation of synapses. Synapses are highly specialized cellular compartments that transmit information from the pre-synaptic axonal terminal to the post-synaptic membrane on the receiving dendrite. The majority of excitatory synaptic contacts in the mammalian brain are made between axon terminals and small dendritic protrusions called dendritic spines (Harris, 1999; Hering and Sheng, 2001), first discovered on cerebellar Purkinje cells by Ramón y Cajal about 120 years ago (Ramón y Cajal, 1888). Prototypical dendritic spines consist of a bulbous spine head, containing the post-synaptic density (PSD), an electron dense region where neurotransmitter receptors and many scaffold and signaling proteins are highly abundant and organized, that is separated from the dendrite by a thin spine neck (Gray, 1959b; Banker et al., 1974; Cotman et al., 1974; Harris and Stevens, 1989; Sorra and Harris, 2000). However, in the brain a wide variety of spine sizes and shapes are observed during development and adulthood, including stubby, thin, cup-shaped and mushroom-like spines as well as filopodia (Peters and Kaiserman-Abramof, 1970) (Figure 8.B, C). Furthermore, dendritic spines are highly motile and dynamic structures that can rapidly change shape during their lifetime (Crick, 1982; Fifkova and Delay, 1982; Matus et al., 1982; Bhatt et al., 2009).

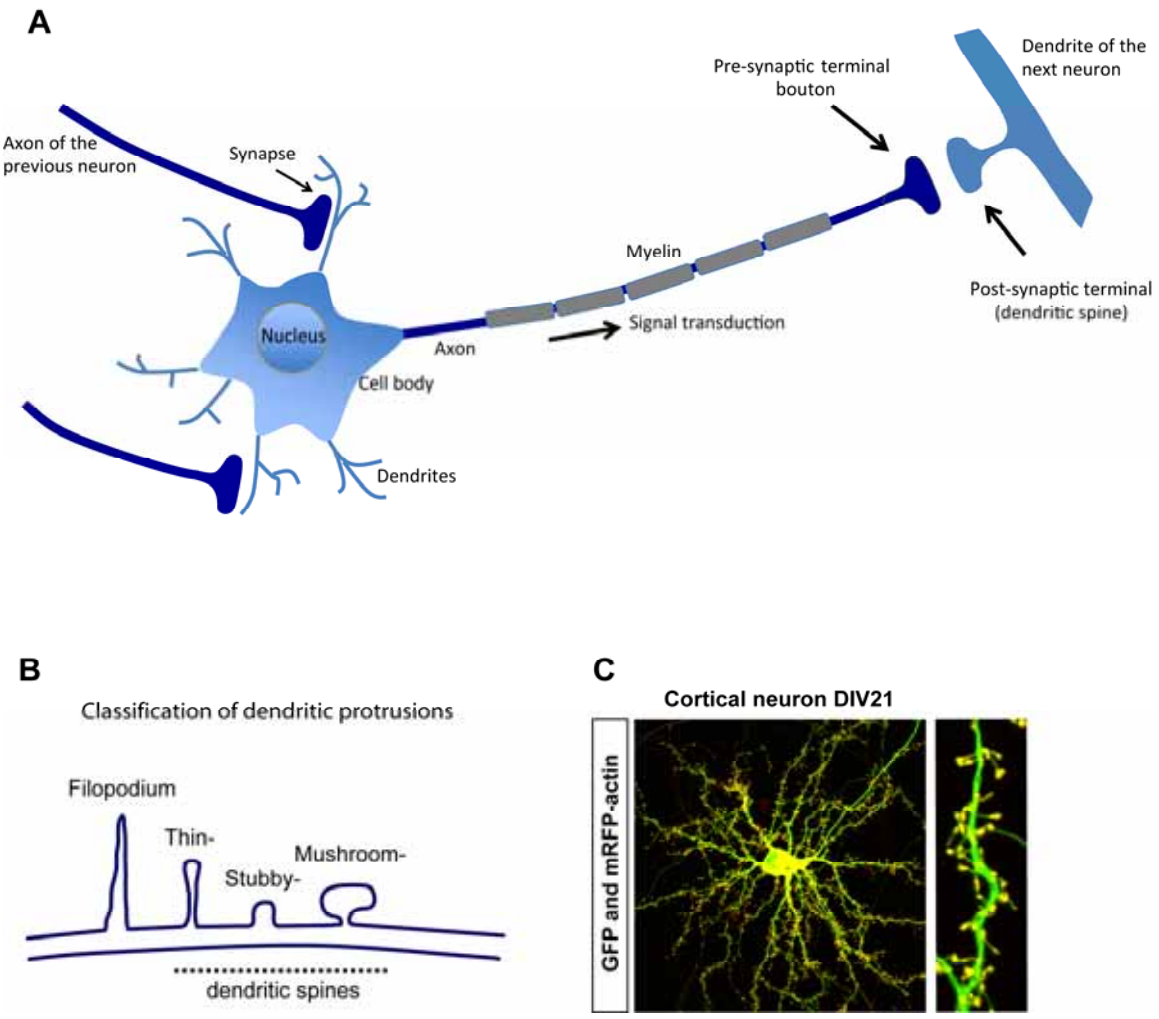


Figure 8: Excitatory synapses in the mammalian brain are formed on dendritic spines.

A) In the mammalian brain neurons communicate via synapses. Excitatory synapses are formed between pre-synaptic axon terminals of one neuron that release the neurotransmitter glutamate and post-synaptic dendritic protrusions, called spines, on the second neuron expressing neurotransmitter-receptors. **B)** Dendritic spines are found in a wide variety of shapes, classified as filopodia, thin, stubby and mushroom spines, accordingly to Peters and Kaiserman-Abramof's classification of 1970. **C)** Dendrites of mature primary cortical neurons, cultured for 21 days, are decorated with dendritic spines of different shapes. Dendritic spines have a high content of actin, as shown by the accumulation of the RFP-actin signal ('yellow') in spine heads of neurons transfected with GFP and mRFP-actin fusion constructs.

The transmission of information from one neuron to another across the synaptic cleft is achieved by transforming electrical signals to chemical signals. In excitatory neurons such as the pyramidal neurons in the hippocampus and cortex, the incoming action potentials moving along the axon cause the fusion of docked pre-synaptic vesicles, carrying the neurotransmitter glutamate. Glutamate is released into the synaptic cleft and activates ligand gated ion channels enriched in the PSD of the opposing post-synaptic spine. Ion flow through the glutamate receptors causes depolarization of the postsynaptic membrane. Multiple depolarization events sum up and propagate to the cell body where, if an electrical threshold is reached, a new action potential is generated in proximity to the cell body at the axon hillock (Figure 8.A).

However the function of dendritic spines is still under debate and several hypotheses are discussed in this competitive field of neuroscience (Spruston, 2008; Yuste, 2011). 1) Dendritic spines could serve to connect with passing axons in order to increase the surface of the postsynaptic cell, maximizing the possible number of synapses per cell (Yuste and Denk, 1995). 2) Spines could also isolate the PSD from the dendrite and act as biochemical compartments, confining active second messengers and proteins close to the activated synapse and to enable input-specific synaptic plasticity (Svoboda et al., 1996; Sabatini et al., 2002; Bloodgood and Sabatini, 2005; Noguchi et al., 2005; Gray et al., 2006). 3) Whether spines could also act as electrical compartments, filtering synaptic potentials and electrically isolating inputs from each other, remains controversial. However recently a new model was proposed by Yuste, suggesting that when viewed from a circuit-based perspective these three putative functions of spines could complement each other to build synaptic circuits that are distributed, linearly integrating and plastic (Yuste, 2010; Yuste, 2011).

1.1.7.1. Spinogenesis and synaptogenesis during brain development

The formation of dendritic spines coincides with the main period of synaptogenesis during the first few weeks after birth in many regions of the developing rodent brain (Yuste and Bonhoeffer, 2004). Progressive changes in the shape of dendritic protrusions are observed during brain development. During early synaptogenesis/spinogenesis, dendritic shafts of immature neurons are covered with thin, headless, elongated, and highly motile filopodia that are gradually replaced by more stable dendritic spines, which acquire a mature morphology with a distinct spine head and reach higher densities on the dendrite, as synapse formation progresses. An early period, the first three postnatal weeks, is characterized by a massive production of an imense number of synapses that is followed by a later period of synapse pruning and elimination, eventually establishing and refining the final number and quality of synaptic networks (Galofre and Ferrer, 1987; Petit et al., 1988; Miller, 1988; Lubke and Albus, 1989; Zecevic et al., 1989; Zecevic and Rakic, 1991; Eckenhoff and Rakic, 1991; Bourgeois and Rakic, 1993; Anderson et al., 1995; Zhang and Benson, 2000).

Based on experimental observations different models have been proposed on how spinogenesis occurs in the developing brain. In the Sotelo model spines emerge from the dendritic shaft independently of the axonal terminal. In the Miller/Peters model the pre-synaptic terminal actually induces the formation of the spine. And finally, in the filopodial model a highly motile dendritic filopodium captures an axonal terminal and becomes a spine (reviewed in Yuste and Bonhoeffer, 2004).

Although synaptogenesis is a continuum from the time of initial axo-dendritic contact to the emergence of a fully functional synaptic contact, this process can be divided into discrete steps, including initial axo-dendritic contact formation, induction and differentiation of pre- and post-synaptic compartments, maturation into functional synaptic contacts followed by stabilization and maintenance (Zhang and Benson, 2000; Garner et al., 2006).

Concentrating on pyramidal neurons found in the forebrain, our current understanding of spine formation suggests the existence of two parallel modes of development (Yoshihara et al., 2009). One is predominantly active during early phases of cortex and hippocampus development and is based on the growth of filopodia and their transformation into mature spine synapses, a process that depends on the regulation of filopodial motility (Dailey and Smith, 1996; Ziv and Smith, 1996; Fiala et al., 1998;

Maletic-Savatic et al., 1999; Marrs et al., 2001; Trachtenberg et al., 2002; Zuo et al., 2005; de Roo et al., 2008). A second mode of spine formation exists, mostly observed in later development and the mature brain, where protrusions grow directly as spines from the dendrite, most likely without initial PSD or partner, and for which activity and specific forms of plasticity such as LTP are probably key factors leading to maturation and persistence of the new synapse (Engert and Bonhoeffer, 1999; Trachtenberg et al., 2002; Jourdain et al., 2003; de Roo et al., 2008).

Time lapse imaging experiments showed that dendritic filopodia are highly dynamic structures, capable of extending and retracting their whole structure in a range of minutes to hours. This dynamic behavior is ideally suited to explore the space around dendrites searching for possible pre-synaptic partners (Dailey and Smith, 1996; Portera-Cailliau et al., 2003).

Although filopodia repeatedly make transient contacts with axons, only a subset of these connections gets stabilized, involving the generation of calcium transients (Lohmann and Bonhoeffer, 2008). Only a small proportion of dendritic filopdia (10-20%) are eventually transformed into spines, as shown by imaging studies in young living mice and hippocampal slice cultures . However, most of these spines disappear within subsequent days due to the high spine turnover taking place during early development (Maletic-Savatic et al., 1999; Marrs et al., 2001; Trachtenberg et al., 2002; Zuo et al., 2005; Holtmaat et al., 2005; de Roo et al., 2008).

By applying time lapse imaging in acute slices of the neocortex during early postnatal development of the mouse brain (P2-12) Portera-Cailliau and colleagues demonstrated that two different types of filopdia exist. At dendritic growth cones filopodia mediate dendritic growth and branching in an activity-independent manner, whereas the dynamic behavior of filopida emerging from the dendritic shaft is regulated by activity and is responsible for synaptogenesis (Portera-Cailliau et al., 2003).

Several molecules, that specifically regulate the formation of flipodia, have been identified. They can be divided into accelerators and brakes of spine maturation (reviewed in Yoshihara et al., 2009). Among the brakes, the cell adhesion molecule Telencephalin (TLCN) is of special interest, as it is expressed exclusively by spiny neurons in the mammalian forebrain, it induces and maintains dendritic filopodia, slows spine formation and it even can cause spine-to-filopdia reversion (Matsuno et al., 2006; Furutani et al., 2007).

Furthermore it was shown that filopodia motility is crucial for synapse formation during development. Elimination of EphBs was found to decrease filopodia motility without affecting spine motility, resulting in a decreased rate of synaptogenesis during early but not late development (Kayser et al., 2008).
Time lapse imaging in more mature tissue revealed the appearance of new protrusions directly as spines, even initially without a discernable PSD or pre-synaptic partner (Engert and Bonhoeffer, 1999; Trachtenberg et al., 2002; Jourdain et al., 2003; de Roo et al., 2008). Cell adhesion molecules and nitric oxide (NO) signalling, depending on PSD-95, were shown to regulate the initial synapse formation and stabilization of newly formed spines (Li et al., 2007; Arikkath and Reichardt, 2008; Nikonenko et al., 2008).

Many important regulators and cellular mechanisms of spine development have been identified that contribute to the formation, remodelling, stabilization and maintenance of newly formed synapses (reviewed inEthell and Pasquale, 2005; Calabrese et al., 2006; Tada and Sheng, 2006).
Trans-synaptic signalling via neurotransmitter receptors, EphB receptors and ephrin-B ligands, cell adhesion molecules, such as Neuroligins and neurexins, cadherins, synaptic cell adhesion molecules of the immunoglobulin superfamily, such as SynCam and NCam, neurotrophins and growth factor receptors, such as BDNF signalling through TrkB receptors regulate spine and synapse development (Butler et al., 1998; Shimada et al., 1998; Maletic-Savatic et al., 1999; Engert and Bonhoeffer, 1999; McKinney et al., 1999; Yuste and Bonhoeffer, 2001; Poo, 2001; Portera-Cailliau et al., 2003; Passafaro et al., 2003; Murai et al., 2003; Penzes et al., 2003; Henkemeyer et al., 2003; Washbourne et al., 2004; Richards et al., 2005; Salinas and Price, 2005; Segura et al., 2007; Arikkath and Reichardt, 2008; Tanaka et al., 2008; Arikkath, 2009). Likewise extracellular matrix proteins and their receptors, such as integrins and syndecans, neuron-glia communication via integrins, EphA receptors and ephrin-A ligands, as well as via secreted molecules from astrocytes, such as cholesterol, were also shown to contribute to synaptogenesis (Ullian et al., 2001; Chavis and Westbrook, 2001; Mauch et al., 2001; Ullian et al., 2004).
Moreover, direct contacts beween astrocytes and neurons contribute to development, maturation and function of synapses (Haber et al., 2006; Nishida and Okabe, 2007; Eroglu and Barres, 2010).

Many of the post-synaptic receptors use CamKII, PKC, Erk1/2, and p38MAP kinase signal transduction pathways to modulate spine development (reviewed in Sheng and Kim, 2002).

Ca^{2+}-signaling through N-methyl-D-aspartate (NMDA) receptors, amino-3-hydroxy-5-methylisoxazole-4- propionic acid (AMPA) receptors, without GluR2 subunits, and voltage-dependent Ca^{2+} channels, or Ca^{2+} release from intracellular stores affect synaptic strength spine shape (Yuste et al., 2000; Rose and Konnerth, 2001).

Moreover, scaffolding proteins of the postsynaptic density, such as Homer, Shank, guanylate kinase-associated protein (GKAP), and MAGUK proteins (see later) play critical roles during spine formation, stabilizing immature spine precursors. The process of synapse formation and especially synapse stabilization is tightly regulated by the MAGUK scaffold protein PSD-95 and neuronal activity, which will be discussed in more detail in chapter 1.2.

Finally, the ultimate machinery that implements the shape and remodelling of spines is the actin cytoskeleton (Fischer et al., 1998; Matus, 2000; Smart and Halpain, 2000; Star et al., 2002; Sekino et al., 2007; Hotulainen and Hoogenraad, 2010; Frost et al., 2010; Tolias et al., 2011). The intrafilopodia and intraspine actin networks are regulated by multiple actin-binding proteins, such as cortactin and cofilin, regulators of actin polymerization and dynamics, such as cortactin, cofilin Arp2/3 and N-WASP, Rho family GTPases, such as Rac, Cdc42, RhoA, and regulators of Rho family GTPases, such as Kalirin 7, which in concert determine the final shape of the spine (reviewed in Ethell and Pasquale, 2005).

Recently, it has been demonstrated that dynamic microtubules are able to enter dendritic spines and thereby control spine morphology and activity-dependent, plasticity-induced spine remodelling (Gu et al., 2008; Hu et al., 2008; Jaworski et al., 2009).

Interestingly, the development of dendritic spines is impaired in neurodevelopmental disorders characterized by mental retardation, such as non-specific X-linked mental retardation, Fragile X syndrome, William's syndrome, Rett syndrome, Down's syndrome, Angelman syndrome and autism (Purpura, 1974; Rudelli et al., 1985; Ferrer and Gullotta, 1990; Kaufmann and Moser, 2000; Nimchinsky et al., 2001; Fiala et al., 2002; Ramakers, 2002; Barnes and Milgram, 2002) as shown in post-mortem studies of human brains as well as in mouse models of these diseases. Patients with these disorders have abnormally long and thin dendritic spines with irregular shapes and

increased or decreased densities. Of note, both decreased and increased spine densities are hallmarks of neurodevelopmental disorders, emphasizing the importance of tight regulation of synapse formation and synapse elimination during brain development.
Moreover changes in dendritic spines, malformation and loss, are associated with a number of neurological conditions, including schizophrenia, bipolar disorder with psychosis and epilepsy, stroke, trauma, dementia, major depression (Garey et al., 1998; Jiang et al., 1998a; Rajkowska et al., 1999; Swann et al., 2000; Rosoklija et al., 2000; Isokawa, 2000; Glantz and Lewis, 2001; Stockmeier et al., 2004).
Furthermore, spine defects are not confined to neurodevelopmental disorders, but are also associated with chronic neurodegenerative diseases. Alzheimer disease (AD) and Parkinson disease (PD) often include spine loss and additional dendritic changes in the early stages of disease progression (Selkoe, 2002).

1.1.7.2. Spine stability and synaptic plasticity in the adult brain

Emerging evidence indicates that once established, synapses and dendrites can be maintained for long periods, if not for an organism's entire lifetime (Grutzendler et al., 2002; Zuo et al., 2005; Holtmaat et al., 2005; Yang et al., 2009a). Many of the cellular and molecular mechanisms that control developmental synaptogenesis were also found to enable long-term synapse stabilization and maintenance (reviewed in Lin and Koleske, 2010).
However dendritic spines and synapses also remain plastic in the adult brain. These changes in synaptic connections are considered essential for learning and memory formation (Changeux and Danchin, 1976; Hubel et al., 1977; Bailey and Kandel, 1993; Buonomano and Merzenich, 1998; Lichtman and Colman, 2000; Lendvai et al., 2000; Trachtenberg et al., 2002; Holtmaat et al., 2006; Hofer et al., 2009). Spine formation, pruning, and remodeling in mature neurons can be induced by many factors, such as certain patterns of synaptic activity, learning and memory formation, stress, hormones, and changes in temperature, normal aging as well as by chronic substance abuse (Fifkova et al., 1982; Woolley et al., 1990; Mcewen et al., 1991; Hosokawa et al., 1995; Maletic-Savatic et al., 1999; Engert and Bonhoeffer, 1999; Kirov and Harris, 1999; Yuste and Bonhoeffer, 2001; Roelandse and Matus, 2004; Fuchs et al., 2006; Radley et al., 2008; Goldwater et al., 2009). Dynamic rearrangements of the spine actin cytoskeleton

are underlying structural and functional synaptic plasticity and dynamic remodelling of dendritic spines (Hotulainen and Hoogenraad, 2010).

Two major forms of synaptic plasticity that represent accepted cellular models for learning and memory in the brain are long-term potentiation (LTP) and long-term depression (LTD). Prolonged enhancement of synaptic transmission at a synapse results in LTP and synapse strengthening, whereas during LTD a persistent weakening of synaptic transmission occurs (Bliss and Collingridge, 1993; reviewed in Malenka and Nicoll, 1999; Malenka and Bear, 2004; Citri and Malenka, 2008). LTP requires Ca^{2+} influx through NMDA receptors, activation of CamKII, and protein synthesis, and is eventually expressed by an increase in the number of AMPA receptors inserted in the post-synaptic membrane. Structural remodeling such as increases in PSD size, the growth of new dendritic spines and the enlargement of existing spines is also associated with LTP. Whereas various forms of LTD require NMDA receptors, group I metabotropic glutamate receptors (mGluRs) and endocannabinoid signaling. LTD depends in general on Ca^{2+} influx through NMDA receptors as well as on protein phosphatase 1, calcineurin, and protein synthesis. In contrast to LTP, LTD is commonly accompanied by a reduction in the number of AMPA receptors that are removed by endocytosis (Malenka and Nicoll, 1999; Malinow and Malenka, 2002; Song and Huganir, 2002; Bredt and Nicoll, 2003; Nagerl et al., 2004; Zhou et al., 2004; Hsieh et al., 2006; Derkach et al., 2007; Holtmaat and Svoboda, 2009).

1.2. The role of the post-synaptic scaffold protein PSD-95 in spine development, maturation and regulation of synapse stability

1.2.1. Organization of the post-synaptic spine compartment, the architecture of the post-synaptic density (PSD)

Excitatory synapses are characterized by an electron-dense thickening, the post-synaptic density (PSD), originally identified by electron microscopy (Gray, 1959a; Gray, 1959b). The PSD appears as an electron-dense protein band located immediately beneath the post-synaptic membrane, in close apposition to the pre-synaptic active zone, from where neurotransmitter vesicles are released. Many different protein complexes were identified as components of the PSD, including neurotransmitter receptors, voltage dependent channels, proteins involved in signalling cascades, scaffolding proteins, actin filaments and actin-binding proteins (Figure 9). The localization of the PSD ensures that post-synaptic receptors are in close proximity to pre-synaptic neurotransmitter release sites. Architecture and organization suggest that the PSD functions as a structural matrix, which clusters ion channels in the postsynaptic membrane (Kennedy et al., 1983; Ehlers et al., 1996; Kennedy, 1997; Kennedy, 2000) and anchors signaling molecules such as kinases and phosphatases at the synapse (Klauck and Scott, 1995). Thereby the PSD serves as a general organizer of the postsynaptic signal transduction machinery, which links regulatory molecules to their targets, coordinates developmental and activity-dependent changes in postsynaptic structures, and establishes the functional topography of the postsynaptic membrane.

At excitatory synapses in the mammalian brain, the main neurotransmitter receptors are AMPA, NMDA and metabotropic glutamate receptors. AMPA receptors are ionotropic receptors composed of GluR 1-4 subunits (Hollmann and Heinemann, 1994; Madden, 2002). Each functional receptor is composed of four subunits (Figure 9). AMPA receptors are permeable to potassium and sodium ions, but AMPA receptors lacking the GluR2 subunit are also permeable to calcium (Burnashev et al., 1992). However, most AMPA receptors in CA1 pyramidal cells contain the GluR2 subunit and are therefore

impermeable to calcium ions (Geiger et al., 1995). NMDA receptors are ionotropic receptors composed of NR1 and NR2A-D subunits (Madden, 2002). The subunit composition determines the magnitude of calcium permeability of the receptor and is regulated during development. At early developmental stages, NMDA receptors are rich in NR2B subunits (high calcium permeability); later in development, these receptors are replaced by NR2A-subunit containing receptors (lower calcium permeability) (Sheng et al., 1994). The activity of NMDA receptors is highly voltage dependent: at resting membrane potential (around -70 mV), NMDA receptors are blocked by magnesium ions in the channel pore. Membrane depolarization relieves this block and the receptors become permeable to calcium, magnesium and potassium (Nowak et al., 1984). These receptors are thought to be the main pathway for calcium entry during synaptic activity (Mainen et al., 1999; Kovalchuk et al., 2000; Sabatini et al., 2002). Moreover, differences in the number and subtype of activated NMDA receptors are thought to be the major determinants regulating spine calcium signals during synaptic activity (Sabatini et al., 2001; Sobczyk et al., 2005).

Prominent scaffolding proteins are Shank, GKAP, Homer and the PSD-MAGUKs, including PSD-95 (Cho et al., 1992), PSD-93 (Mcgee et al., 2001), SAP97 (Muller et al., 1995) and SAP102 (Muller et al., 1996). Post-synaptic scaffolds link membrane glutamate receptors to the underlying spine actin cytoskeleton and provide the spatial organization of multiple downstream signalling pathways (Pak et al., 2001; Lee et al., 2006; Chen et al., 2008; Feng and Zhang, 2009).

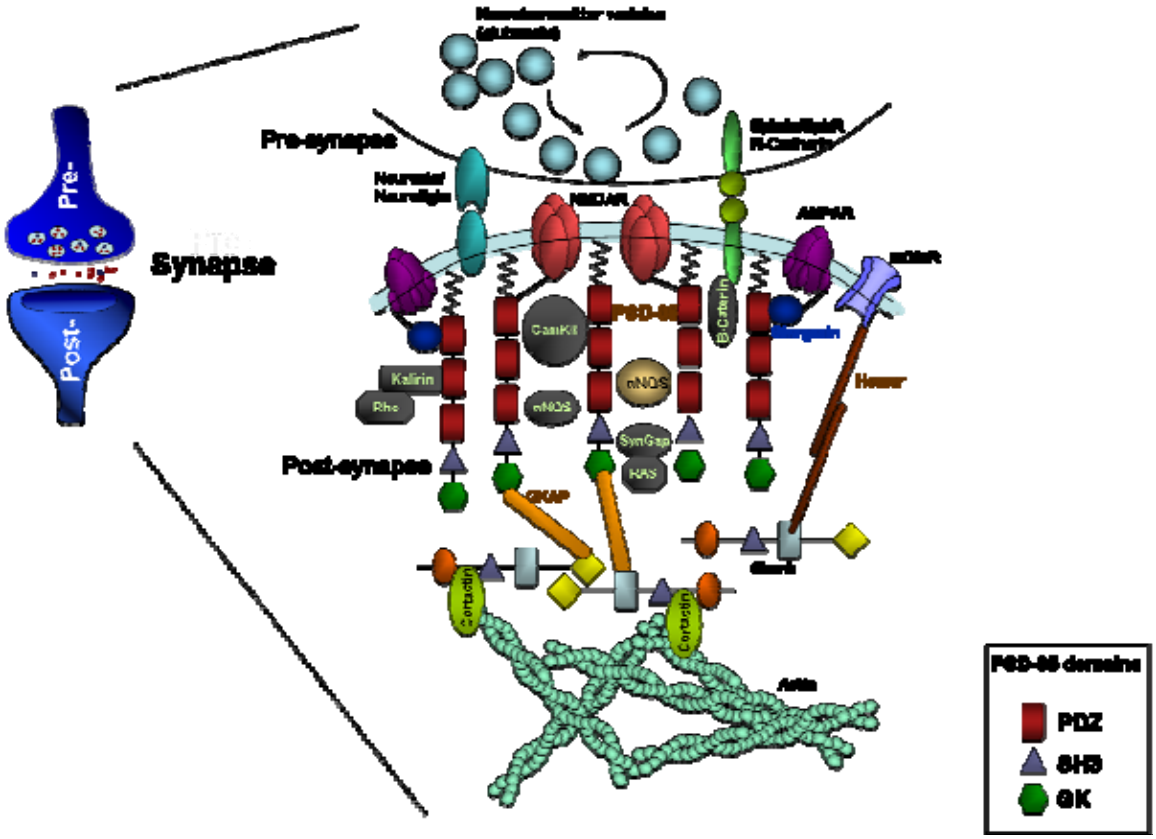


Figure 9: Protein complex organization in the post-synaptic density (PSD) of spines.

The postsynaptic density is comprised of membrane receptors and ion channels, scaffold and adaptor proteins, signalling proteins, cell-adhesion molecules and components of the cytoskeleton. Glutamate receptors, such as NMDARs (N-methyl-d-aspartate receptors) and AMPARs (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors), are inserted into the postsynaptic membrane, with the NMDARs at the centre and the AMPARs more peripheral. The PDZ-domain-containing scaffold proteins PSD-95 (also known as DLG4) and the Src-homology domain 3 (SH3) and multiple ankyrin repeat domains (Shank) family form a two-layer protein network below the postsynaptic membrane, which is bridged by guanylate kinase-associated protein (GKAP). PSD95 forms filamentous structures, with its amino terminus attached to the membrane. Other signalling molecules occupy the spaces in the PSD95–GKAP–Shank protein web. Shank-family scaffolds are further linked to actin filaments. The domains of PSD-95 are shown in the box on the right; other proteins are represented by simple shapes and are labelled. The presynaptic and postsynaptic membranes are connected by cell-adhesion molecules. CaMKII, calcium/calmodulin-dependent protein kinase II; EphR, ephrin receptor; GKAP, guanylate kinase-associated protein; mGluR, metabotropic glutamate receptor; nNOS, neuronal nitric oxide synthase.

1.2.2. The role of PSD-95 and other scaffold proteins in dendritic spine and synapse development

Scaffolding proteins of the postsynaptic density, including Homer, Shank and GKAP were shown to regulate synapse development, structural and functional organization of dendritic spines and modulate synaptic plasticity (Rao et al., 1998; Naisbitt et al., 1999; Sala et al., 2001; Usui et al., 2003; Romorini et al., 2004; Sala et al., 2005; Gerrow et al., 2006; Hung et al., 2008; Verpelli et al., 2011). Among the different scaffold proteins in the spine, the family of PSD-MAGUKs (Membrane associated guanylate kinases), and especially the post-synaptic density protein 95 (PSD-95), organizes glutamate receptors, AMPA and NMDA receptors, and their associated signalling proteins determining the size and strength of synapses (Xu, 2011; Zheng et al., 2011). The modular structure of these proteins and in particular their PDZ domains are critical apects necessary to be understood in order to fully comprehend the post-synaptic functions of this family of proteins.

1.2.2.1. The modular structure of PDZ-MAGUK proteins

PSD-95-like-MAGUKs constitute a major family of multidomain scaffold proteins, including PSD-95, PSD-93, SAP102 and SAP97, that shares a multi-modular protein structure. Three PSD-95/Discs large/zona occludens-1 (PDZ) domains, followed by a Src-homolgy-3 (SH3) domain and a catalytically inactive guanylate kinase (GK) domain (Montgomery et al., 2004; Kim and Sheng, 2004) (see also Figure 9) mediate protein-protein interactions to numerous other PSD proteins, including neurotransmitter receptors, such as the NMDAR; adhesion molecules, such as neuroligin and signaling enzymes, like nNOS (Kim et al., 1997; Kawashima et al., 1997; Kornau et al., 1997; Craven and Bredt, 1998; Kennedy, 2000; Sheng and Lee, 2000; Garner et al., 2000; Sheng and Sala, 2001).

PSD-95 directly interacts with NMDARs (Kornau et al., 1995; Niethammer et al., 1996; Hoe et al., 2006), while it indirectly interacts with AMPA receptors by recruiting the tetraspanning membrane protein stargazin to synapses, that binds directly to AMPAR subunits (Schnell et al., 2002). Stargazin and its relatives are essential for the surface expression and synaptic accumulation of AMPARs, and the AMPAR activity depends on

an interaction of the stargazin C terminus with the PDZ domains of PSD-95 (Chen et al., 2000a; Schnell et al., 2002; Chen et al., 2003a).

1.2.2.2. Developmental regulation of MAGUKs

During neuronal development in mammals, expression of PSD-95 family members occurs in a regulated manner. SAP102 is highly expressed before postnatal day 10 in the rodent brain and then gradually decreases. In contrast, little PSD-95, PSD-93 and SAP97 are found in the brain of newborn mice and rats, but their expression dramatically increases during later postnatal development (Sans et al., 2000; van Zundert et al., 2004; Petralia et al., 2005; Elias et al., 2006; Elias et al., 2008). It was shown that the switch from SAP102 to PSD-95 drives synaptic development by inducing a replacement of NR2B-containing NMDRs with NR2A-containing NMDRs as well as a developmental increase in AMPA receptor transmission (Elias et al., 2008).

The PSD-MAGUKs N-terminus is highly variable. Multiple splice variants have been identified for PSD-95 (Chetkovich et al., 2002; Funke et al., 2005; Schluter et al., 2006), PSD-93 and SAP97 (Muller et al., 1995; Lue et al., 1996; Nikandrova et al., 2010; Zheng et al., 2011). Furthermore, the predominant isoform of PSD-95 (α-isoform) has an N-terminal domain, which is palmitoylated and also capable of multimerizing PSD-95 with itself and other MAGUKs with a palmitoylated N-terminus (Kim et al., 1996; Hsueh et al., 1997; Firestein et al., 2000; Fukata et al., 2004; Schluter et al., 2006; Fukata and Fukata, 2010). Palmitoylation motifs consist of specific cysteine residues that get modified with the fatty acid palmitate (Dunphy and Linder, 1998; Resh, 1999; El-Husseini and Bredt, 2002; Smotrys and Linder, 2004) by palmitoyl transferases (Fukata et al., 2004). Palmitoylation of PSD-95 in neurons is especially necessary for synaptic targeting and receptor clustering (Topinka and Bredt, 1998; Craven et al., 1999; El-Husseini et al., 2000a). N-terminal splice variants containing suitable cysteine residues occur for each PSD-MAGUK (Muller et al., 1996; Brenman et al., 1996b; Tiffany et al., 2000; Firestein et al., 2000; Schluter et al., 2006). PSD-95 and PSD-93 were found palmitoylated *in vivo*, whereas the N-terminal region of SAP102 forms a zinc finger. A small proportion of PSD-95 in the brain contains an N-terminal L27 domain (β-isoform) (Chetkovich et al., 2002). A recent study demonstrated that the PSD-95 α-isoform influences AMPAR-mediated synaptic strength independent of activity, whereas the delivery AMPAR

through the β-isoforms is activity-dependent and involves NMDAR and CamKII activity (Schluter et al., 2006)

1.2.2.3. PSD-95 in synapse development and maturation

PSD-95 was found to regulate synapse development and maturation. Roles for MAGUKs in synapse formation were derived first from studies of Drosophila melanogaster disk large (dlg) mutants (Lahey et al., 1994; Budnik et al., 1996). In the mammalian brain, PSD-95 and related PSD-MAGUKs are also suggested to play roles in postsynaptic development. A synaptogenic role for PSD-95 was suggested, since it was shown to cluster at synapses prior to any of its associated protein partners, thus suggesting a developmental role (Rao et al., 1998). Furthermore, overexpression of PSD-95 accelerates development and increases the size of neuronal synapses (El-Husseini et al., 2000b). This overexpression enhances the postsynaptic accumulation of certain PSD-95 binding proteins such as GKAP, and also promotes the development of the pre-synaptic terminals that innervate PSD-95 expressing cells (El-Husseini et al., 2000b). This triggering of pre-synaptic differentiation implies that PSD-95 induces a retrograde signal. The retrograde signal is likely mediated by NO production, triggered by PSD-95, and neuroligin which binds to PSD-95 and by itself can trigger pre-synaptic differentiation via binding to neurexin on nerve terminals (Scheiffele et al., 2000; Futai et al., 2007; Nikonenko et al., 2008). Due to its modular structure, PSD-95 was found to interact with many proteins such as kalirin-7, SPAR, synGAP, SPIN90/WISH and GKAP, that eventually would affect spine and synapse development (Kim et al., 1997; Naisbitt et al., 1997; Naisbitt et al., 1999; Pak et al., 2001; Sala et al., 2001; Penzes et al., 2001; Vazquez et al., 2004; Lee et al., 2006; Xie et al., 2007). In general, overexpression of PSD-95 promotes synaptic maturation (El-Husseini et al., 2000b), while knock-down of PSD-95 results in decreased synaptic strength and spine density (Ehrlich et al., 2007).

1.2.2.4. PSD-95 in basal synaptic transmission

PSD-95 controls synaptic neurotransmission at excitatory synapses. Acute overexpression of PSD-95 potentiates AMPAR-mediated excitatory post-synaptic currents (EPSCs), whereas no enhancement of NMDAR-mediated EPSCs occurs in response to PSD-95 overexpression. In addition, acute knock-down of PSD-95 via RNAi

decreases AMAPR EPSCs but, in most studies, not NMDAR EPSCs. Furthermore, knock-down of PSD-95 was shown to reduce spine growth during LTP and to arrest normal spine maturation (Schnell et al., 2002; Beique and Andrade, 2003; Ehrlich and Malinow, 2004; Nakagawa et al., 2004; Elias et al., 2006; Schluter et al., 2006; Beique et al., 2006; Ehrlich et al., 2007; Futai et al., 2007; Elias et al., 2008; Xu et al., 2008).
The regulation of basal synaptic strength by PSD-95 was shown to require both N-terminal and C-terminal domains, two palmitoylated N-terminal cysteine residues in PSD-95 as well as the C-terminal SH3 and GK domains, both confering synaptic localization of PSD-95 (Xu et al., 2008).

1.2.2.5. PSD-95 in synaptic plasticity

PSD-95 also regulates synaptic plasticity. Synaptic potentiation induced by the overexpression of PSD-95 seems to mimic LTP, in that it converts silent synapses into functional synapses and drives GluR1-containing AMPARs into synapses, thereby dramatically increasing the synaptic density of AMPARs (El-Husseini et al., 2000b). This overexpression prevents the further recruitment of additional AMPARs that normally occurs during LTP, probably due to the saturation of the AMPAR trafficking machinery (Stein et al., 2003; Ehrlich and Malinow, 2004). This might also explain why PSD-95 overexpression was found to occlude LTP (Stein et al., 2003). To be an effective learning mechanism, synaptic plasticity has to be a reversible process. Indeed, prolonged low intensity activation of NMDARs leads to a decreased synaptic AMPAR density, a process known as long-term depression (LTD) (Dudek and Bear, 1992). Interestingly, overexpression of PSD-95 augments the magnitude of LTD induction, while acute knock-down of PSD-95 imapirs LTD, suggesting a role for PSD-95 in this plasticity process (Stein et al., 2003; Beique and Andrade, 2003; Xu et al., 2008).

Two general hypotheses exist for how PSD-95 regulates NMDAR-dependent synaptic plasticity.
The first theory supports PSD-95 as a slot protein for anchoring AMPARs in the PSD, acting as the target of signalling during synaptic plasticity (Schnell et al., 2002; Colledge et al., 2003; Ehrlich and Malinow, 2004). In this role a reduction in synaptic PSD-95 would directly affect the levels of synaptic AMPARs leading to a long-term loss during LTD. Consistent with this hypothesis, two biochemical modifications of PSD-95 were

described, palmitoylation (El-Husseini et al., 2000a; El-Husseini and Bredt, 2002) and ubiquitination (Colledge et al., 2003), that are necessary for removal of synaptic PSD-95 resulting in AMPAR endocytosis. However, the direct polyubiquitination of PSD-95 in response to NMDAR activation is controversial (Patrick et al., 2003). Moreover, it has been shown that membrane detachment of PSD-95 is not crucial for mediating synaptically induced LTD (Xu et al., 2008; Bhattacharyya et al., 2009). Different experimental approaches, e.g. primary neurons vs. acute brain slices, might account for the different mechanisms observed for PSD-95 functioning during LTD (Carroll et al., 1999a; Carroll et al., 1999b; Sheng and Kim, 2002).

Alternatively, the second hypothesis establishes PSD-95 as a signalling scaffold connecting NMDAR-mediated Ca^{2+} influx to downsream intracellular signalling cascades that trigger LTD. In support of this idea, PDZ domains of PSD-95 are required for binding to NMDARs and to AMPARs via stargazing (Kornau et al., 1995; Niethammer et al., 1996; Chen et al., 2000a; Schnell et al., 2002; Chen et al., 2003a), while the SH3 and GK domains were found to interact with various signalling complexes, including AKAP79/150-PKA-PP2B signalling (Mulkey et al., 1994; Kameyama et al., 1998; Tavalin et al., 2002; Bhattacharyya et al., 2009), GKAP and spine-associated Rap guanosine triphosphatase activating protein (SPAR) (Kim et al., 1997; Naisbitt et al., 1997; Pak et al., 2001; Montgomery et al., 2004; Kim and Sheng, 2004).

These two hypotheses are not mutually exclusive and might infact co-exist, but the the selective importance of each mechanism remains to be clarified (reviewed in Xu, 2011).

1.2.2.6. Dynamic regulation of synaptic PSD-95

The function of PSD-95 was found to be regulated by several modifications that will be briefly discussed below.

As described above, synaptic accumulation of PSD-95 requires the palmitoylation of two N-terminal cysteines (Cys3 and Cys5) (Craven et al., 1999). Neuronal activity promotes the dispersal of PSD-95 from synapses, in part by depalmitoylating these two residues (El-Husseini et al., 2002). However, it was demonstrated that LTD induction is not dependent on membrane detachment of PSD-95 (Xu et al., 2008; Bhattacharyya et al., 2009).

An important downstream effector of NMDAR signaling is the neuronal isoform of nitric oxide synthase (nNOS). PSD-95 PDZ domains bind to both the C-terminus of the

NMDAR and to an N-terminal PDZ domain from nNOS (Brenman and Bredt, 1996; Brenman et al., 1996a; Christopherson et al., 1999). This interaction ensures that Ca^{2+}-influx through NMDARs activates nNOS in a calcium/calmodulin-regulated manner causing NO production (Bredt and Snyder, 1990; Bredt et al., 1990; Christopherson et al., 1999; Sattler et al., 1999).

A recent study showed that in addition to palmitoylation, PSD-95 is physiologically S-nitrosylated on Cys3 and Cys5, suggesting a competition between these two modifications. In response to NMDAR activation, synaptic production of NO is enhanced, that increases nitrosylation of PSD-95 (Ho et al., 2011). As a consequence, PSD-95 would be maintained in a depalmitoylated state resulting in its removal from the spine.

Furthermore, the ubiquitin-proteasome system is implicated in activity-dependent regulation of synaptic PSD-95. Ca^{2+}-influx through NMDARs during LTD causes loss of synaptic AMPAR due to polyubiqutination and proteasome-mediated degradation of PSD-95 (Colledge et al., 2003; Pak and Sheng, 2003). This mechanism could involve direct ubiquitylation of PSD-95 (Colledge et al., 2003) or could be indirect, through the ubiquitylation and degradation of other postsynaptic regulatory proteins such as SPAR (Patrick et al., 2003; Pak and Sheng, 2003).

The function of PSD-95 is regulated more acutely by phosphorylation via different kinases. Cyclin-dependent kinase 5 (CDK5), a serine–threonine kinase that is essential for brain development, phosphorylates the N-terminal region of PSD-95, inhibiting its multimerization, channel clustering activity and possibly its synaptic localization (Morabito et al., 2004). In addition, a recent study provided evidence that CDK5-dependent phosphorylation of PSD-95 regulates nonproteolytic ubiquitination of PSD-95. Reduced CDK5 activity results in increased murine double minute 2 (Mdm2)-dependent ubiquitination of PSD-95 leading to increased interaction with β-adaptin, a subunit of the clathrin adaptor protein complex AP-2, suggesting a mechanism by which PSD-95 might regulate NMDAR-induced AMPAR endocytosis (Bianchetta et al., 2011).

Rac1-JNK-dependent phosphorylation of Serine residue 295 promotes synaptic accumulation of PSD-95, thereby influencing synaptic plasticity (Kim et al., 2007b). Induction of LTD by NMDA treatment decreases S295 phosphorylation, whereas chemical-induced LTP was found to increase S295 phosphorylation. Moreover, dephosphorylation of S295 is required for LTD induction and expression, presumably by regulation of synaptic PSD-95 levels (Kim et al., 2007b).

Steiner *et al.* identified a mechanism by which destabilization of the PSD by CamKII-dependent phosphorylation of PSD-95 at serine 73 inhibits spine growth and synaptic plasticity (LTP) (Steiner et al., 2008).

Time-lapse imaging has confirmed that PSD-95 tagged with green fluorescent protein undergoes dynamic turnover, although at a slower rate than several other synaptic proteins (Inoue and Okabe, 2003; Sharma et al., 2006; Sturgill et al., 2009). In summary, PSD-95 is regulated in a dynamic fashion by subcellular redistribution, protein phosphorylation, degradation and probably by additional, so far unknown modifications.

1.2.2.7. *In vivo* functions of PSD-95

Targeted disruption of PSD-95 produces synaptic defects that partially correspond to its proposed role in regulating synaptic AMPARs (Migaud et al., 1998a; Beique et al., 2006), however, important issues remain unsolved. Mice lacking PSD-95 show an absence of LTD as might be expected (Migaud et al., 1998b). On the other hand, these mice show enhanced LTP, which is not easily reconciled with the proposed roles for PSD-95 in controlling synaptic plasticity (Migaud et al., 1998a). Nevertheless, these mice show impairments in behavioral tests of learning and memory, which likely reflect alterations in their activity-dependent control of synaptic AMPARs (Migaud et al., 1998a). An important unsolved issue is why basal synaptic strength remains intact in mice lacking PSD-MAGUKs. No changes in baseline AMPAR function are noted in mice lacking functional PSD-95, PSD-93, or SAP97 (Migaud et al., 1998a; Mcgee et al., 2001; Klocker et al., 2002). This seems inconsistent with the prevalent model that PSD-95, via its interaction with stargazin and other transmembrane AMPA receptor regulatory proteins (TARPs), control AMPAR density at synapses. This discrepancy might be explained by gene redundancy. The neuronal MAGUKs are highly similar in sequence and the C-terminus of stargazin can bind to multiple members of this family (Dakoji et al., 2003). Defining the essential roles for PSD-MAGUKs in the brain remains a critical challenge and may require inter-breeding of these mutant mice. This seems to be true at least for PSD-93-mediated compensations (Elias et al., 2008). Furthermore, it was shown that PSD-95 controls activity-dependent AMPAR incorporation at synapses via PDZ interactions not only during LTP *in vitro* but also during experience-driven synaptic strengthening by natural stimuli *in vivo* (Ehrlich and Malinow, 2004).

1.3. Ubiquitin, Ubiquitin-like proteins and protein degradation in neuronal function and development

Ubiquitylation is a post-translational protein modification whereby target proteins are covalently conjugated to the small protein ubquitin via a thightly regulated sequence of enzymatic steps, thereby leading either to the proteasome-mediated degradation of the ubiquitylated substrate or inducing a change in the function of the protein (reviewed in Hershko and Ciechanover, 1998). Ubiquitylation was shown to play important roles in controlling various steps of neuron development and function, and dysregulation of the ubiquitin-proteasome system is implicated in multiple CNS diseases (Tai and Schuman, 2008). In recent years many more ubiquitin-like proteins (UBLs) were discovered that now comprise the family of UBLs, including NEDD8, SUMO, ISG15, ATG8, ATG12, URM1 and UFM1, whose functions are just beginning to be elucidated (Schwartz and Hochstrasser, 2003; Kerscher et al., 2006). Almost all UBLs were found to be conjugated to their substrates via enzymatic cascades in a manner analogous to ubiquitin (Kerscher et al., 2006; Dye and Schulman, 2007). Among the UBLs, Nedd8 is one of the most studied, because of its relevance in cancer, and shares the highest homology (~80%) to ubiquitin (Watson et al., 2011).

1.3.1. Ubiquitin, UBLs and the Nedd8 pathway

1.3.1.1. Ubiquitin and UBLs conjugation cascades

Ubiquitin and ubiquitin-like proteins (UBLs) are attached to substrates via related enzymatic pathways (Haas and Siepmann, 1997; Hershko and Ciechanover, 1998; Huang et al., 2004; Pickart and Eddins, 2004; Kerscher et al., 2006; Dye and Schulman, 2007).

Ubiquitin and most of the Ubls are synthesized as inactive precursors that need to be processed at their C termini to expose the glycine carboxylate that is the site of substrate conjugation. Specific proteases called deubiquitinating enzymes (DUBs) are responsible for the processing in the case of ubiquitin, whereas, UBLs-specific proteases (ULPs)

process the other UBLs. To be capable of substrate modification, the UBL must first be activated (Haas et al., 1982). A specific activating enzyme (called E1, or E1-like enzyme) uses ATP to adenylate the Ubl modifier at its C terminus. The high-energy mixed anhydride bond formed is quickly attacked by the sulfhydryl group of the E1 active-site cysteine, forming a high-energy thioester bond between the E1 and the UBL and expelling AMP. From the E1, the modifier is passed to the active-site cysteine of an E2, or E2-like enzyme, also known as an Ub-conjugating or Ubl-conjugating enzyme. Finally, the ubiquitin modifier is conjugated to its substrate with help of an E3 protein ligase, resulting in the covalent isopeptide linkage of the modifier's C terminus to the ε-amino group of a lysine in the substrate. Structural overlap between E1 and E3 binding sites found in E2s results in mutually exclusive E1-E2 and E2-E3 interactions, ensuring the unidirectional progression of the E1-E2-E3 cascade (Huang et al., 2005; Eletr et al., 2005). The types of ubiquitin chains linked to a given substrate play crucial roles in dictating the functional outcome. Formation of polyubiquitin chains via lysine 48 (K48) conjugation of ubiquitin on substrate proteins typically targets them for degradation by the 26S proteasome (Chau et al., 1989; Thrower et al., 2000), whereas, mono-, oligo-, or polyubiquitylation via alternate ubiquitin lysine residues, e.g. K63, does not specify degradation but serves other cellular functions, such as intracellular targeting, endocytosis, signaling and DNA repair (Chan and Hill, 2001; Haglund et al., 2003; Pickart and Fushman, 2004; Welchman et al., 2005; Hurley et al., 2006; Kirkin and Dikic, 2007; Mukhopadhyay and Riezman, 2007) (Figure 10). Nevertheless, the ubiquitin chain-type specificity for the proteasomal targeting is still poorly understood, especially *in vivo*. In this line, a recent study showed that K63-linked polyubiquitin chains might also serve as a targeting signal for the 26S proteasome (Saeki et al., 2009).

There are two main types of E3 ligases for ubiquitin, the really interesting new gene (RING) finger domain-containing class and the homologous to E6-AP carboxyl terminus (HECT) class. The largest family of RING E3s consists of cullin RING ligases (CRLs), E3 ligases that contain a subunit or domain with a RING motif, a cullin scaffold protein, and one or more additional cullin-specific subunit(s) that have substrate recognition and adaptor functions. E3 ligase activity of CRLs depends on the covalent modification of cullin scaffolds with another UBL Nedd8 (will be discussed in more detail below). RING E3s function by binding the ubiquitin-thioester-linked E2 and substrate protein simultaneously and positioning the substrate lysine nucleophile in close proximity to the

reactive E2-Ubl-thioester bond, facilitating the transfer of ubiquitin (Cardozo and Pagano, 2004; Petroski and Deshaies, 2005; Ardley and Robinson, 2005; Hotton and Callis, 2008; Deshaies and Joazeiro, 2009). In contrast, catalysis of ubiquitin-substrate modification by the HECT E3s follows a different mechanism compared to RING E3s. In HECT E3s, the ubiquitin is first transferred from the E2 to an active-site cysteine in the conserved HECT domain of the E3 ligase. In a subsequent step, the thioester-linked ubiquitin is transferred to the substrate protein (Huibregtse et al., 1995; Huang et al., 1999; Verdecia et al., 2003; Wang et al., 2006a; Scheffner and Staub, 2007; Kee and Huibregtse, 2007; Bernassola et al., 2008; Rotin and Kumar, 2009).

The E3 ligases are largely responsible for target recognition in the ubiquitin-proteasome system (UPS), through their physical interactions with the final substrates. The large number of E3 genes in eukaryotic genomes, e.g. in humans more than 700 putative E3s are predicted, reflects the highly specific nature of substrate recognition in UPS-mediated protein degradation (Semple, 2003; Ardley and Robinson, 2005; Tai and Schuman, 2008; Deshaies and Joazeiro, 2009).

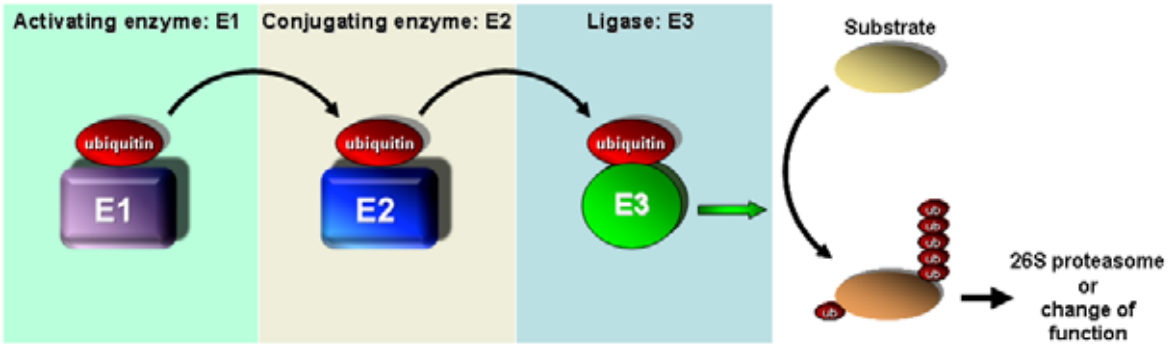


Figure 10: The ubiquitin conjugation pathway.

Ubiquitylation is a post-translational protein modification carried out by an E1-E2-E3 enzyme casdcase, which conjugates the 8.5 kDa protein ubiquitin to substrate proteins. First, ubiquitin is covalently conjugated to the E1 (ubiquitin-activating enzyme) in an ATP-dependent reaction, and is then transferred to the E2 (ubiquitin-conjugating enzyme). The E3 (ubiquitin–protein ligase) transfers the ubiquitin from the E2 to the substrate protein. After the first ubiquitin has been attached (monoubiquitylation), the E3 can elongate the ubiquitin chain by creating ubiquitin–ubiquitin isopeptide bonds. K48 chains, which are the most abundant once, lead to degradation of the substrate by the 26S proteasome, whereas monoubiquitylation and K63 chains do not specify degradation but have other biological functions, including marking proteins for endocytosis. In the UPS, E3 enzymes are largely responsible for target recognition via physical interactions with the final substrates. Thus, the large number of E3 genes in eukaryotic genomes reflects the highly specific nature of substrate recognition in UPS-mediated degradation.

Ubiquitylation is a reversible modification which is removed by deubiquitinating enzymes (DUBs). DUBs are proteases that process ubiquitin or ubiquitin-like gene products, reverse the modification of proteins by a single ubiquitin (or ubiquitin-like protein), and remodel polyubiquitin (or ubiquitin-like) chains on target proteins. The human genome encodes nearly 100 DUBs with specificity for ubiquitin in five families: the ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumor superfamily of ubiquitin isopeptidases (OTUs), Machado-Josephin domain-containing ubiquitin peptidases and the JAMM family of proteases. The first four families are cysteine proteases, while the later is a family of zinc metalloproteases (reviewed in D'Andrea and Pellman, 1998; Kim et al., 2003; Reyes-Turcu et al., 2009).

1.3.1.2. The Nedd8 conjugation pathway

Nedd8 was originally identified in 1992 as a neural precursor cell-expressed, developmentally downregulated (Nedd) gene in a cDNA library screen (Kumar et al., 1992). Nedd8 shares approximately 60% amino acid identity with ubiquitin and its conjugation to substrates resembles the three-step reaction of the ubiquitylation pathway (Kumar et al., 1993; Kamitani et al., 1997; Gong and Yeh, 1999; Yeh et al., 2000).

The Nedd8 conjugation cascade or neddylation involves E1, E2, E3, and deneddylating enzymes (Liakopoulos et al., 1998; Osaka et al., 1998; reviewed in Rabut and Peter, 2008; Xirodimas, 2008). Like ubiquitin, Nedd8 is first synthesized as a (81 amino acid) precursor that is processed at the conserved C-terminal Gly76 residue by the hydrolase activity of deneddylating enzymes, including NEDP1 (also known as DEN1 and SENP8) and UCH-L3, which can also process ubiquitin, exposing a glycine-glycine motif that serves as the attachment site for target substrates (Kamitani et al., 1997). The exposed C-terminal glycine of Nedd8 is adenylated in an ATP-dependent reaction by the heterodimeric E1 Nedd8-activating enzyme (Nae), which is composed of Nae1, also known as Appbp1, and ubiquitin-activating enzyme (Uba) 3 subunits. Next Nedd8 is transferred to an Uba3 cysteine side chain (C216) via a thiolester linkage (Gong and Yeh, 1999; reviewed in Pan et al., 2004; Huang and Schulman, 2005). The activated Nedd8 is subsequently transferred to the E2 Nedd8-conjugating enzyme Ubc12, also called Ube2m, forming another thiolester linkage (Gong and Yeh, 1999). Recently, another Nedd8 E2 was identified, Ube2f, which preferentially promotes neddylation of Cul 5 (Huang et al., 2009). Via the help of E3 Nedd8 ligases, Nedd8 is then transferred

to the ε-amino group of lysyl residues on substrates forming an isopeptide bond. The classical targets of Nedd8 conjugation are the cullin scaffolds of Cullin RING E3 ubiquitin ligases (cullin 1 through 5) which are neddylated on a conserved C-terminal lysine residue (reviewed in Pan et al., 2004; Watson et al., 2011) (Figure 11). In contrast to the great knowledge about ubiquitin E3 ligases, little is known about Nedd8 E3 ligases, and only a few have been described so far. Nedd8 E3s contain really interesting novel gene (RING) finger domains. This is the case for ring-box protein (Rbx) 1 and Rbx2 (also known as ROC1 and ROC2, respectively) that neddylate cullins, murine double minute 2 (Mdm2), casitas b-lineage lymphoma (c-CBL) protein, and Skp1-Cullin-F box protein SCFFBX011 (Kamura et al., 1999a; Morimoto et al., 2003; Xirodimas et al., 2004; Oved et al., 2006; Abida et al., 2007; Huang et al., 2009) with the sole exception being defective in Cullin neddylation 1 (Dcn1) protein (Kurz et al., 2005; Kurz et al., 2008). Interestingly, Dcn1 does not require any of its cysteines for its catalytic activity; rather Dcn1 in conjunction with the yeast Rbx1 homolog, Hrt1, functions synergistically as a dual Nedd8 E3 ligase to promote ligation to cyclin-dependent kinase 53 (Cdc53), a yeast cullin RING ligase (Scott et al., 2010). There are five Dcn1-like proteins in humans termed Dcnl1-5, of which DcnL1-3 have been shown to promote cullin 3 neddylation (Meyer-Schaller et al., 2009). The possible types of Nedd8 chains are only beginning to be elucidated (Ohki et al., 2009). Recently, Jones *et al.* discerned from mass spectrometry analyses that Lys11, Lys22, Lys48, and Lys60 can be linked to form Nedd8 chains *in vivo* (Jones et al., 2008). Interestingly, ubiquitin was also found in proteomic studies identifying Nedd8 substrates, although the prevalence of such mixed Nedd8-ubiquitin chains appears minor and their function is unknown (Jones et al., 2008; Xirodimas et al., 2008). As ubiquitylation, neddylation is reversible, and Nedd8 moieties can be removed from substrates by de-neddylating enzymes. The constitutive photomorphogenesis 9 (COP9) signalosome (CSN) is a zinc metalloprotease and the most studied of the Nedd8 deconjugating enzymes. CSN is an 8 subunit complex, in which the CSN5 subunit possesses the catalytic activity (Schwechheimer et al., 2001; Lyapina et al., 2001). Nedd8-specific protease 1 (Nedp1) was recently identified as another Nedd8-specific protease (Yamoah et al., 2005; reviewed in Rabut and Peter, 2008; Xirodimas, 2008). However, compared to CSN, Nedp1 exhibits three orders of magnitude less activity in cleaving neddylated Cul1 (Yamoah et al., 2005). Interestingly, deletion of the Nedp1 ortholog in Schizosaccharomyces pombe and Drosophila melanogaster did not result in accumulation of neddylated Cul 1 or Cul 3, although a

significant accumulation of still unidentified neddylated substrates was observed, suggesting roles for Nedp1 in the regulation of cullin-independent substrates (Zhou and Watts, 2005; Chan et al., 2008). Additional proteases with dual protease activity for ubiquitin and Nedd8 have been identified (reviewed in Rabut and Peter, 2008; Xirodimas, 2008).

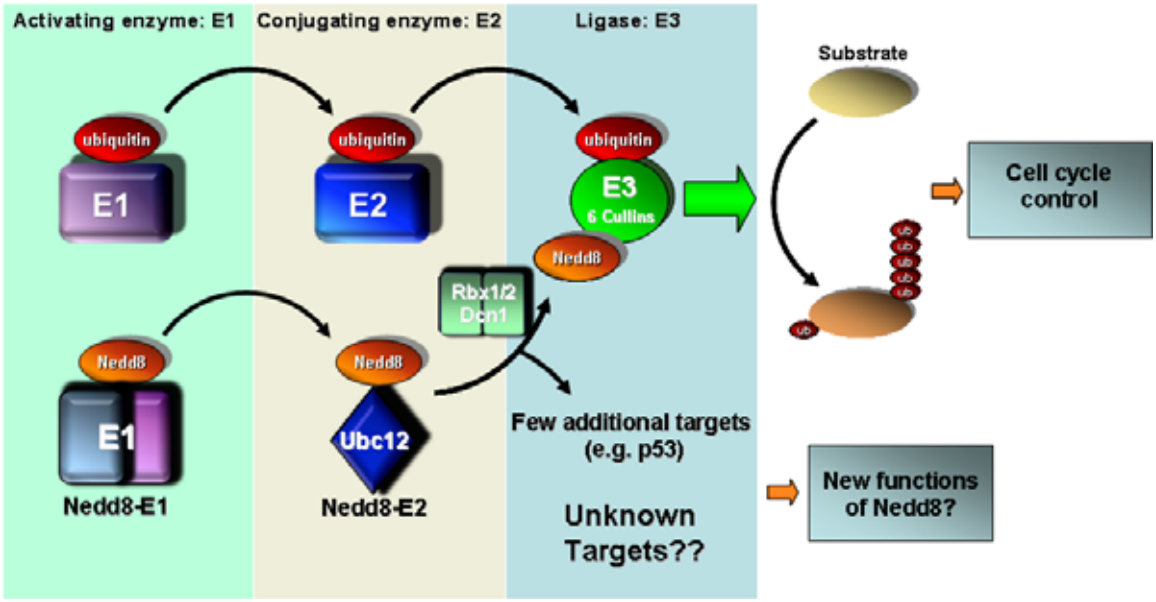


Figure 11: Interaction of the Nedd8 conjugation pathway with the ubiquitylation system.

The neddylation pathway is analogous to ubiquitin conjugation, but relies on its own specific enzymes. Nedd8 is activated by the Nedd8-E1 activating enzyme, a heterodimer composed of Nedd8-activating enzyme1 (Nae1, Appbp1) and a UBA3 subunit, and then conjugated to the Nedd8-E2, Ubc12. Neddylation is best studied as a post-translational modification of the cullin subunits of cullin RING-type E3 ubiquitin ligases (CRLs). Rbx1 (RING box protein 1) and Dcn1 (Defective in Cullin Neddylation 1) function as E3 ligases for neddylation of cullins. Neddylation is important for the proper enzymatic function of CRLs, thereby controlling the UPS-mediated degradation of various proteins involved in cell cycle progression and regulation. Only a few additional targets of Nedd8 were identified until now, including the tumor suppressor p53, which is neddylated via the E3 ligase Mdm2. More and more Nedd8 targets are identified via proteomic approaches uncovering new functions of Nedd8 beyond cell cycle control.

1.3.1.3. Specificity of the Nedd8 cascade

Schulman and colleagues have elucidated many of the molecular mechanisms that mediate the E1-E2-E3 cascade reaction for the Nedd8 pathway, and identified specific molecular determinants that distinguish the ubiquitin and Nedd8 conjugation cascades (Walden et al., 2003b; Schulman and Harper, 2009). First, Uba1 and the Nedd8-activating enzyme component, Uba3, differ in their ability to interact with ubiquitin and Nedd8, respectively, due to amino acid dissimilarity at residue 72 (Ala in Nedd8 and Arg in ubiquitin) (Walden et al., 2003a). The selectivity is attributed to a unique Arg in Uba3, which repels Arg72 of ubiquitin.

Nevertheless, recent studies conducted by Dimitris Xirodimas (personal communication) and Timo Kurz (Hjerpe et al., 2011) indicate that under specific circumstances like cellular stress, the level of free Nedd8 increases significantly and Nedd8 can be charged by Uba1 and neddylation proceeds in part by the ubiquitylation cascade. Additional experiments are required to clarify the physiological relevance of this apparently promiscuous cross-talk.

The selectivity of the Nedd8 cascade is also dictated by interactions of the N-terminal sequence of the E2 catalytic domain and the ubiquitin fold domain of Uba3. A unique 26 amino acid N-terminal extension of Ubc12 docks in a groove exclusively found in Uba3, further ensuring pathway specific E1-E2 interactions (Huang et al., 2005). Interestingly, when the Nae1/Uba3 heterodimer is doubly loaded with Nedd8 linked by a thiolester bond and an adenylated Nedd8, the ubiquitin-fold domain (UFD) undergoes a striking conformational change that unmasks a cryptic Ubc12 binding site, and as a result, promotes E2 binding when the E1 is ready to transfer Nedd8 (Huang et al., 2007). Thus, the transfer of Nedd8 to Ubc12 may eliminate the second binding site, which would promote Ubc12-Nedd8 dissociation (Schulman and Harper, 2009). Finally, as demonstrated for ubiquitin and SUMO E1 and E2 enzymes, the Nedd8 E1 and E3 binding sites overlap on Ubc12, which ensures progression of the E1-E2-E3 cascade (Huang et al., 2005).

1.3.1.4. Nedd8 substrates and biological roles of Nedd8

The first and best characterized Nedd8 substrates are the cullins, which are structurally related proteins that function as molecular scaffolds of CRLs (Hori et al., 1999). The cullin family is composed of CUL1, 2, 3, 4A, 4B, 5, and 7, PARC, a parkin E3 ubiquitin ligase, and Apc2, a component of the anaphase promoting complex/cyclosome (APC/C) (reviewed in Petroski and Deshaies, 2005). Neddylation of cullins 1 through 5 occurs on a conserved C-terminal Nedd8 consensus sequence (IVRIMKMR) (Pan et al., 2004; Skaar et al., 2007). Neddylation activates the related ubiquitin E3 complex by inducing conformational rearrangements of the cullin C-terminus and the Ring-domain of Rbx1 thereby promoting its interaction with ubiquitin-E2 ligase and catalyzing downstream target protein ubiquitination (Wu et al., 2000; Kawakami et al., 2001; Sakata et al., 2007). Cullin neddylation results in an increase in the ubiquitylation activity of CRLs, thereby stimulating proteasome-mediated degradation of CRLs substrates (Saha and Deshaies, 2008; Merlet et al., 2009; Boh et al., 2011). Neddylation of cullins was shown to control important signaling targets such as nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB), NF-κB inhibitor alpha (IκB), $p27^{Kip1}$ and cyclin E, thereby regulating cell cycle progression, signaling cascades, and developmental programming processes (Podust et al., 2000; Morimoto et al., 2000; Read et al., 2000; Ou et al., 2002; Amir et al., 2002; Cardozo and Pagano, 2004; Herrmann et al., 2007).

On the structural level cullin neddylation promotes conformational changes that increase binding of Rbx1 to ubiquitin E2s, reduces the distance between E2 and the substrate recognition component, thus bringing ubiquitin closer to its target, and increases ubiquitin chain extension by allowing greater E2 access to the nascent polyubiquitin chain (Pan et al., 2004; Duda et al., 2008; Saha and Deshaies, 2008; Yamoah et al., 2008; Duda et al., 2011; Boh et al., 2011). Neddylation also abrogates binding of cullin-associated and neddylation-dissociated 1 (CAND1), which associates with unneddylated cullins to inhibit CRL activity by preventing cullin binding to adaptor and substrate recognition components (Liu et al., 2002; Zheng et al., 2002; Chua et al., 2011). CSN-mediated deneddylation has the reverse effect on the activity of CRL-based E3s, and the CSN also recruits the deubiquitinating enzyme Ubp12/USP15 to prevent ubiquitin chain assembly by CRLs (Schwechheimer et al., 2001; Lyapina et al., 2001; Zhou et al., 2003). However, dynamic cycling of Nedd8 conjugation and deconjugation was shown to be

critical in maintaining CRL activity, as deletion of any CSN subunit or CAND1 leads to decreased CRL function (Petroski and Deshaies, 2005; Hotton and Callis, 2008; Merlet et al., 2009). Nevertheless, by using a general NAE-inhibitor MLN4924, recently developed by Millenium Pharmaceuticals (Soucy et al., 2009), and a quantitative mass spectrometry based methodology, Harper and colleagues demonstrated that prolonged global dennedylation does not convert CRL complexes to cullin-CAND1 complexes (Bennett et al., 2010). The study indicates that irrespective of their neddylation status, most cullins are bound to adaptors, whereas, a small fraction is associated with CAND1, suggesting that the abundance of adaptor modules, rather than cycles of neddylation and CAND1 binding, drives global CRL network organization (Bennett et al., 2010).
To date, only a few additional cullin-independent Nedd8 targets were identified by proteomic approaches, including the tumor suppressors p53, p73, Mdm2, von Hippel Lindau (pVHL) protein, breast cancer associated gene (BCA) 3, epidermal-growth-factor-receptor (EGFR), amyloid precursor protein (APP) intracellular domain, hypoxia inducible factor (HIF) α, L11 and other ribosomal proteins (Xirodimas et al., 2004; Stickle et al., 2004; Oved et al., 2006; Watson et al., 2006; Gao et al., 2006; Abida et al., 2007; Xirodimas et al., 2008; Russell and Ohh, 2008; Lee et al., 2008; Ryu et al., 2011) (reviewed in Rabut and Peter, 2008; Xirodimas, 2008). A recent study found that *Drosophila* and human inhibitor of apoptosis (IAP) proteins can function as E3 ligases of the Nedd8 conjugation pathway, targeting effector caspases for neddylation and inactivation (Broemer et al., 2010).

The neddylation pathway is expressed in most eukaryotes (plants, slime molds, fungi and animals), where it is found to be ubiquitously expressed and it was shown that an intact Nedd8 pathway is required for viability in *Schizosaccharomyces pombe*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Arabidopsis* and mouse (reviewed in Pan et al., 2004; Rabut and Peter, 2008). Inactivation of the Nedd8 pathway in the ts41 CHO cell line possessing a temperature-sensitive mutation in the SMC gene (the hamster ortholog of human Nae1 (Appbp1)) caused multiple rounds of S-phase DNA replication without intervening mitosis (Hirschberg and Marcus, 1982; Handeli and Weintraub, 1992). RNAi-mediated knockdown of NED-8, or NED-8 E1 and E2 enzymes in *C. elegans* caused hypersensitivity to N-ethyl-N-nitrosourea (ENU)-induced apoptosis in germ cells (Gao et al., 2008), and other developmental abnormalities including defects in cytoskeleton regulation (Jones and Candido, 2000; Kurz et al., 2002). A recent study

showed that inhibition of Nedd8 conjugation in cell lines causes abnormalities in the actin cytoskeleton, partially due to the accumulation of the small GTPase RhoA, a recently identified CRL (cullin 3) substrate (Leck et al., 2010). In *Drosophila,* Nedd8 null mutants were found to be growth-arrested in the first-instar larval stage and die within several days without further growth (Ou et al., 2002). Furthermore, in Nedd8 and Cul1 *Drosophila* mutants, protein levels of the signal transduction effectors, Cubitus interruptus (Ci) and Armadillo (Arm), and the cell cycle regulator, Cyclin E (CycE) were found to be highly accumulated, eventually resulting in defects in eye development (Ou et al., 2002). Another study in *Drosophila*, demonstrated that the *Drosophila* ortholog of the Appbp1 subunit of the Nedd8-activating enzyme is required for normal development of imaginal disc cells (Kim et al., 2007a). In the plant *Arabidopsis thaliana* Nedd8 E2 ortholog RCE1 mutants had a reduced growth phenotype (Dharmasiri et al., 2003). A general elevation in the level of Nedd8 conjugation has been observed in oral squamous cell carcinoma cell lines where Nedd8 pathway inhibition decreased cell proliferation (Chairatvit and Ngamkitidechakul, 2007). The components of cullin-RING ligases (CRLs) are overexpressed, amplified or mutated in several human cancers, and many CRLs regulate the activity of numerous proteins involved in tumorigenesis and cell cycle control and cell survival (Guardavaccaro and Pagano, 2004; Watson et al., 2011). Furthermore, several of the newly discovered Nedd8 substrates are established tumor suppressors or oncoproteins, including VHL, p53 and Mdm2. Therefore, deregulation of neddylation was implicated in the aetiology of cancers in humans (Chairatvit and Ngamkitidechakul, 2007; Salon et al., 2007) and components of the neddylation pathway represent promising new therapeutic targets in anti-cancer treatments. Accordingly, the specific inhibitor of the Nedd8 pathway that blocks NAE activity, MLN4924 (Langston et al., 2007), was recently shown to possess tumor-inhibiting properties (Soucy et al., 2007; Soucy et al., 2009) and is currently under investigation in several preclinical tumour models as well as in clinical trials as a drug for treating patients with myeloma, lymphoma, leukemia and other nonhematologic malignancies.

Despite its prominent role in cell cycle regulation and cancer, recent evidence suggests that the functions of the neddylation system might be more diverse as previously thought.

1.3.2. Role of ubiquitin and UBL proteins in the central nervous system

Ubiquitylation and proteasome-mediated protein degradation were shown to play important roles in controlling various steps of neuron development, function and plasticity (reviewed in Mabb and Ehlers, 2010; Kawabe and Brose, 2011) and dysregulation of the ubiquitin-proteasome system has been implicated in multiple diseases of the central nervous system (Ciechanover and Brundin, 2003; reviewed in Tai and Schuman, 2008; Ding and Shen, 2008; Schwartz and Ciechanover, 2009).

The ubiquitin-proteasome system (UPS) was found to critically regulate various aspects of neuronal development and function, including neurogenesis, axon and dendrite growth, synaptogenesis and synapse elimination, pre-synaptic function as well as post-synaptic function during activity-dependent plasticity and remodelling (reviewed in Yi and Ehlers, 2007; Haas et al., 2007; Haas and Broadie, 2008; Segref and Hoppe, 2009; Mabb and Ehlers, 2010; Kawabe and Brose, 2011). Moreover, the continuous isolation and identification of ubiquitin conjugates from nervous tissue extends the role of ubiquitylation during neuronal development (Franco et al., 2011). From the wide variety of functions of ubiquitylation in the CNS, few selected examples will be described below.

During very early brain development, self renewal of progenitor cells and their transition into neurogenic and gliogenic fates are tightly controlled by extracellular cues and cell-intrinsic signalling pathways, including WNT, Notch, Hedgehog, receptor type serine/threonine kinase signaling, like transforming growth factor (TGF) β, and receptor-type tyrosine kinase signaling, like Trks and fibroblast growth factor (FGF). All these signalling cascades are themselves regulated by the UPS (Gerhart, 1999; Kikuchi, 2000; Yoon and Gaiano, 2005; Yun et al., 2010; Inestrosa and Arenas, 2010).
The Notch pathway whose activation inhibits neuronal differentiation, was identified as a major target of regulation by ubiquitylation. Notch signalling is triggered by the intercellular interaction between Notch ligands Delta-like (DLL) proteins or jagged 1, which are induced by the proneuronal gene neurogenin 1 (*Ngn1;* also known as *Neurog1*) and the Notch receptor, which is expressed on the surface of RGCs. This interaction induces γ-secretase activity, which cleaves the intramembrane domain of the

Notch receptor to release the Notch 1 intracellular domain (NICD) into the RGC cytosol. NICD then activates genes of the HES (hairy and enhancer of split) family of bHLH transcriptional repressors, which then downregulate proneuronal bHLH genes, that encode for NGN protein family members and achaete-scute homologue 1 (ASH1). This process maintains the developmental potential of RGCs as the neural and glial precursor cells, and prevents them from differentiating into neurons or intermediate progenitor cells (Yoon and Gaiano, 2005). Recent studies have implicated the RING finger E3 ubqiuitin mind bomb 1 (MIB1) ligase and the HECT E3 ligase HUWE1 (also known as HECT, Uba and WWE domain-containing protein 1) in Notch function. Experiments indicated that *MIB1* is required in signalling cells for efficient activation of Notch in neighbouring cells. Mouse MIB1 is strongly expressed in neurons and intermediate progenitor cells, which generate Notch signals for RGCs during their migration. Mice with a brain-specific deletion of MIB1 showed premature differentiation of RGCs into intermediate progenitor cells and neurons (Yoon et al., 2008). MIB1 mediates endocytosis of Delta via ubiquitylation and hereby facilitates Notch cleavage and signaling (Itoh et al., 2003). Interestingly, MIB1 itself is subjected to UPS-mediated degradation upon phosphorylation by protein kinase PAR1, resulting in down-regulation of Notch signaling and induction of neurogenesis (Ossipova et al., 2009).

In contrast, the HECT-type E3 ligase HUWE1 was recently implicated in negative regulation of the Notch pathway, involving the transcription factor NMYC. The HECT-type E3 ligase HUWE1 binds and ubiquitylates NMYC, thus targeting it for UPS-mediated degradation. This pathway is a crucial determinant of neuronal differentiation *in vivo*, as RNAi-mediated knockdown of HUWE1 led to an increase of the fraction of proliferating cells in the developing brain and blockade of neuronal differentiation. Interestingly, the Notch ligand DLL3 is also a downstream component of the NMYC pathway that controls proliferation and neuronal differentiation (Zhao et al., 2008b; Zhao et al., 2009). Thus, HUWE1–NMYC signalling may act through two pathways, one involving cell-autonomous downregulation of CCND2 in RGCs and the other causing downregulation of Notch signalling through repression of DLL3 in neurons and intermediate progenitor cells.

The ubiquitin ligase Nedd4-1 was shown to promote neurite growth and dendritic arborization via ubiquitylation of Rap2A thereby reducing the activity of Rap2 effector

kinases of the TNIK family (Kawabe et al., 2010). Furthermore, Nedd4 enhances axon branching by downregulating phosphatase and tensin homolog (PTEN) (Drinjakovic et al., 2010).

The E3 ubiquitin ligase $Cul7^{Fbxw8}$ controls Golgi morphology, secretory trafficking and dendrite patterning in granule neurons of the cerebellum. $Cul7^{Fbxw8}$ was found to target the substrate Grasp65, a protein controlling golgi stack biogenesis, for UPS-mediated degradation (Litterman et al., 2011).
The Anaphase promoting complexes (APCs), RING finger-type E3 ligase complexes, using CDH1 (WD40 domain-containing proteins cadherin 1) or CDC20 as adaptor proteins to recognize substrates, were originally identified as regulators of the cell cycle targeting cyclins for degradation. However, APC is also strongly expressed in post-mitotic neurons. CDH1-APC acts as a negative regulator of the transcriptional repressor SnoN by promoting its degradation, thereby yet unknown genes become upregulated that have inhibitory roles in axon growth (Konishi et al., 2004; Stegmuller et al., 2006). In contrast, CDC20-APC promotes dendritic growth via ubiquitylation of the centrosomal protein Id1 (Kim et al., 2009). In addition CDC20-APC indirectly controls the expression of the pre-synaptic regulatory protein complexin 2 by triggering the degradation of the transcription factor neurogenic differentiation factor 2 (NEUROD2), thereby influencing pre-synaptic differentiation and synapse function (Yang et al., 2009b). Moreover, in *Drosophila* and *C. elegans* the multisubunit E3 ligase APC was shown to be a negative regulator of synaptic development (Juo and Kaplan, 2004; van Roessel et al., 2004). Liprin-α/SYD2 was identified as a substrate of APC, and was shown to act as a scaffold molecule involved in pre-synaptic differentiation in *C. elegans*. On the other hand, liprin-α/SYD2 regulates membrane targeting of AMPA receptors in the post-synaptic compartment in mammalian neurons (van Roessel et al., 2004).

In *C. elegans* and *Drosophila*, the pre-synaptic E3 ligase RPM-1 or highwire regulates synaptic growth and synapse formation (Zhen et al., 2000; Schaefer et al., 2000; Wan et al., 2000; DiAntonio et al., 2001; Wu et al., 2005; Wu et al., 2007; Grill et al., 2007). Aditionally, in the mouse its homolog Phr1 was found to control axon growth and guidance via regulation of the microtubular cytoskeleton (Lewcock et al., 2007; Bloom et al., 2007).

Within the synapse, the UPS has emerged as an important mediator of synaptic protein degradation (Bingol and Schuman, 2005; Yi and Ehlers, 2005; Yi and Ehlers, 2007; Tai and Schuman, 2008; Haas and Broadie, 2008).

In mammals, one of the first genetic links between the UPS pathway and synaptic plasticity was the discovery that mice deficient in Ube3a, a HECT domain ubiquitin E3 ligase encoded by the UBE3A gene, that is mutated in Angelmann syndrome, are seizure prone, have deficits in contextual learning, exhibit impaired LTP (Jiang et al., 1998b) and show widespread deficits in synaptic plasticity and learning (Weeber et al., 2003; van Woerden et al., 2007; Yashiro et al., 2009). Further evidence for a role of ubiquitylation in learning and memory was provided by mice deficient in the ubiquitin E3 ligase CDH1-APC, which show defects in LTP and are impaired in contextual fear conditioning (Li et al., 2008). Both, protein synthesis and proteasome-mediated degradation were shown to be required for the maintenance of LTP during synaptic potentiation (Frey et al., 1993; Fonseca et al., 2006; Dong et al., 2008). In addition, the UPS was also implicated in the regulation of some forms of LTD (Colledge et al., 2003; Hou et al., 2006; Citri et al., 2009). Furthermore, sets of post-synaptic proteins are ubiquitinated and degraded in response to neuronal activity in glutamatergic neurons, including PSD-95, Shank, GKAP/SAPAP and AKAP79/150 (Colledge et al., 2003; Ehlers, 2003). As discussed above, at mammalian synapses, the insertion and removal of AMPA receptors is regulated by the PSD scaffold molecules SAP97 and PSD-95 (Rumbaugh et al., 2003; Schluter et al., 2006), which themselves are targets of the UPS. PSD-95 is ubiquitylated and degraded by the E3 ligase Mdm2 in response to direct stimulation of NMDA receptors inducing LTD (Colledge et al., 2003). PSD-95 was also found to be degraded in response to AMPA receptor activation (Colledge et al., 2003; Bingol and Schuman, 2004). Furthermore, it has recently been shown that the proteasome itself is sequestered in dendritic spines in response to neuronal activity (Bingol and Schuman, 2006; Bingol et al., 2010).

Deficits in proteasomal degradation have been implicated in multiple neurological disorders, including Alzheimer disease, Down syndrome, Parkinson disease, Huntington's disease, amyotrophic lateral sclerosis, frontotemporal dementias and neurodevelopmental disorders including autism and Angelmann syndrome (Lehman, 2009). Furthermore, mutations and deletions in a variety of ubiquitin pathway proteins are associated with neurological and psychiatric diseases (Jiang and Beaudet, 2004).

Ubiquitin-positive protein aggregates, resulting from decreased degradation not increased synthesis, have been found in the neuropathology of many neurodegenerative diseases (Kuzuhara et al., 1988; DiFiglia et al., 1997; Ross and Poirier, 2004; Ross and Pickart, 2004; Ding et al., 2007).

However, much less is known about the roles of other UBL proteins in neuronal function. For instance, sumoylation was originally thought to only target nuclear proteins, but strong evidence has emerged, indicating that the role of sumoylation is much more diverse (Scheschonka et al., 2007; Wilkinson et al., 2008; Wilkinson and Henley, 2010; Wilkinson et al., 2010). In neurons, different membrane proteins, including glucose transporters, K^+-channels and metabotropic glutamate receptors (Giorgino et al., 2000; Boudin et al., 2000; Lalioti et al., 2002; Perroy et al., 2002; Rajan et al., 2005), as well as transcription factors, such as myocyte enhancer factor 2 (MEF2), important for neuronal and synaptic differentiation and survival (Gocke et al., 2005; Gregoire and Yang, 2005; Shalizi et al., 2006), and PAX6 regulating brain development (Yan et al., 2010) were found to be sumoylated (reviewed in Scheschonka et al., 2007). Furthermore, sumyolation was shown to regulate calcium influx and glutamate release from pre-synaptic terminals in primary hippocampal neurons (Feligioni et al., 2009). In addition, alterations in global levels of sumoylated proteins have been recently reported in a number of neurodegenerative diseases. In this sense, several Alzheimer disease (AD)- and Parkinson disease (PD)-associated proteins, including APP, tau, and parkin, have been shown to be sumoylated (Gocke et al., 2005; Um and Chung, 2006; Dorval and Fraser, 2006; Zhang and Sarge, 2008; Anderson et al., 2009; McMillan et al., 2011).

In contrast to ubiquitin and SUMO, very little is known about the function of the Nedd8 pathway in the brain. However, some evidence exists that neddylation might be involved in brain development and neuronal morphogenesis. This is mainly derived from studies in invertebrates, suggesting that the functions of neddylation in the CNS could be more diverse than previously thought.

1.3.3. Is there a role for neddylation in neuronal development and functioning in the central nervous system? Little evidence, great potential.

To date, virtually all functions of Nedd8 in the central nervous system were described as dependent on cullin RING-based ubiquitin ligases. As one of the central components of the ubiquitin-proteasome system, Cullin-based E3 ubiquitin ligases ubiquitylate substrate proteins and thereby facilitate their proteasome-mediated degradation. The cullin scaffold proteins themselves are known targets of Nedd8 conjugation, and cullin ligase activity is controlled by neddylation as well as de-neddylation mediated by the COP9-signalosome (as described in 1.3.1.4).

First solid evidence for a role of cullin E3 ubiquitin ligases in neuronal morphogenesis was provided by studies in *Drosophila*. Djagaeva and Doronkin showed that the COP9 signalosome has a dual role, both stimulating and repressing dendritic growth in larval PNS (peripheral nervous system) neurons through cullin 1 and cullin 3, respectively (Djagaeva and Doronkin, 2009a; Djagaeva and Doronkin, 2009b). Furthermore, cullin 1 was shown to act with the substrate-specific F-box protein Slimb to target the Cubitus interruptus protein for degradation by the proteasome, thereby stimulating dendritic growth. Whereas cullin 3 was shown to reduce dendritic branching via proteasome-mediated degradation of the actin-crosslinking BTB-domain protein Kelch (Djagaeva and Doronkin, 2009a; Djagaeva and Doronkin, 2009b).

Additionally, an earlier study showed the involvement of cullin 3 in axonal arborization and dendritic elaboration in mushroom-body (mb) neurons of *Drosophila* (Zhu et al., 2005). In contrast to the findings of Djagaeva and Doronkin in PNS neurons, loss-of-function of cullin 3 in mb neurons, resulted in reduced axonal arborization and decreased dendritic elaboration. Similar phenotypes were observed in Nedd8 mutant neurons, suggesting that Cul 3-based ubiquitin ligase is required for neurite arborization, and its proper function might require neddylation.

Two recent studies, performed in mouse embryos, highlighted a role of cullin 5 in regulating neuron layering in the developing cortex by stimulating degradation of Dab1, a protein that mediates the cellular effects of reelin signaling and thereby controls migration speed and stopping point of developing neurons (Feng et al., 2007; Simo et al., 2010). However, the role of neddylation of cullin 5 in these processes was not

addressed. In the same line, another study in *Drosophila* implicated cullin 5 to play an important role in cell fate specification and synapse formation of motor neurons during development (Ayyub et al., 2005).
Finally, several substrates of cullin E3 ligases were identified in non-neural tissues that regulate the cytoskeleton, including RhoA and Katanin (Kurz et al., 2002; Chen et al., 2009), which might be also relevant in neurons, although this hypothesis has not been experimentally addressed so far.

Today we know that Nae1 (or Appbp1) is one of the two subunits that constitute the Nedd8-activating enzyme, together with Uba3. Nevertheless, as its name indicates, Appbp1 was originally described as a protein that binds to the β-amyloid precursor protein (APP) carboxyl-terminal domain (Chow et al., 1996) and acts as one component of the bipartite activating enzyme of Nedd8 (Osaka et al., 1998; Gong and Yeh, 1999; Walden et al., 2003a). Overexpression of Appbp1 in post-mitotic primary neurons induces APP-mediated apoptosis by reactivation of the dormant cell cycle machinery that stimulates DNA synthesis (Chen et al., 2000b; Chen et al., 2003c; Chen, 2004). Moreover, recently a second mechanism has been reported in which overexpression of Appbp1 in primary neurons increases Rab5-mediated endocytosis in an APP-binding-dependent manner, thereby causing apoptosis (Laifenfeld et al., 2007). Appbp1 function is also critically required for cell cycle progression in dividing neural stem cells (Joo et al., 2010) and it has been demonstrated that Appbp1 rescues the cell cycle S-M checkpoint defect in ts41 hamster cells and that this rescue is dependent on the interaction of Appbp1 with Uba3 (Hirschberg and Marcus, 1982; Handeli and Weintraub, 1992; Chen et al., 2000b). All in all, eventhough both Appbp1 features, the APP binding and the Uba3 interaction are very well established and documented, it is so far not entirely clear whether (and if yes to what extend) these two functions are somehow related. Furthermore, upregulated Appbp1 expression has been observed in lipid rafts in hippocampi of Alzheimer disease brains (Chen et al., 2003d) as well as in brains of a Alzheimer disease mouse model (Tg2576 mice) (Yang et al., 2010) and accumulations of Nedd8 were found in neuronal and glial inclusions of neurodegenerative disorders (Dil et al., 2003; Mori et al., 2005), providing a putative link of the neddylation system and amyloid β (Aβ)-induced Alzheimer pathology.

Null mutations in the *Drosophila* ortholog of Appbp1 block the neddylation pathway and cause apoptosis in imaginal disc cells, confirming the requirement of neddylation during development (Kim et al., 2007a).

In addition to Appbp1, Uba3, the catalytic subunit of the Nedd8-activating enzyme is also crucial for cell survivial. Mice with a deletion in Uba3 die *in utero*, due to defects in both mitotic and endoreduplicative cell cycle progression and show accumulation of cyclin E and β-catenin (Tateishi et al., 2001).
Unfortunately, to date only conventional KO animals were generated, that die very early during embryogenesis due to cell cycle defects, precluding the analysis of putative functions of Nedd8 in the central nervous system. To overcome this limitation, conditional mouse models are needed, in which the neddylation pathway can be inhibited in postnatal and adult mice via the use of both Cre and inducible Cre-ERT2/loxP systems.

Finally, it is worth to remark that the results obtained in our lab during the last years and that will be presented in this thesis (although they are still unpublished) provide the most comprehensive analysis of the role of the neddylation pathway in CNS development and function done until now. Thus, throughout this thesis we will describe these novel findings. In summary we found high and ubiquitous expression of Nedd8 and enzymes of the Nedd8 pathway in the mouse brain both during embryogenesis and in adulthood. Additionally, we observed many proteins to be differentially neddylated in the brain in embryo-, postnatal- and adult stages as well as in mature primary neurons (results will be presented in 4.1). These observations led to the hypothesis that there might be additional functions of Nedd8 in post-mitotic neurons apart from the regulation of cell cycle progression and independent of cullin E3 RING ligases. To disentangle these discrepancies we performed a detailed dissection of functions of neddylation during brain development and in the mature neuron of the mammalian central nervous system. Furthermore, we seeked for putative novel targets of Nedd8-conjugation in neurons beyond the classical cullin proteins.

2. AIMS OF THE PROJECT

In this study we focused on the comprehensive examination of the role of the Nedd8 pathway, an ubiquitin-like protein pathway, in the mammalian central nervous system. We used combinational approaches to dissect the functions of neddylation during neuronal development and in the adult brain as well as to identify the underlying molecular mechanisms. In the course of this work we will present the analysis of the following experimental questions.

2.1. When and in which cells is the neddylation pathway expressed and active in the mammalian central nervous system?

Our first aim was to characterize in detail the pattern of neddylated substrates and the expression profiles of different members of the Nedd8 pathway in the mouse brain during development and in adulthood. Therefore, complementary approaches were combined to study mRNA and protein expression in primary neurons and in brain tissue, to deduce the brain region- and cell type-specific expression of Nedd8 and the Nedd8-conjugating enzyme Ubc12, and to analyze the subcelluar distribution of Nedd8, Ubc12 and neddylated target proteins during brain development and in the adult brain.

2.2. What are the functions of neddylation during neuronal development in vitro and in vivo?

To gain insight into the distinct functions of neddylation during neuronal development, we studied the requirement of neddylation during axon, dendrite and spine formation in primary neuronal cultures as well as in the living mouse on the morphological and functional level by applying different loss-of-function approaches. To unravel the specific

functions of neddylation during different steps of neuronal development we manipulated the neddylation pathway in primary hippocampal and cortical neurons by means of pharmacological inhibition, RNAi-mediated knockdown or expression of dominant-negative versions of the Nedd8-conjugating enzyme Ubc12. To validate the findings obtained in primary neurons and to further investigate the physiological relevance of Nedd8 in neuronal development in the intact living brain, we utilized different *in vivo* approaches, including *in utero* microinjection and electroporation of mouse embryos and injection of retroviruses to study newborn neurons in the dentate gyrus of the adult mouse.

2.3. Is the Nedd8 pathway required in the adult brain? Does neddylation control synapse stability in mature neurons?

Beside the function of neddylation during neuronal development, we were interested in the putative functions of Nedd8 in the adult brain. Morphological analysis combined with functional data will provide detailed information about the role of Nedd8 in functioning of mature neurons in the brain. In the work presented here, we tested whether neddylation controls spine maintenance and synapse stability in adult neurons. Therefore, we combined live cell imaging approaches in primary neurons and morphological analysis of brain sections from *in utero* electroporated animals. To analyze effects on spine stability and synapse maintencance it is necessary to perform experiments in mature and fully developed neurons. In this regard, we applied the Nedd8 inhibitor MLN4924 to mature neurons in culture and moreover, we established an inducible genetic approach, based on the Cre-ERT2/loxP system to disrupt the neddylation pathway in the adult neuron *in vitro* and *in vivo* in the intact living brain.

2.4. Is there a cullin-independent role of Nedd8? Which proteins are neddylated in neurons and what is the functional consequence of neddylation for each specific target?

The identification of neuron-specific targets of Nedd8 conjugation is essential to explain the molecular mechanisms underlying the observed phenotypes during development and in adulthood. In our group unbiased screening approaches are combined with hypothesis-based analysis of candidate proteins. We found that PSD-95, a major scaffold protein of the post-synaptic density of spines, is modified by Nedd8 conjugation. One aim of this project was to confirm PSD-95 as a novel neuronal target of Nedd8, to unravel the functional consequences of Nedd8-conjugation to PSD-95 (on the protein level) in neurons and to examine the role of PSD-95 neddylation in spine development, maturation and stability.

2.5. What are the functions of neddylation in vivo?

The generation of mouse models of the neddylation pathway will allow a comprehensive analysis of Nedd8 functions in the central nervous system *in vivo*. In the scope of this project we planned to generate conditional knockout mice for *Ubc12* (Nedd8-conjugating enzyme) and *Appbp1* (one subunit of the Nedd8-activating enzyme (Nae1)) as well as a knockin mouse conditionally overexpressing a dominant-negative version of Ubc12 in the *ROSA26* locus.

This study places neddylation as a key regulator of neuronal development, controlling axon, dendrite and synapse formation on the one hand, and synaptic stability on the other. This work also identified Nedd8 conjugation to PSD-95 as an important cellular mechanism regulating synapse development and maintenance.

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
CAG-IRES-GFP	Retroviral expression vector GFP	Chichung Lie
CAG-IRES-mRFP	Retroviral expression vector mRFP	Chichung Lie
CAG-RFP-actin	RFP-actin reporter	T. Soderling
CAG-stop-Ubc12-C111S-IRES-LacZ	Tamoxifen-inducible expression vector	cloned
CAG-stop-IRES-LacZ	Tamoxifen-inducible expression vector	cloned
CAG-Ubc12-C111S-IRES-GFP	Retroviral expression vector, FLAG-humanUbc12-C111S	cloned
EB3-GFP	EB3-GFP expression vector	Reinhard Köster
GKAP-eGFP	GKAP-eGFP expression plasmid	Peter Scheiffele
Homer-1c-eGFP	Homer-1c-eGFP expression plasmid	Peter Scheiffele
Myr-Venus	Membrane-bound Venus reporter	Victor Tarabykin
Lifeact-meGFP	Visualization of actin dynamics	R. Wedlich-Soldner
pCALNL-dsRED	Tamoxifen-inducible expression vector	Matsuda & Cepko
pCALNL-eGFP	Tamoxifen-inducible expression vector	Matsuda & Cepko
pcDNA3/pcDNA3.1	Expression vector/CMV promoter	Invitrogen
pcDNA3-3xFLAG	Expression of 3xFLAG-tagged fusion proetins	cloned
pcDNA3-3xFLAG-Nedd8	3xFLAG-Nedd8 expression vector	cloned
pcDNA3-3xFLAG-Rbx1 wt, Rbx1 C42S C45S, Rbx1 C75S	Rbx1 wt and dominant-negative Rbx1 C42S C45S, and C75S expression vector	from J. W. Conaway, subcloned into pcDNA3-3xFLAG
pcDNA3-dn cul1 to 5	dominant-negative cullin 1 to 5 expression plasmids	Wade Harper
pcDNA3-FLAG-Ubc12 wt	Ubc12 wt expression vector	Yosef Yarden
pcDNA3-FLAG-Ubc12-C111S	Ubc12-C111S expression vector	Yosef Yarden

Plasmid	Purpose	Source
pcDNA3-mouse PSD-95	PSD-95 expression plasmid	cloned
pCi-PSD-95-eGFP	PSD-95-eGFP expression plasmid	Valentin Stein
pcRII-mouse Nedd8	In vitro transcription (Sp6, T7 polym.)	cloned
pcRII-mouse Ubc12wt	In vitro transcription (Sp6, T7 polym.)	cloned
pcRII-TOPO	TOPO cloning vector	Invitrogen
pEGFP-C1 (Clontech)	Cloning of eGFP-fusion proteins (eGFP N-terminal)	Clontech
pEGFP-N1 (Clontech)	Cloning of eGFP-fusion proteins (eGFP C-terminal)	Clontech
pEGFP-Nedd8	eGFP-Nedd8 fusion protein	cloned
pEGFP-Ubc12	eGFP-Ubc12 fusion protein	cloned
pLKO.1-puro	Expression of shRNAs (U6 promoter)	Sigma Aldrich
pSUPER-CMVIE4-Venus	Expression of shRNAs (H1 promoter)	Bill Snider
ROSA26-targeting vector	ES cell targeting vector, conditional overexpression of Ubc12-C111S, based on pROSA26-1 (Soriano, 1999)	cloned, Jan Deussing

Table 1: Plasmids.

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

Primary and Secondary Antibodies and Fluorescent Dyes

Antibody	Company, cat. #	Species	Dilution
DAPI	Sigma Aldrich, #D8417, 20 mg/ml	-	1:10.000
Phalloidin-405	Gentaur, # 00034	-	5 Units/ 200 µl
α-Acet.-Tubulin	Sigma Aldrich, #T6793	mouse, 6-11B-1	1:3000
α-Cullin1	Invitrogen, #322400	mouse	1:1000
α-DCX	Santa Cruz, #sc-8066	goat, C-18	1:300-1:500
α-EB3	Abcam, #53360	rat, KT36	1:1000-1:3000
α-FLAG M2	Sigma Aldrich, #F3165	mouse	1:2000
α-GFAP	Dako, # Z0334	rabbit	1:1000-1:3000
α-GFP	Abcam, #ab6556	rabbit	1:2000-1:5000
α-GFP	Abcam, #ab13970	chicken	1:3000
α-GFP	Aves Labs, #GFP-1020	chicken	1:2000
α-MAP2	Chemicon, #AB5622	rabbit	1:2000
α-MAP2	Abcam, #ab5392	chicken	1:1000-1:2000
α-Myc	Abcam, #ab9106	rabbit	1:5000
α-Nedd8	Epitomics, #1571-1	rabbit	1:500-1:1000
α-Nedd8	Alexis, #ALX-210-194	rabbit	1:500-1:1000
α-Neurofilament	Abcam #24574	mouse, SMI-312	1:1000
α-PSD-95	Neuromab, #75-028	mouse, clone 28/43	1:500 (IF)/ 1:10.000 (WB)
α-PSD-95	Cell Signaling Technology, #2507	rabbit	1:1000
α-Synapsin	SySy, #106 001	mouse	1:500-1:1000
α-Synaptophysin	Abcam, #ab14692	rabbit	1:500-1:1000
α-Synaptophysin	Sigma Aldrich, #S5768	mouse, SVP-38	1:1000
α-TYR-Tubulin	Abcam, #ab6160	rat, YL1/2	1:5000 (IF)/ 1:15.000 (WB)
α-Ubc12	Lifespan BioSciences, #LS-B432	rabbit	1:1000
α-α-Tubulin	Sigma Aldrich, #T5168	mouse, clone B-5-1-2	1:2000
α-β-Actin	Cell Signaling Technology, #4967	rabbit	1:5000

Table 2: Primary antibodies and fluorescent dyes.

WB, Western blot; IF, immunofluorescence staining.

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 donkey α-goat IgG	Invitrogen, #A11058	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
Alexa Fluor 647 chicken α-rabbit IgG	Invitrogen, #A A21449	1:1000
Alexa Fluor 647 donkey α-goat IgG	Invitrogen, #A21447	1:1000
Alexa Fluor 647 goat α-mouse IgG	Invitrogen, #A21236	1:1000
Alexa Fluor 647 goat α-rabbit IgG	Invitrogen, #A21244	1:1000
α-mouse-IgG HRP	CST, #7076	1:2000-1:4000
α-rabbit-IgG HRP	CST, #7074	1:2000-1:4000
α-rat-IgG HRP	CST, #7077	1:2000-1:4000
Biotinylated-goat α-rabbit IgG	Jackson Lab, #BA-1000	1:700

Table 3: Secondary antibodies.

General Buffers and Solutions

1x PBS

137 mM	NaCl
2.7 mM	KCl
20 mM	Na_2HPO_4
2 mM	KH_2PO_4

Adjust pH 7.4

20x SSC

3 M	NaCl
0.3 M	Soudium Citrate

Adjust pH 7.4

LB medium

1% (w/v)	Bacto-tryptone
0.5% (w/v)	Bacto-yeast extract
1.5% (w/v)	NaCl

Adjust Adjust pH 7.4

LB agar plates

1% (w/v)	Bacto-tryptone
0.5% (w/v)	Bacto-yeast extract
1.5% (w/v)	NaCl
1.5% (w/v)	Bacto-agar

Adjust pH 7.4

6x DNA Loading buffer (Orange)

1g	Orange G
10 ml	2 M Tris/HCl, pH7.5
150 ml	Glycerol

50x TAE buffer

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

Adjust pH 8.1

10x SDS-PAGE running buffer

1% (w/v)	SDS
250 mM	Tris
1920 mM	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
125 mM	Tris-HCl, pH 6.8
4%	SDS
0.08% (w/v)	Bromophenol blue
5%	β-Mercaptoethanol

1x TBS

50 mM	Tris
150 mM	NaCl

Adjust pH 7.6

Reagents

MLN4924 was purchased at Active Biochem or kindly provided by Lak Shin Jeong from the Department of Bioinspired Science and Laboratory of Medicinal Chemistry, College of Pharmacy at the Ewha Womans University in Seoul, South Korea. MLN4924 was dissolved in DMSO at a 10 mM stock concentration and frozen at -80 ºC.

Taxol (LC laboratories) was dissolved in DMSO at a 10 mM stock concentration and frozen at -20 ºC.

Cytochalasin D (Sigma Aldrich) was dissolved in DMSO at a 10 mM stock concentration and frozen at -20 ºC.

The proteasome inhibitor MG-132 (Merck) was dissolved in absolute Ethanol at a stock concentration of 20 mM and frozen at -20 ºC.

Tamoxifen was purchased at Sigma Aldrich; for cell culture experiments Tamoxifen was dissolved in absolute Ethanol at a 10 mM stock concentration and frozen at -20 ºC. For *in vivo* experiments Tamoxifen was first dissolved in absolute Ethanol (100 mg/ml) and then diluted 1:10 with sunflower seed oil (Sigma Aldrich) to reach a final concentration of 10 mg/ml. Diluted Tamoxifen was stored at 4 ºC and used for maximal three consecutive days.

All other reagents were purchased at Sigma-Aldrich or Roth, except otherwise stated.

DNA oligos were ordered at Sigma Aldrich or MWG.

3.2. Animal experiments

3.2.1. Animals and housing

In all experiments mice were housed under standard laboratory conditions (22±1 °C, 55±5% humidity, 12 h light :12 h 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 (see below).
For staging of embryos, noon on the day of the appearance of a vaginal plug was treated as embryonic day 0.5 (E0.5), and the day of birth was considered postnatal day 0 (P0).
If required, Tamoxifen, 1 mg twice (every 12h) per day for 5 consecutive days, was administered by intraperitoneal (i.p.) injections in sunflower seed oil (10 mg/ml).
Animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Government of Bavaria, Germany and of the University of Buenos Aires, Argentina.

3.2.2. Mouse and rat strains as well as transgenic mouse lines used/generated for the presented projects

- Female C57BL/6 for retroviral injection experiments to study adult neurogenesis.
- Timed CD1 breedings for standard *in utero* electroporation experiments.
- CamKIIα-Cre-ERT2xCD1 breedings for *in utero* electroporation experiments with tamoxifen-inducible plasmids to study spine and synapse stability in the adult mouse brain (Erdmann et al., 2007).
- Timed pregnant CD1 mice and Sprague Dawley rats for preparation of primary neuronal cell cultures from mouse and rat embryos, respectively.

- Ubc12-LacZ reporter mice, Nae1-LacZ reporter mice, *ROSA26*-Ubc12-C111S conditional overexpressing mice were generated during the current project.
- hACTB::Flp-deleter mice to excise genomic DNA flanked by frt sites (Dymecki, 1996; Rodriguez et al., 2000) .

3.2.3. Genotyping

Genomic DNA was prepared from mouse tail biopsies, taken at the age of 3 to 4 weeks, by alkaline lysis of the tissue. 100 µl of 50 mM NaOH was added to a mouse tail of approximately 1-2 mm length and heated to 95 °C for 30 min until the tissue was lysed. After lysis, samples were cooled to 4°C and neutralized by addition of 30 µl 1 M Tris-HCl (pH 7.5, containing 4 mM EDTA), vortexed at slow intensity and centrifuged at 4°C with maximum speed in a table top centrifuge for 1 min. Samples were stored at 4°C and were used as PCR templates. For genotyping, 1 µl of the preparation of genomic DNA was used in a 25 µl-PCR reaction, containing 2.5 µl 10x PCR buffer (Abgene), 1.5 µl 25 mM $MgCl_2$, 0.5 µl dNTPs (10 mM each, Roche), 0.5 µl of each primer (1, 2 and 3) and 0.5 µl Taq DNA polymerase (5 units (U)/µl, Abgene). Routinely a standard PCR program, 95 ºC 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. PCR samples were subsequently 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.

3.3. Cloning and work with plasmid DNA

3.3.1. Production and transformation of electrocompetent bacteria

For cloning of plasmid DNA, electrocompetent E. coli bacteria were used. For normal plasmids, the conventional DH5α strain was used; for more complicated plasmids (i.e. DNA fragments containing hairpins or inverted terminal repeats), the recombination deficient strain ElectroMAX Stabl4™ (Invitrogen) was applied. Electrocompetent bacteria were prepared as follows. An overnight (o.n.) 50 ml culture was inoculated with the frozen glycerol-stock of DH5α. After vigorous shaking o.n. at 37 °C, the pre-culture was transferred to 500 mL LB medium and incubated on an orbital shaker at 37 °C. The extinction of the bacteria solution was checked regularly with a spectrophotometer (DU 530 Life Science UV/Vis Spectrophotometer, Beckman) at 600 nm wavelength until it reached 0.5-0.6. The bacteria suspension was split in four 250 mL centrifugation bottles and cooled down on ice for 30 minutes. Since here, all procedures were performed at 4 °C. The tubes were centrifuged at 4000x g for 15 min at 4 °C. The supernatants were discarded and each of the pellets was carefully resolved in 150 mL of ice-cold H_2O (Ampuwa). Bacterias were centrifuged again at 4000x g for 15 min at 4 °C. Each of the bacteria pellets were resolved the second time in 80 ml of ice cold H_2O and centrifuged again at 4000x g for 15 min at 4 °C. Each pellet was then carefully resolved in 6 ml of 10% glycerol diluted in water. Bacteria suspensions were then transferred to two 30 ml centrifugation plastic tubes and centrifuged at 4000x g for 15 min at 4 °C. Each pellet was dissolved in 1 ml of 10% glycerol in water, bacteria suspensions were combined and splitted in 20 µl aliquots, frozen on dry-ice and stored at -80 ºC. The transformation efficiency (# of colonies/µg plasmid DNA) was checked for each batch by transformation of 10 pg and 100 pg pUC18 control plasmid. Usually, preparations of electrocompetent bacterias had a transformation efficiency of 10^8-10^9 colonies/ 1 µg plasmid DNA.

For one transformation, one aliquot of electrocompetent E. coli was thawed on ice and 1-2 µl of a ligation reaction or 10-100 pg of circular plasmid DNA were added. The suspension was mixed carefully and then transferred into a 1 mm electroporation cuvette (VWR). Electroporation was carried out using a Biorad electroporation system following

the manufacturer's instructions (1800 V, 25 µF capacitance, 200 Ω resistance). After the electroporation pulse bacterias were transferred immediately to 1 ml prewarmed LB or SOC (superoptimal broth with catabolite repression, containing 2% (w/v) bacto-tryptone, 0.5% w/v bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) medium and incubated at 37 °C, shaking vigorously, for 30-60 min. Afterwards dilutions of the electroporated bacterias were plated on LB agar plates containing the appropriate antibiotic for selection and incubated over night at 37 °C. Typically, 100 µg ampicillin or 50 µg kanamycin per ml LB medium were used for selection of transformed clones.

3.3.2. Preparation of plasmid DNA

Plasmid DNA was isolated from transformed bacteria using plasmid isolation kits from Qiagen following the manufacturer's instructions. The Qiagen MiniPrep Kit was used for screening of correctly transformed clones and the Qiagen Plasmid Maxi Kit, Qiagen HiSpeed Plasmid Maxi Kit, or Qiagen Plasmid Maxi Kit Plus were used for higher yield plasmid preparations.
For MiniPreps, a single colony was inoculated in 5 mL LB medium with the appropriate antibiotic o.n. at 37 °C. For MaxiPreps, either 500 µL of an o.n. MiniPrep culture, or the appropriate glycerol-stock of bacterias was added to 250 mL LB medium with antibiotic and incubated o.n. at 37 °C with vigorous shaking.

3.3.3. DNA and RNA concentration measurement

DNA or RNA concentration was measured in duplicates in a UV photometer (Gene Quant II, Pharmacia Biotech) at 260 nm. Samples were diluted in distilled water. At a wavelength of 260 nm and a cuvette thickness of 1 cm an OD of 1 corresponds to a concentration of 50 µg/ml for double stranded DNA, 33 µg/ml for single stranded DNA and 40 µg/ml for RNA. The concentration of the sample (X) was therefore $OD_{260} \times T \times$

dilution factor = X µg/ml, with T being 50 µg/ml for double stranded DNA, 33 µg/ml for single stranded DNA and 40 µg/ml for RNA.

3.3.4. Amplification of DNA sequences from genomic DNA, cDNA or plasmid DNA via PCR

To amplify DNA sequences for further cloning into vectors, PCRs were carried out using genomic DNA, cDNA or plasmid DNA as template sequences. 0.5-5 µg of genomic DNA, 0.1-1 µg of cDNA, or 20-200 ng of plasmid DNA were used in a 25 µl-PCR reaction, containing 2.5 µl 10x PCR buffer (Abgene), 1.5 µl 25 mM $MgCl_2$, 0.5 µl dNTPs (10 mM each, Roche), 0.5 µl of each primer (1 and 2) and 0.5 µl Taq DNA polymerase (5 U/µl, Abgene) or a high fidelity DNA polymerase, e.g. Accuzyme (Bioline). Routinely a standard PCR program, 95 ºC 5 min, 35 cycles of 98 ºC for 45 sec, 58-60 ºC for 30 sec, 72 ºC for 1 min per 1 kb of DNA, 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. To analyze the PCR, small aliquots of the PCR samples (1-5 µl) were subsequently mixed with 6x Orange loading buffer and PCR products were analyzed by gel electrophoresis in a 1-2% agarose gel (1x TAE), containing ethidium bromide. After electrophoresis, gels were analyzed using a UV-transilluminator and a BioDoc II gel documentation system from Biometra.

3.3.5. Restriction digest of plasmid DNA and PCR products

For digestion of plasmid DNA or PCR products, 2-4 U of restriction enzyme (Fermentas or New England Biolabs (NEB)) were used per µg of DNA or per 25 µl PCR reaction. The reaction conditions and the type of buffer were chosen following the manufacturer's instructions. The restriction digest was incubated for 3-4 hours at 37 °C (unless a different temperature was recommended for the used enzyme). For the generation of blunt ends, the digested DNA was incubated with Klenow fragment of DNA polymerase I

(NEB). Therefore, 5 U Klenow fragment and 25 nM dNTPs were added and incubated at RT for 20 min. Afterwards, Klenow fragment was inactivated by incubation at 75 °C for 20 min.
In order to prevent unwanted religation of the opened plasmid, the terminal phosphates of the digested vector were removed by adding 10 U Fast phosphatase (CIP, Fermentas) and 1x CIP Buffer. For dephosphorylation samples were incubated at 37 °C for 45 min, and afterwards phosphatase activity was inactivated by incubation at 75 °C for 20 min.

3.3.6. Isolation of DNA fragments

After restriction digest, the DNA fragments were separated by gel electrophore-sis. DNA samples were supplemented with 6x Orange buffer and loaded on agarose gels containing ethidium bromide for visualisation of the DNA. For gen-eral purpose, 0.9% agarose gels and for larger fragments 2-4% agarose gels were used. The gel run was performed in 1x TAE buffer at a voltage of 100 V for 30-120 min dependent on the size of the fragments. After separation of the bands, the DNA was visualized using UV light and the appropriated bands were cut out using a scalpel.
The DNA was extracted from the gel slices using the Qiagen Gel Extraction Kit following the manufacturer's instructions. After elution, the DNA concentration was determined by spectrophotometry and DNA qualtity was checked by gel electrophoresis of a small aliquot of the eluted DNA.

3.3.7. Ligation of DNA fragments

For the ligation of the linearized vector and the insert, a molar ratio of 1:3, 1:6 or 1:9 of vector:insert was used. In general, 100 ng of vector DNA and 3x, 6x or 9x of insert, in molar ratio, were mixed. For very short inserts (<500 bp) a molar ratio of 1:15 was used. T4 DNA ligase buffer and 5 U of T4 DNA ligase (Fermentas) were added in a total volume of 10-15 µl. The reaction was incubated o.n. at 16 °C. Afterwards, 1-2 µl of the

ligation reaction were used for transformation into electrocompetent bacterias. Usually 5-10 colonies were inoculated for MiniPreps and the recovered plasmid DNA was subjected to restriction digestion with appropriate restriction enzymes. To verify the sequence of the cloned insert, positive clones were sent for sequencing (Sequiserve).

3.3.8. Direct cloning of PCR products

For direct cloning of PCR products, e.g. cloning of *in situ* hybridization probes, the TOPO TA kit from Invitrogen was used. PCR products, amplified with Taq DNA polymerase (5 U/µl, Abgene), therfore containing 3'-A overhangs, were mixed with TOPO pcRII vector and salt solution, following the manufacturer's protocol. The reaction was incubated 30 min at RT, and subsequently transformed into electrocompetent bacteria. For white-blue selection (LacZ complementation assay) bacterias were plated on LB agar plates containing X-Gal (1 mg/ml LB agar) and ampicilin or kanamycin as antibiotic selection. Usually 5-10 white or light blue colonies were analyzed by MiniPrep and restriction digestion of the recovered plasmid DNA with EcoRI, releasing the inserted PCR product. To determine the orientation of the insert in the pcRII vector, either a restriction digestion with an appropriate enzyme was performed or a positive colony was sent for sequencing (Sequiserve) with T7 (5'-TAA TAC GAC TCA CTA TAG G-3') or Sp6 (5'-ATT TAG GTG ACA CTA TAG-3') primers.

3.3.9. Cloning of shRNAs into the pSUPER vector

shRNA sequences were cloned into the pSUPER vector according to the oligoengine protocol (Brummelkamp et al., 2002). In the pSUPER vector the human RNA polymerase III H1 promoter drives the expression of a hairpin structure in which the sense and antisense strands of the small interfering RNA are connected by a 9-bp long loop sequence. shRNA sequences were ordered as DNA oligos in MWG carrying BglII and HindIII restriction overhangs, as described in the pSUPER manual. Oligos were annealed by heating to 98 ºC for 10 min and slow cooling down at RT. Annealed oligos

were ligated into the previously BglII- and HindIII-linearized pSUPER vector. shRNAs for Ubc12 and Nedd8 were cloned into a modified pSUPER/CMVIE94-Venus (pSUPER-Venus) vector kindly provided by W. Snider, in which the green fluorescent Venus protein is expressed from the CMVIE94 promoter, which allows the identification of transfected eukaryotic cells (Nagai et al., 2002; Kim et al., 2006b). Successful cloning was confirmed by restriction digestion and sequencing (Sequiserve).
To knockdown mouse Ubc12 and Nedd8, hairpin shRNAs were cloned into pSUPER-Venus vector, targeting the following Nedd8 and Ubc12 sequences.

Nedd8: shRNA 1, CTG CCA ATC ATA ATG TGG CAT; shRNA 2, GAG ATT GAG ATA GAC ATC GAA; shRNA 3, GCG GCT CAT CTA CAG TGG CAA; shRNA 4, CAA GCA AAT GAA TGA TGA GAA; shRNA 5, CCA CAG ACA AGG TGG AGC GAA;
Ubc12: shRNA 1, CCT TAC GAT AAA CTC CAT AAT; shRNA 2, CCT CAA CTT CAA GCT GGT GAT; shRNA 3, GCA AGT TTG TAT TCA GCT TTA; shRNA 4, GCG CTC CAT GAG AGG TGG TTA; shRNA 5, CCA AGG TGA AGT GTG AAA CAA;

In the case of Rbx1, shRNAs and a control shRNA were purchased at Sigma Aldrich. shRNAs are expressed from the human U6 promoter and target the following mouse Rbx1 sequences.

Rbx1: shRNA 1, CCG TGT TCA ATT GCT GGC ATA; shRNA 2, CCT GGG ACA TTG TGG TTG ATA; shRNA 3, CCA CAT TAT GGA TCT TTG TAT; shRNA 4, GAG TGG GAG TTC CAG AAG TAT; shRNA 5, CCG AAG AGT GTA CGG TTG CAT;

3.3.10. Site directed mutagenesis

To introduce point mutations, small deletions or insertions into plasmid DNA the QuikChange Lightning Site Directed Mutagenesis-Kit from Stratagene was used accordingly to the manufacterer's protocol. Successful mutagenesis was confirmed by sequencing (Sequiserve).

3.4. Radioactive in situ hybridization (ISH)

Single *in situ* hybridization (ISH) procedures were performed as previously described (Lu et al., 2008). Embryos and young postnatal mice were sacrificed by decapitation, adult mice were sacrificed by an overdose of isoflurane (Forene®, Abbott). Brains were carefully removed and immediately shock-frozen on dry ice. Frozen brains were cut on a cryostat in 20-μm thick sections and mounted on SuperFrost Plus slides (Menzel GmbH). All sections were processed for in situ hybridization as described. The following riboprobes for Ubc12 and Nedd8 were used: Probe Ubc12: nucleotides 248-799 of GenBank accession no. NM_145578.3; probe Nedd8: nucleotides 95-340 of GenBank accession no. NM_008683.3 and probe Appbp1: nucleotides 942-1551 of GenBank accession no. NM_144931.3. 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 o.n. with a probe concentration of 7 x 10^6 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 captured with Zeiss AxioCam MRm and AxioCam MRc5 digital cameras adapted to a Zeiss axioplan 2 imaging microscope and a stereomicroscope (Leica). Images were digitalized using Axio Vision 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 or white background.

3.5. LacZ staining (X-Gal staining of β-Galactosidase activity) of brain sections

Mice were anesthezized with isoflurane (Forene®, Abbott) and transcardially perfused with a peristaltic pump for 1 min with PBS, 5 min with LacZ fix solution (4% PFA (w/v), 0,005M EGTA, 0,001M MgCl in PBS, pH7.4), and 1 min with PBS at a flow of 10 ml/min. Brains were removed, post-fixed for 1 h in LacZ fix solution at 4ºC and immersed in 15% (w/v) Saccharose in PBS, pH7.6 o.n. at 4 ºC. Brains were washed with PBS and schock-frozen on dry-ice for cryo-sections or embedded in warm 4% (w/v) agarose (Invitrogen) in PBS for vibratome-sections. Brain sections were washed 3x 5min in LacZ wash solution (0,01% Deoxycholate, 0,02% NP40 in PBS, pH7.4) and stained 3-24 h in LacZ staining solution (0,1% X-GAL (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, Genaxxon Bioscience) (w/v), 0.005 M Potassium-Ferrocyanide, 0,005 M Potassium-Ferricyanide in LacZ wash solution) at 37 ºC with gentle shaking. Sections were then washed in PBS, post-fixed in 4% PFA for 1 h at RT, washed in PBS, dehydrated and mounted in DPX (Roth). When required a DAB-immunohistochemical staining was performed directly after the LacZ staining using the VECTASTAIN-ABC-kit (Vector Lab), combined with anti-biotinylated secondary antibodies (Jackson Lab) and SIGMAFAST^{TM} 3,3'-Diaminobenzidine (DAB) tablets (Sigma Aldrich).

3.6. RNA isolation and qRT-PCRs

Total RNA was prepared from cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was derived from this RNA using Reverse Trancriptase Superscript II from Invitrogen and oligo-dT primers following the manufacturer's protocol. Aliquots of the cDNA were utilized as templates for (quantitative) real-time PCR.
Quantitative real-time PCR was performed with a Light Cycler (Roche) using QuantiFast SYBR Green PCR Kit (Qiagen) according to manufacturer´s protocol and relative expression was calculated via the 2Δ method and results were normalized to the internal control *Gapdh*, *Hprt* or *β-Actin*.
The following primers were used:

Nae1 (rat)-for: 5'-CAA GGG ATT TTA AAG AAT GAG AAT G-3'
Nae1 (rat)-rev: 5'-ATA TAT CTT CAA TAC TGC TTG GGA TCT-3'
Uba3 (rat)-for: 5'-AGG AGC CGG AGA AGA AAA GA-3'
Uba3 (rat)-rev: 5'-CCA CCA TCA ACA GCC ATT TT-3'
Ubc12 (rat)-for: 5'-AGT CGG CCG GCG GCA CCA AG-3'
Ubc12 (rat)-rev: 5'-GGG AGG GTC ATG TGG GTA AC-3'
Nedd8 (rat)-for: 5'-CAT CTA CAG TGG CAA ACA AAT GA-3'
Nedd8 (rat)-rev: 5'-CAA GTT TCT TCA TTG CCC AAG-3'
Senp8 (rat)-for: 5'-TTT AGA CGA CAG CCA GAA TCC-3'
Senp8 (rat)-rev: 5'-CAT CAC AAG CATT CTT CAG TAG C-3'
Cul1 (rat)-for: 5'-TTA CAG CAG AAC CCA GTT ACT GAA-3'
Cul1 (rat)-rev: 5'-ACT CTC CGC TGT TCC TCA AG-3'
Cul2 (rat)-for: 5'-AAC CAC TTA TGG AAA TAG GAG AGC-3'
Cul2 (rat)-rev: 5'- TTC TGA AGG GGT TCA ACC AT-3'
Cul3 (rat)-for: 5'-CAG AGA GCG GAA AGG AGA AGT-3'
Cul3 (rat)-rev: 5'-TCA TTA ACA TCT GGC AAG CAT T-3'
Cul4a (rat)-for: 5'-GAC TAA CTG TCT ATA CGC TGC TGA A-3'
Cul4a (rat)-rev: 5'-TCA CAT GGT TAA GGT ATT CTG GAA C-3'
Cul4b (rat)-for: 5'-TCG TCG CAT GAA TAT TAG AAC AA-3'
Cul4b (rat)-rev: 5'-AAC GTG ACA TCC TCT TTT TGC-3'
Cul5 (rat)-for: 5'-CTG CTG ATA GCG AGA TTG AAG A-3'
Cul5 (rat)-rev: 5'-CTG CTG GCA TAC CAA CTT CTC-3'

As control, mRNA expression of the housekeeping genes *Gapdh*, *Hprt* or *β-Actin* was measured using the primers Gapdh (mouse/rat)-for 5´-CCA TCA CCA TCT TCC AGG AGC GAG-3´ and Gapdh (mouse/rat)-rev 5´-GAT GGC ATG GAC TGT GGT CAT GAG-3´, Hprt (mouse)-for 5'-ACC TCT CGA AGT GTT GGA TAC AGG-3' and HPRT (mouse)-rev 5'-CTT GCG CTC ATC TTA GGC TTT G-3', or β-Actin (rat)-for 5'-CCC GCG AGT ACA ACC TTC T-3' and β-Actin (rat)-rev 5'-CGT CAT CCA TGG CGA ACT-3'.

3.7. Primary neuronal cell cultures, transfection of neurons, primary astrocytes and neuron-astrocyte co-cultures

All cell culture reagents were purchased from Invitrogen except as otherwise stated, and all cell culture dishes were purchased from Nunc as otherwise stated.

Primary hippocampal and cortical neurons were prepared from mouse or rat embryos as described (Dotti et al., 1988; Goslin and Banker, 1991; de Hoop et al., 1997; Kaech and Banker, 2006). Once plated neurons were transfected via a modified calcium phosphate protocol (Jiang and Chen, 2006) or neurons were transfected before plating with the Nucleofector system (Amaxa, LONZA) using highly purified DNA as described (Zeitelhofer et al., 2007; Zeitelhofer et al., 2009).

3.7.1. Preparation of hippocampal and cortical neuronal cultures

Hippocampi and cerebral cortices from embryonic day (E) 17.5-18.5 CD1 mice or E18.5–19.5 Sprague Dawley rats 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 3x with DMEM supplemented with 10% FCS and afterwards triturated with a fire-polished Pasteur-pipette to yield dissociated cells. Cells were centrifuged at 90x g for 5 min, cell pellet was carefully resuspended in Neurobasal-A medium supplemented with B27, cell number and viability was assessed by counting the number of living cells of 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 (0.05 mg/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 0.5 mM GlutaMAXI at 37 ºC and

5% CO_2. For qRT-PCR analysis and Western blot analysis of the Nedd8 pathway in primary neuronal cultures, neurons were grown in the presence of 5 µM Ara-C (Arabinofuranosyl Cytidine, Sigma Aldrich) to suppress proliferation of glial cells.

3.7.2. Transfection of primary neurons

Once plated neurons were transfected with expression plasmids at the desired DIVs via a modified calcium phosphate protocol (Jiang and Chen, 2006). 1 ml transfection mixtures (5-8 µg plasmid DNA, adjusted with Ampuwa H_2O to 50 µl, 12.5 µl 1 M $CaCl_2$, 50 µl 2x BBS (50 mM BES, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 7.26) and 900 µl Neurobasal-A medium supplemented with 2% B27-supplement and 0.5 mM GlutaMAXI) were prepared in sterile 1.5 ml 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, pre-incubated 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 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. To avoid any damage of the neurons, the time outside of the incubator was minimized during and after transfection. Expression efficiency, usually between 0.5 and 1% of neurons, was checked 24 h after transfection and was evaluated by expression of fluorescent proteins.

To study the very early steps of polarization, neurite formation and axonal outgrowth, neurons were transfected before plating with the Nucleofector system (Amaxa, LONZA) using highly purified DNA as described (Zeitelhofer et al., 2007).

3.7.3. Neuron – astrocyte co-cultures

For electrophysiological recordings, synapse stainings, and if necessary for live-cell-imaging experiments, neurons were cultivated on an astrocyte-monolayer. Primary cerebral astrocytes were isolated from cerebral cortices of CD1 postnatal day 1 (P1) mouse pubs, similar to the neuron protocol described above, and cultivated in DMEM supplemented with 10% FCS, 2mM GlutaMAXI, Penicillin (100 units/ml), Streptomycin (100 μg/ml) and 0.1 mM MEM Non Essential Amino Acids on gelantine- and Poly-D-Lysine-coated 10-cm plates as described (Allen et al., 2000; Cole and de Vellis, 2001). When astrocyte-cultures reached 80-90% confluency, they were washed three times with PBS, gently shaked to deattach non-astrocytic cells, trypsinized with 0.25% trypsin containing 1 mM EDTA and splitted 1:3 or 1:4 onto new plates, or directly seeded at a low density for astrocyte-neuron co-cultures. For co-cultures, atrocytes of passage 1 or 2 were plated two to four days prior to neuron preparation. On the day of neuron preparation the astrocyte growth medium was changed to Neurobasal-A supplemented with 2% B27-supplement and 0.5 mM GlutaMAXI 4-6 h before plating of neurons.

3.8. Electrophysiology

Whole-cell recordings of miniature excitatory postsynaptic currents (mEPSC) were recorded at room temperature (20-22 °C) from dissociated hippocampal neurons (DIV 15-19). The recording chamber was continuously perfused with carbogenated artificial cerebrospinal fluid (ACSF) containing (in mM): 119 NaCl; 2.5 KCl; 1.3 $MgSO_4$; 2.5 $CaCl_2$; 1 NaH_2PO_4; 26.2 $NaHCO_3$ and 11.1 D-glucose supplemented with tetrodotoxin (TTX, 0.2 μM), picrotoxin (PTX, 100 μM) and trichlormethiazide (TCM, 250 μM). mEPSC were recorded at a holding potential of -70 mV. Patch pipettes (borosilicate glass; Science Products, Hofheim, Germany) had a resistance of 3-5 MΩ and were filled with internal solution (150 mM CsGluc; 8 mM NaCl; 10 mM HEPES; 2 mM NaATP; 0.2 mM EGTA and 5 mM QX-314). Recordings were obtained with an Axon Instruments 700B patch clamp amplifier (filter at 1 kHz) and digitized at 10 kHz via Digidata 1322A (Axon Instruments). Data were collected using Clampex 10 (Axon Instruments, Molecular

Devices). mEPSCs were detected off-line and statistically analyzed with a custom written Matlab routine (MathWorks).

3.9. 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 room temperature, washed 3x with PBS and permeabilized with PBS-TritonX-100 0.1% 3x 5 min. For cytoskeletal stainings, neurons were fixed and extracted with Triton X-100 simultanously in PHEM buffer previously heated to 37 ºC (60 mM PIPES, 25 mM HEPES, 5 mM EGTA, 1 mM MgCl, pH 6.9, containing 0.25% Glutaraldehyde 3.7% PFA, 3.7% Sucrose, 0.1% Triton X-100), then washed 1x with PBS, quenched with 50 mM Ammonium chloride in PBS, pH7.4 for 5 min, 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, 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 o.n.. 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 room temperature for 2 h. Coverslips were washed 3x 10 min with PBS-TritonX-100 0.01% and mounted with anti-fading VectaShield medium (Vector) if desired containig DAPI to stain the nucleus.

3.10. Image acquisition and analysis of neuronal morphology

To analyze the morphology and synapses of neurons in culture and in 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 W

Olympus objective. Labeled neurons were excited at 405 nm (DAPI, Alexa-405), 488 nm (GFP, Venus, Alexa-488), 559 nm (RFP, dsRed, cherry, Alexa-594) and 635 nm (Alexa-647), and emission was collected at 425-475 nm, 500-545 nm, 575-675 and 680-750 nm, respectively. Usually a Z-stack of pictures was collected with 0.4-1.2 µm 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 plug-in (http://www.imagescience.org/meijering/software/neuronj/) in ImageJ, as well as the Sholl analysis plug-in (http://www-biology.ucsd.edu/labs/ghosh/software/) to measure dendrite arborization. Axons and dendrites of neurons were identified based on morphology and selective expression of MAP2 in dendrites and neurofilament (NF, SMI-312) in axons. Total dendrite or total axon length was determined by summing the lengths of all dendrite or axon 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 (10 µm step size) (Sholl, 1953). For spine analysis dendritic protrusions were counted per 10 or 20 µm segments of primary, secondary or tertiary dendrites of pyramidal excitatory neurons. Dendritic spines and filopodia were classified using the following criteria. Filopodia were defined as headless filamentous thin protrusions longer than 1.0 µm; spines (including mushroom-shaped, bulbous/stubby and thin spines) were defined as protrusions of 0.5–2.5 µm length that had distinct heads. Synapses were identified by post-synaptic PSD-95 and pre-synaptic synaptophysin clusters. PSD-95 and Synaptophysin puncta were analyzed by counting the number of puncta per 20 µm segments of dendrites. 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 in brain slices, including total dendrite length, number of nodes and Sholl analysis, neurons were directly traced on a Zeiss Axioplan microscope equipped with a Zeiss AxioCam camera and analyzed with Neurolucida software (mbf Bioscience).

3.11. Time-lapse analysis of neuronal development and filopodia and spine dynamics

For live-cell confocal imaging analysis, primary neurons were plated on glass-bottom dishes (35 mm MatTEK) or 35 µm ibidi plates (ibidi) and transfected via nucleofection (Amaxa) or calcium phosphate transfection at the desired DIVs. The confocal microscope was equipped with an environment-controlled chamber (Pecon) maintaining the neurons at 37 ºC and 5% CO_2. Neurons were chosen randomly and their coordinates were saved on the motorized stage for repeated re-localiztaion using the Multi-Area-Time-Lapse feature of Fluoview1000 software. To analyze development of young neurons, images were repeatedly acquired with the 20x or 40x objective across hours or days. For analysis of filopodia and spine dynamics, fast time lapse movies were recorded by repeated imaging of one neuron with a defined time interval (60 to 120 sec) with the 60x objective and zoom 2.0 to 3.0. To minimize photodamage to neurons and extend their lifetime during imaging, Z-stacks with step sizes between 0.4-0.6 µm and 512x512 to 800x800 pixels picture size were acquired with the minimal laser adjustments possible.

3.12. Long-term live cell imaging of spine stability

For imaging of spine stability in mature neurons, the Olympus IX81 epifluorescence microscope was equipped with a HAMAMATSU digital camera C10600 ORCA-R^2 and neurons were kept in the environment-controlled chamber (Pecon) at 37 ºC and 5% CO_2. Coordinates of neurons were saved on the automated stage for relocalization and images were taken every 12 to 36 h with cellSens digital imaging software (Olympus). In between image acquisition neurons were kept in the cell culture incubator at 37 ºC and 5% CO_2. For each neuron a Z-stack of pictures was taken with a step size of 0.6 µm that was subjected to deconvolution software (cellSens digital imaging software, Olympus) during image analysis.

3.13. Analysis of activity-dependent accumulation of Venus-Ubc12 in spines: Quantification of fluorescence intensities in spines and dendrites

To address if Ubc12 accumulates in dendritic spines in response to neuronal activity, mature hippocampal neurons, transfected with Venus-Ubc12 and mRFP, were stimulated with 10 µM glutamate for 10 min. Neurons were repeatedly imaged with the confocal microscope, before and 1 h after Glutamate treatment, recording Venus and mRFP fluorescence with constant laser settings for each neuron.

For analysis spines were divided into two groups based on the mRFP fluorescence picture. Spines that increased in their size 1 h after glutamate treatment by at least 1.3 fold and all remaining spines that did not change in their size, or showed minimal change in size after glutamate application, calculated as the area of spine^{basal}/area of spine$^{1h\ Glu}$ in the single *Z*-stack image slice in which the spine appeared brightest and biggest.

For immunofluorescence quantification, integrated red (mRFP) and green (Venus) fluorescence intensities were measured from boxed regions of interest (ROIs) on the dendrites and from boxed ROIs surrounding the spine heads in the single *Z*-stack image slice in which the spine appeared brightest and biggest. For correction all spine and dendrite fluorescence intensities were background subtracted. Background subtraction for dendrites was performed by subtracting “distant background” (mean pixel intensity from an ROI distant from the labeled dendrites multiplied by the number of pixels in the dendrite ROI) from the integrated dendritic ROI intensity. To account for higher background fluorescence near the dendritic shaft, background subtraction for spines was performed by subtracting “adjacent background” (integrated pixel intensity from a neighboring ROI of equal dimensions proximal to the dendritic shaft) from the integrated spine ROI intensity. Finally, corrected Venus intensities were normalized to their respective corrected mRFP intensities to calculate the fold change in Venus intensity in spines and dendrites.

3.14. Cell culture of cell lines and transient transfections

All cell culture reagents were purchased from Invitrogen except as otherwise stated, all cell culture dishes were purchased from Nunc as otherwise stated. HEK293 (human embryonic kidney), HT22 (mouse neuroblastoma) and Neuro-2a (mouse neuroblastoma) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FCS, Penicillin (100 units/ml), Streptomycin (100 μg/ml) and 2 mM L-Glutamine at 37 ºC, 5% CO_2. For transient transfection experiments cells were seeded at a desired density, if necessary on Poly-D-Lysine (Sigma Aldrich)-coated cell culture plates and 24 hours later transfected with the respective expression vectors using lipofectamine 2000 according to the manufacturer protocol. Neuronal differentiation of Neuro-2a cells was induced by serum-withdrawal for 24 to 48 h.

3.15. In vivo experiments

3.15.1. *In utero* intraventricular injection and electroporation of plasmid constructs

Intraventricular injection and electroporation

Timed pregnant female CD1 mice were anesthetized via i.p. injection with a mixture of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight). The eyes of the pregnant dam were protected from drying during the surgery by a drop of eye cream (Bepanthen eye and nose cream). After shaving, the abdomen was cleaned with 70% ethanol and 3-cm long midline lapratomy was performed. The uterine horns were carefully exposed, placed on sterile gauze and hydrated with saline (0.9% NaCl solution), prewarmed to 37 ºC. 1-2 µl 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 E13.5-E15.5 mouse embryos using a glass micropipet and plunger (Drummond PCR micropipets, 1-10 µl). After DNA injection, electroporations

were performed using an Electro Square Porator ECM830 and tweezertrodes (BTX Genetronics). Five pulses with 40V, 50 ms duration and with 950 ms intervals, were delivered to each embryo. The developing cortex or hippocampus was targeted by electroporations with 7-mm diameter tweezertrodes (BTX Genetronics) as described in (Nakahira and Yuasa, 2005). After the embryos had been 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 chance of infection. The abdominal wall and skin were sewed up with surgical sutures (Johnson & Johnson), and 7.5% povidone-idonine 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. Then it was placed back into a new clean cage. The embryos were allowed to develop up to two month after birth (Tabata and Nakajima, 2001; Saito and Nakatsuji, 2001; Noctor et al., 2001; Saito, 2006; Saito, 2010).

Genotyping of CamKIIα-Cre-ERT2 mice

In case of CamKIIα-Cre-ERT2 breedings, the litters were genotyped by PCR using the following primers,

iCre-for1: 5'-GGT TCT CCG TTT GCA CTC AGG A-3'

iCre-rev1: 5'-CTG CAT GCA CGG GAC AGC TCT-3'

iCre-rev2: 5'-GCT TGC AGG TAC AGG AGG TAG T-3'.

Standard PCR conditions resulted in a 290-bp wild-type and a 375-bp Cre product.

3.15.2. Analysis of adult neurogenesis in the dentate gyrus

Retroviral constructs, virus preparation and stereotactic injections into the DG were performed in collaboration with Chichung Lie's lab at the IDG at the Helmholtz-Zentrum München.

Preparation of retroviruses

Retroviral vectors CAG-GFP (cytomegalovirus immediate early enhancer-chicken β-actin hybrid-green fluorescent protein), CAG-IRES-GFP, and CAG-RFP (red fluorescent protein) were previously decribed (Zhao et al., 2006). Ubc12-C111S was cloned into CAG-IRES-GFP (via PmeI and SfiI restriction sites).

Retroviruses were generated using an all-transient transfection approach. 293T cells were transfected with a mixture containing three separate plasmids, including capsid (CMV-VsVg), viral proteins (CMV-gag/pol), and retroviral plasmid (CAG-vectors) using Lipofectamine 2000 (Invitrogen). Virus-containing supernatant was harvested twice, 48 and 96 h after transfection, and concentrated by two rounds of ultracentrifugation (Tashiro et al., 2006; Jagasia et al., 2009). Viral titers ranged between 0.5 and 5×10^7 colony-forming units (cfu) ml^{-1}.

Stereotactic injections of retroviruses into the DG

For the site-specific gene delivery into restricted brain areas of mice, we used stereotactic injections of viral vectors. In order to label newborn neurons in the dentate gyrus of adult mice, recombinant retroviral vectors expressing CAG-GFP/CAG-RFP, CAG-Ubc12-C111S-IRES-GFP/CAG-RFP were injected. Appropriate coordinates for the different brain regions were taken from the stereotactic atlas "The mouse brain in stereotaxic coordinates" (Paxinos and Franklin, 2001) and the surgery procedure was adapted from Cetin et al. (2006).

For all stereotactic injection experiments of retroviruses 8- to 11-week-old female C57BL/6 mice were used. Mice were anesthetized with a mixture of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight). Mice were put back into their home cage until they were completely sedated. Then the animal was fixed in the stereotactic apparatus (Stoelting) using ear bars and a mouse nose clamp. Eye ointment was applied to prevent corneal drying during the surgery. The fur on the skull was

cleaned with 70% ethanol and the scalp was opened with a sterile scalpel along the midline. Small surgical clamps were used to keep the area open and the skull was moistened regularly with PBS. The coordinates of Bregma and Lambda were measured and the target injection coordinates were calculated. Mice were stereotactically injected with 1.5 µl of 1:1 mixtures of CAG-GFP/CAG-RFP, CAG-Ubc12-C111S-IRES-GFP/CAG-RFP retroviruses into the dentate gyrus [coordinates from bregma were (in mm): -1.9 anterior/posterior, ±1.6 medial/lateral, -1.9 dorsal/ventral from dura]. Titers of injected viruses were adjusted for each experiment to the retroviral preparation with the lower titer. For the injection small holes were drilled with a hand-held drill and then the micropipette was positioned at the correct coordinates. With a motorised Nanoliter injector (World Precision Instruments), 1.5 µl of the viral vector solution was injected within 4 min. Afterwards the micropipette remained for additional 4 min in the brain to allow spreading of the virus and was then pulled out slowly and carefully. The injection procedure was repeated at the second hemisphere to obtain symmetrical gene delivery. Finally, the surgery area was cleaned and was subsequently sutured using an absorbable thread. The animal was placed on a heating plate at 37 °C for recovery from anesthesia.

3.15.3. Preparation of brain slices

Electroporated and stereotactically injected animals were anesthezized 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, pH7.4, and 1 min with PBS at a flow of 10 ml/min. Brains were removed, post-fixed for 1 h in 4% PFA at 4ºC and cryoprotected in 15% (w/v) Saccharose in PBS, pH7.6 o.n. at 4 ºC. Brains were washed with PBS and schock-frozen 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). 20-40 µm thick cryo-sections and 50-75 µm thick vibratome-sections were stored at -20 ºC in cryopreservation solution (25% (v/v) glycerol, 25% (v/v) Ethylenglycol, 50% (v/v) PBS, pH7.4) until immunohistochemistry, DAPI staining and mounting.

3.15.4. 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 10 min 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 2 h at RT. 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).

3.16. Immunoprecipitation analyses

Cells were lysed in radio-immune precipitation buffer (RIPA, 50 mM Tris, pH 8.0, 150 mM NaCl, 0,1% SDS, 1.0% NP-40 or Triton X-100 and 0.5% Sodium deoxycholate) or NP40 (150 mM NaCl, 1.0% NP-40 and 50 mM Tris, pH 8.0) buffer containing protease inhibitors (complete protease iInhibitor tablets, Roche). When protein neddylation was analyzed 20 mM N-Ethylmaleimide (NEM) (Sigma Aldrich), an inhibitor of cysteine peptidases, and 2 mM 1,10-Phenanthroline monohydrate (OPT) (Sigma Aldrich), an inhibitor of metalloproteases were added to the lysis buffer. Lysates were briefly precleared with Dynabeads Protein G (Invitrogen) for 30 min at 4 ºC and then incubated with either the appropriate antibody or antibody-conjugated magnetic beads, o.n. at 4 ºC, or 2 h at RT for M2-FLAG-beads (Sigma Aldrich). For non-conjugated antibodies, the antibody-protein complexes were immunoprecipitated with Dynabeads Protein G (Invitrogen) for 2-3 h at 4 ºC. Immunoprecipitated proteins bound to beads were washed 3 to 5x for 10 min with washing buffer (10 mM Tris, pH 7.4, 1 mM EDTA, pH 8.0, 1mM EGTA, pH 8.0, 150 mM NaCl, 0.5% Triton X-100, containing protease inhibitors) and then proteins were eluted by boiling in 1x Lämmli-buffer or in case of the M2-FLAG-beads by incubating with 3x FLAG peptide (Sigma Aldrich) at a final concentration of 150

ng/µl in TBS-TritonX-100 0.1% at 4 ºC for 30 min. Samples were analyzed by SDS-PAGE and transferred to PVDF membrane for immunoblotting analysis.

3.17. Immunoblotting

Cells and tissue was lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0,1% SDS, 1.0% NP-40 or Triton X-100 and 0.5% sodium deoxycholate) containing protease inhibitors (complete protease iInhibitor tablets, Roche). When protein neddylation was analyzed 20 mM N-Ethylmaleimide (NEM) (Sigma Aldrich), an inhibitor of cysteine peptidases, and 2 mM 1,10-Phenanthroline monohydrate (OPT) (Sigma Aldrich), an inhibitor of metalloproteases were added to the lysis buffer. Protein concentrations were measued using Bradford assay. For immunoblotting, protein samples were separated by 8-14% SDS-PAGE (Laemmli, 1970) and transferred to 0.45-µm PVDF membranes (Millipore). Membranes were blocked in 5% nonfat milk (Roth) in TBS-Tween 20 0.01% (Sigma Aldrich) for 1h 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 chemiluminescence (Millipore) and membranes were exposed to X-ray films (Kodak). Developed X-ray films were scanned, transformed to 8-bit gray pictures and quantified by Image J software, measuring integrated intensities of gray values. All signals were normalized to their respective house-keeping protein control bands.

3.18. Brain fractionation

The preparation of synaptosomes, synaptic vescicles, plasma membranes and post-synaptic densities was performed as previously described (Cho et al., 1992; Blackstone et al., 1992; Lau et al., 1996). A ultracentrifuge Beckman L70 equipped with casing for 5 ml polyallomer tubes was used in the procedure. All procedures were performed at 4 °C; all buffers and reagents included complete protease inhibitors cocktail (Roche) and

phosphatase inhibitor cocktails I and II (Sigma Aldrich), plus 5 mM of Sodium Orthovanadate. C57BL/6 mice were sacrificed by decapitation, the brains were quickly dissected and the cerebral cortex were isolated and homogenized in ice-cold HEPES-sucrose buffer (Sucrose 0.32 M, 4 mM HEPES, pH 7.4) in a manual glass Dounce homogenizer (10 strokes). The homogenate was centrifuged at 1000x g for 5 min to remove the nuclear fraction and tissue debris (P1). The supernatant (S1) was centrifuged at 10,000x g for 15 min to precipitate the crude synaptosomal fraction (P2). The P2 pellet was resuspended in 10 volumes of 0.32 M sucrose-HEPES and centrifuged again (10,000x g for 15 min) to obtain the washed synaptosomal fraction P2'). Synaptosomes were further purified layering the P2' (resuspended in 0.32 M sucrose) onto 4 ml of 1.2 M sucrose and centrifuging at 230,000x g for 15 min. The gradient interphase was collected, diluted in 8 volumes of 0.32 M sucrose, layered on 4 ml of 0.8 M sucrose and centrifuged again at 230,000x g for 15 min. The resulting pellet, P2'', contained purified synaptosomes. To further fraction the synaptosomal pellet, synaptosomes were lysed by hypoosmotic shock in 9 volumes of H_2O plus protease and phosphatase inhibitors and three passes through a G32 syringe needle. pH was rapidly adjusted to pH7.4 by adding HEPES to a concentration of 4 mM; the suspension was incubated at 4 °C with constant mixing to ensure the complete lysis. The lysate was then centrifuged at 25,000x g for 20 min to recover a supernatant (S3) containing a crude synaptic vesicles fraction and a pellet (P3) containing lysed synaptosomal membranes (pre- and post-synaptic membranes). Synaptic vesicles were recovered by centrifugation at 165,000x g for 2 h. Synaptic membranes were further purified by resuspension in 0.32 M sucrose and centrifugation on a discontinuous sucrose gradient 0.8 to 1 to 1.2 M sucrose (pH7.4) at 150,000x g for 30 min. Synaptic plasma membranes were recovered between layers 1 and 1.2 M sucrose; sucrose concentration was adjusted to 0.32 M with 4 mM HEPES solution and the synaptic plasma membranes were pelleted (P4) by centrifugation at 150,000x g for 30 min. Post-synaptic densities were further purified according to Cho et al. (Neuron, 1992). Synaptic plasma membranes (P4) were resuspended in 5 ml ice cold 50 mM HEPES (plus protease and phosphatase inhibitors, plus 2 mM EDTA plus 0.5% Triton X-100) and rotated in cold room for 15 min. The resulting suspension was centrifuged at 32,000x g for 20 min to obtain the PSD-1T fraction. The PSD-1T was resuspended in 50 mM HEPES, 2 mM EDTA plus 0.5% Triton X-100 and incubated for 15 min with continuous mixing. The suspension was thereafter centrifuged at 200,000x g for 20 min to obtain the PSD-2T fraction. Samples from P2,

P3, P4, synaptic vescicles, PSD-1T and PSD-2T were collected and directly lysed in SDS-containing lysis buffer and subjected to immunoblotting.

3.19. Generation of mouse models of the Nedd8 pathway

3.19.1. Ubc12-LacZ reporter mouse (from German Gene Trap Consortium – GGTC)

A Ubc12-GGTC-ES cell clone was generated by the German Gene Trap Consortium (GGTC) via random insertion of a genetrap vector consisting of a reporter (β-Galactosidase)/selector (Neomycin-resistance) cassette – rsFlipROSAßgeo(Cre)∗ – into intron 1 of the mouse Ubc12 gene (ENSMUSG00000005575) in E14 mouse ES cells (http://tikus.gsf.de/) (for details see targeting construct in Figure 68.A in 4.8.1). The integration site was confirmed by Splinklerette-PCRs and mutant ES cells were used to generate chimeric mice by BALB/c blastocyst injection in collaboration with the IDG at the Helmholtz-Zentrum München. Germline transmission of the modified Ubc12 allele was confirmed in offspring from male chimeras bred to wild-type C57BL/6J mice.
Genotyping was performed by PCR using the following primers,
Ubc12-for: 5'-GAT AAG TAT GAC ATC ATC AAG GAA AC-3'
GTVint1-rev: 5'-TTG TAA TAA GAT GGA GAC GAG GAA GGA C-3'
Ubc12-rev: 5'-TAC GTG GAC TCT TCT GGA TAC ACA GTA TC-3'
and standard PCR conditions resulted in a 333-bp wild-type and a 847-bp Ubc12-reporter PCR product.

3.19.2. Nae1 (Appbp1)-conditional KO mouse

Conditional Nae1 ES cell clones were generated by Eucomm via targeted gene trapping (Project: 33210, http://www.knockoutmouse.org/about/eucomm). In these ES cell clones (EPD0441_1_B07, _F07, and _C08) a gene trap cassette, consisting of a engrailed 2 splice acceptor-IRES-LacZ sequence followed by a pGK promoter-neomycin-resistance gene construct, was inserted by homologous recombination in JM8A3.N1 ES cells into intron 3 of mouse Nae1 gene, leading to disruption of the Nae1 gene and to splicing of exon 3 of Appbp1 to the SA-IRES-LacZ sequence. The whole gene trap cassette is flanked by frt sites for FLP-mediated excision, resulting in the floxed *Appbp1* allele, in which exon 4 of *Nae1* is flanked by loxP sites (for details see targeting construct in Figure 69 in 4.8.2). Correct targeting of the *Nae1* locus was confirmed by long range PCRs in the quality control step of the Eucomm pipeline. Mutant ES cells were used to generate chimeric mice by BALB/c blastocyst injection in collaboration with the IDG at the Helmholtz-Zentrum München. Germ-line transmission of the modified Nae1 allele was confirmed in offspring from male chimeras bred to wild-type C57BL/6J mice. Offspring carrying the mutant *Nae1* allele were further bred among each other to establish the conventional Nae1-KO mouse line.

Genotyping of conventional Nae1-KO mice: Genotyping was performed by PCR using the following primers.
Nae1-for: 5'-AGG AAA ATC AGG ATT TAG TTC ATA TTT CC-3'
GTV_int2-rev: 5'-CAC AAC GGG TTC TTC TGT TAG TCC-3'
GTV_int3-for: 5'-ATC CGG GGG TAC CGC GTC GAG-3'
Nae1-rev: 5'-GGC TAT TAA CCG CCT ACT GCG ACT ATA GAG-3'
LacZ-for: 5'-GGT GGC GCT GGA TGG TAA GCC GCT GGC AAG-3'
LacZ-rev: 5'-CGC CAT TTG ACC ACT ACC ATC AAT CCG GTA G-3'
Standard PCR conditions resulted in a 532-bp wild-type (Nae1-for/Nae1-rev) product, as well as in a 572-bp mutant Nae1 (Nae1-for/ GTV_int2-rev), 208-bp mutant Nae1 (Nae1 GTV_int3-for/ Nae1-rev) and a 650-bp LacZ (LacZ-for/ LacZ-rev) PCR product.

To obtain a conditional KO of Nae1, conventional heterozygous KO mice of Nae1 were first bred to hACTB::Flp-deleter mice (Rodriguez et al., 2000) resulting in the floxed wild-

type allele of Nae1 ($Nae1^{flox}$) after excision of the gene trap cassette, and then they are further bred to Cre, e.g. Nestin-Cre (Tronche et al., 1999), or tamoxifen-inducible Cre-ERT2, e.g. CamKIIα-Cre-ERT2 (Erdmann et al., 2007) mouse lines.

Genotyping of conditional Nae1-KO mice: Genotyping was performed by PCR using the following primers,
Nae1-for: 5'-AGG AAA ATC AGG ATT TAG TTC ATA TTT CC-3'
Nae1-rev: 5'-CAT GAA ATC CAC TGT TCC ATT TAA TGG C-3'
Cre-for: 5'-GAT CGC TGC CAG GAT ATA CG-3'
Cre-rev: 5'-AAT CGC CAT CTT CCA GCA G-3'
iCre-for1: 5'-GGT TCT CCG TTT GCA CTC AGG A-3'
iCre-rev1: 5'-CTG CAT GCA CGG GAC AGC TCT-3'
iCre-rev2: 5'-GCT TGC AGG TAC AGG AGG TAG T-3'
Standard PCR conditions with primers Nae1-for and Nae1-rev resulted in a 532-bp wild-type and a 620-bp floxed Nae1 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 CamKIIα-Cre-ERT2 mouse line genotyping with iCre primers resulted in a 290-bp wild-type and a 375-bp Cre product.

3.19.3. Conditional overexpression of Ubc12-C111S in the *ROSA26* locus

Generation of the targeting vector

The FLAG-tagged human Ubc12-C111S cDNA sequence was cloned 5' to the IRES-LacZ sequence into the PacI and FseI restriction sites in the *ROSA26* targeting vector, which is based on the pROSA26-1 vector (Soriano, 1999). A minimal CAG promoter sequence (cytomegalovirus immediate-early enhancer and chicken β-actin promoter) flanked by frt sites was introduced 5' of the floxed STOP cassette, which contains the neomycin-resistance gene under the control of the pGK promoter. The whole construct (frt – CAG – frt – Splice Acceptor – loxP – STOP – loxP – FLAG-hUbc12-C111S – IRES – LacZ) was flanked 5' and 3' by homology arms consisting of genomic DNA sequence

of the *ROSA26* locus to achieve homologous recombination in ES cells (see also targeting strategy in Figure 70 in 4.8.3).

ES cell culture

All cell culture reagents were purchased from Invitrogen except as otherwise stated, and all cell culture dishes were purchased from Nunc as otherwise stated.

Targeting via homologous recombination was performed in the IDG3.2 ES cell line kindly provided by Ralf Kühn from the IDG at the Helmholtz-Zentrum München. Neomycin-resistant feeder cells were kindly provided by Susanne Bourier from the IDG at the Helmholtz-Zentrum München. For ES cells the medium was exchanged every 24 h.

Medium for IDG3.2 ES cells

500 ml DMEM (high glucose, 21969035)
75 ml FCS (PAN Biotech, 2602P242901)
5 ml L-glutamine (200 mM, 25030-024)
5 ml MEM nonessential amino acids (100x, 11140-035)
1 ml mercaptoethanol (50 mM, 31350-010)
10 ml HEPES (1 M, 15630-056)
90 µl LIF (107 U/ml) (Chemicon, ESG 1107)
for selection, 180 µg/ml geneticin (G418) was added

Medium for EMFI feeder cells

500 ml DMEM (high glucose, 21969035)
50 ml FCS (PAA, A15041PAA)
5 ml L-glutamine (200 mM, 25030-024)
6 ml MEM nonessential amino acids (100x, 11140-035)

2x Freezing medium for ES cells

5 ml FCS (PAN Biotech)
3 ml ES cell medium
2 ml DMSO

0.05% Trypsin in Tris Saline/EDTA buffer

GENETICIN, G418 Sulphate (11811-098), potency 703 µg/mg

0.1% (w/v) Gelantine (BDH Laboratory Supplies, 44045B) in Ampuwa H_2O, autoclaved

Preparation of feeder cells

For targeting via homologous recombination, IDG3.2 ES cells were cultured on inactivated feeder cells (passage 2-4). Feeder cells are embryonic mouse fibroblasts (EMFI) that were isolated from mice expressing the neomycin-resistance gene. Feeder cells prevent differentiation of ES cells and provide support and growth factors. Each aliquot of feeder cells (passage 2 to 3), containing ~$5x10^6$ cells, was unfrozen, plated on 3x 15 cm plates each containing a total of 25 ml EMFI medium and incubated at 37°C, 5% CO_2 for 3 or 4 days. For expansion, every feeder plate was splitted up to two passages 1:3 on new 15 cm plates. For inactivation of feeder cells, 70%-80% confluent 15 cm plates were used. Directly before inactivation medium was replaced by 15 ml fresh EMFI medium. Cells were then treated with 0.01 mg/ml (150 µl in 15 ml medium) Mitomycin C (1 mg/ml in PBS, Sigma Aldrich) during their exponential growth phase for mitotic inactivation for 2-3 hrs at 37°C. Feeder cells were trypsinized, counted and an amount of $1x10^4$ cells/cm^2 was plated on new dishes containing EMFI medium. 96-, 48-, 24- and 12-well plates were shortly centrifuged to allow better cell adhesion. EMFI cells were allowed to attach o.n. before ES cells were plated onto them.

Culturing of ES cells

One aliquot of xxMio IDG3.2 ES cells was unfrozen, plated on 2x 10 cm feeder plates containing ES cell medium and incubated at 37°C, 5% CO_2. 1-2 days later, ES cells were passaged on new feeder plates in a 1:4 dilution and grown for about 2-3 days.

Electroporation and selection of ES cells

ES cells were trypsinized, counted and a dilution of $1x10^7$ cells/800 µl per electroporation cuvette was prepared. 20 µg of the linearized targeting vector in a volume of 20 µl was added to the cell suspension. The mix was transferred to a 4mm electroporation cuvette (Bio Rad) and electroporated using the Bio Rad gene pulser at 0.24 kV, 500 µF. The cuvette was removed and incubated on ice for 10-20 min. Then, the cell suspension from one cuvette was carefully transferred into 2 ml ES cell medium containing LIF and the suspension from one cuvette was distributed to 2x 10 cm feeder

plates containing already 9 ml medium with LIF. Two days after electroporation, the ES cell medium was exchanged for selection medium containing 180 µg/ml of the antibiotic geneticin (G418). In addition to the G418-resistant feeder cells, only those ES cells can survive and expand which carry the transgene containing the neomycin selection marker. After about 6-8 days of selection, drug resistant colonies should have appeared and be ready for picking.

Picking of ES cell colonies

Before picking, medium in the original electroporation plate was removed and 10 ml PBS were added. With the aid of a binocular, single ES cell colonies were carefully scraped off the plate using a pipette, aspirated and transferred to a conical 96-well plate. After trypsination, cells were plated on fresh 96-well feeder plates containing 100 µl ES medium.

Splitting of ES cell colonies

After 3-5 days, each ES cell clone was analysed using a binocular and passaged in a 1:2, 1:3 or 1:4 dilution depending on the respective cell density. This synchronisation step is necessary to avoid differential growth of the single clones. Cells were trypsinized and resuspended in an appropriate amount of medium. Then the cells were plated on fresh 96-well feeder plates containing 150 µl/well ES cell medium. After two days, one plate was splitted again on two feeder plates and the other one on two gelatine-coated plates for Southern blot analysis. Therefore, 96-well plates were previously incubated with 50 µl of 0.1% autoclaved gelatine for 20 min. Since inactivated EMFI cells are not able to expand on gelatine plates, the DNA portion of feeder cells in the Southern blot analysis can be neglected.

Freezing of ES cell colonies

For freezing of ES cell clones grown on feeder plates, cells were trypsinized in 35 µl Trypsin solution and resuspended in 75 µl cold ES cell medium. 110 µl 2x concentrated freezing medium was added and mixed with the cell suspension. Plates were sealed with parafilm, wrapped in tissue towels, transferred into a paperbox, and frozen at -80 °C. Gelatine plates for Southern Blot analysis were incubated 1-2 days longer until the cells became very dense to obtain high amounts/yields of genomic DNA. Then, cells

were washed 2x with PBS and frozen at -20 ºC without medium for Southern blot analysis.

Isolation of genomic DNA and Southern Blot analysis of ES cell clones

Genomic DNA of frozen ES cells was extracted as follows. Cells were incubated o.n. with DNA lysis buffer (10 mM Tris HCl pH 7.5 , 10 mM EDTA pH 8.0, 10 mM NaCl, 0.5% Sarcosyl, containing Proteinase K 1mg/ml) at 56 °C. DNA was precipitated by addition of 2 volumes of ice cold 100% Ethanol, containing 75 mM NaCl and centrifugation at 2000xg for 15 min. DNA pellets were washed 3x with 70% Ethanol, air-dried and solved in H_2O (Ampuwa).

For Southern blot analysis, approximately 6 µg of genomic DNA or DNA yield of one well of a 96-well plate were digested o.n. with 10 to 30 U of the appropriate restriction enzyme in a total volume of 30 µl. Then, additional 10 U of enzyme were added and again incubated for 4 hours. Digested DNA was separated on a 0.8 % agarose gel with TBE as running buffer for 15 to 18 hours at 30-55 V, depending on the size of the expected bands. After the run, the DNA in the gel was denatured by shaking for 1 hour in denaturing solution (1.5 M NaCl, 0.5 M NaOH). Then, neutralisation was performed by shaking the gel for 1 hour in neutralising solution (0.5 M NaCl, 0.1 M Tris-HCl, pH 7.5) and subsequent rinsing with 2x SSC. Blotting was performed o.n. via capillary transfer using 20x SSC and a nylon membrane (Hybond N^+, GE Healthcare). After the transfer, the membrane was rinsed briefly with 2x SSC and crosslinked by UV radiation (UV-Stratalinker, Stratagene) before proceeding with the hybridization.

50 to 100 ng of DNA probe were radioactively labelled with α-32P-dCTP (Perkin Elmer) using the Roche HighPrime Kit following manufacturer's instructions. To remove unincorporated radioactive nucleotides, Microspin S-300 (GE Healthcare) columns were used. The labelling efficiency was determined by measuring 1 µl of the probe in a liquid scintillation counter.

Before hybridization, the membrane was preincubated with Church buffer (0.5 M Na_2HPO_4, 0.5 M NaH_2PO_4, 1 % BSA, 7 % SDS, 1mM EDTA, pH 8.0, 0.1 mg/ml salmon sperm DNA) for 1 hour at 65 °C in a hybridization bottle. Meanwhile, the labelled probe was denatured for 5 min at 95 °C, chilled on ice, and then added to the Church buffer to a final activity of 100,000 to 900,000 cpm/ml. The membrane was hybridized at 65 °C for 5 hours to o.n.. Afterwards, in order to remove unbound and unspecifically hybridized probe, the membrane was washed two times with pre-warmed wash solution I (2x SSC,

0.5% SDS) at 65 °C for 30 min. If necessary, a third washing step with wash solution II (1x SSC, 0.5% SDS) at 65 °C for 30 min was added.

For detection of the signal, the hybridized membrane was wrapped in saran wrap and exposed to an autoradiography film for 1-2 days at −80 °C. To in-crease the intensity of the signal, Biomax MS films together with Biomax screens were used. After exposure, the films were developed using a developing machine (Kodak).

If the membrane should be used for hybridization with a second probe after the first detection, the first probe could be washed away with stripping solution (0.1x SSC, 1% SDS). Therefore, the membrane was boiled 5-10 min in stripping solution and briefly rinsed with 2x SSC before the prehybridization could be started.

Expansion of positive ES cell clones for blastocyst injection

IDG3.2 ES cell plates containing the positive clones were quickly unfrozen in a waterbath at 37ºC. Plates were centrifuged 2 min at 1200 rpm. Freezing medium was carefully removed from the wells containing the positive ES cell clones. Cells were resuspended in 100 µl fresh selection medium and transferred to a new 96-well feeder plate containing already 100 µl selection medium. Cells were expanded consecutively on 24-well plates, 6-well plates, 6 cm plates, 2x 10 cm plates. After this expansion several aliquots of the positive ES cell clones were frozen in liquid nitrogen for conservation and future experiments. ES cell clones for injection were splitted 1-2 days before blastocyst injection onto gelantine-coated plates to minimize the contribution of feeder cells. On the day of blastocyst injection, ES cells plates were trypsinized, centrifuged and the cell pellet was resuspended in 2-3 ml ES cell medium and stored on ice until injection.

Generation of chimeric mice by blastocyst injection

The blastocyst injection was carried out in collaboration with Dr. Ralf Kühn at the Institute of Developmental Genetics (IDG), Helmholtz-Zentrum München applying standard techniques using laser-microinjection. Superovulation of blastocyst donor female mice was achieved by PMSG and HCG i.p. injection. (In the case of the ROSA26-Ubc12-C111S IDG3.2 ES cells, F1 intercrosses of C57BL/6 and DBA mice were used as donor females). The injected blastocysts were then transferred to CD1 pseudo-pregnant foster mothers. Chimeric mice were identified according to their coat colours.

Analysis of germline-transmission by PCR

Chimeric mice were bred to C57BL/6 females and offspring was genotyped by PCR with specific primers to amplify a 3'-fragment of the β-Galactosidase/LacZ sequence (LacZ-for 5'-GGT GGC GCT GGA TGG TAA GCC GCT GGC AAG-3' and LacZ-rev 5'-CGC CAT TTG ACC ACT ACC ATC AAT CCG GTA G-3') or a 5'-band with ROSA-for (5'-AAA GTC GCT CTG AGT TGT TAT-3') and CAG-rev (5'-GCG TTA CTA TGG GAA CAT ACG TCA TTA TTG-3') primers. Standard PCR conditions resulted in a 650-bp 3'-LacZ and 450-bp 5'-ROSA-for/CAG-rev PCR product.

3.20. Statistics

Each set of numerical data shown was obtained in two to five independent experiments. Statistical analysis was carried out using GraphPad Prism 5 software. All values are given as mean ± s.e.m. 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 ANOVA followed by Bonferroni post hoc test. Differences were considered statistically significant at *$p<0.05$, **$p<0.01$.

4. RESULTS

4.1. Expression analysis of the neddylation pathway in the central nervous system of the mouse

The first aim was to characterize the expression profiles of the members of the Nedd8-conjugation pathway in the central nervous system (CNS) during development and adulthood on the mRNA and protein level. By the use of double (co-)-staining techniques we deduced the cell-type specific expression and the subcellular localization of Nedd8 and the Nedd8-conjugating enzyme (E2) Ubc12. Furthermore, we analyzed the activity and targets of neddylation in the developing mouse brain and during development of primary neurons in culture.

4.1.1. Expression pattern analysis of Ubc12 and Nedd8 mRNA in the mouse brain by *in situ* hybridization

To study the mRNA expression pattern of key components of the neddylation pathway in the mouse brain we used *in situ* hybridization (ISH). Therefore, radioactively-labeled riboprobes detecting specifically mouse *Ubc12* and *Nedd8* mRNA were generated and hybridized to mouse brain sections of different developmental stages. Darkfield photomicrographs show that Ubc12 and Nedd8 are highly and ubiquitously expressed in the central nervous system during embryonic and early postnatal development as well as in the adult brain (Figure 12, Figure 13.A, B). To address the cell type-specific expression of Ubc12 and Nedd8, higher magnification brightfield photomicrographs of hippocampal sections with cresyl-violet counterstaining were taken. These highlight the enrichment of Ubc12 and Nedd8 mRNA in neurons, shown for the cell bodies of pyramidal neurons and interneurons in the stratum pyramidale of CA1 of the hippocampus in Figure 13.C, D.

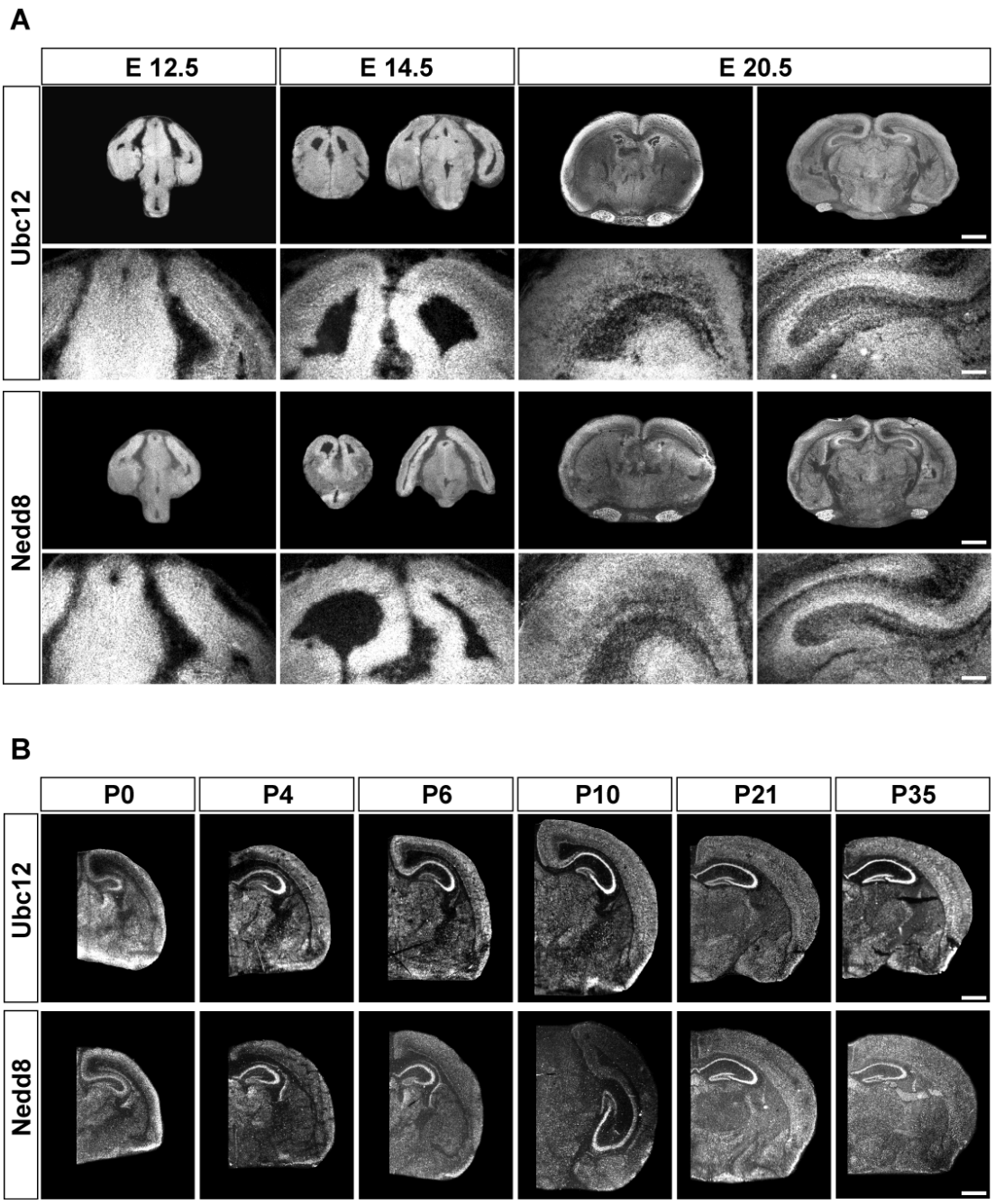


Figure 12: *In situ* hybridization for Ubc12 and Nedd8 in the developing mouse brain.

Darkfield photomicrographs of radioactive *in situ* hybridizations (ISH) revealed high and ubiquitous mRNA expression of Ubc12 and Nedd8 in the brain during embryonic (**A**) and postnatal (**B**) development. Scale bars represent 1 mm.

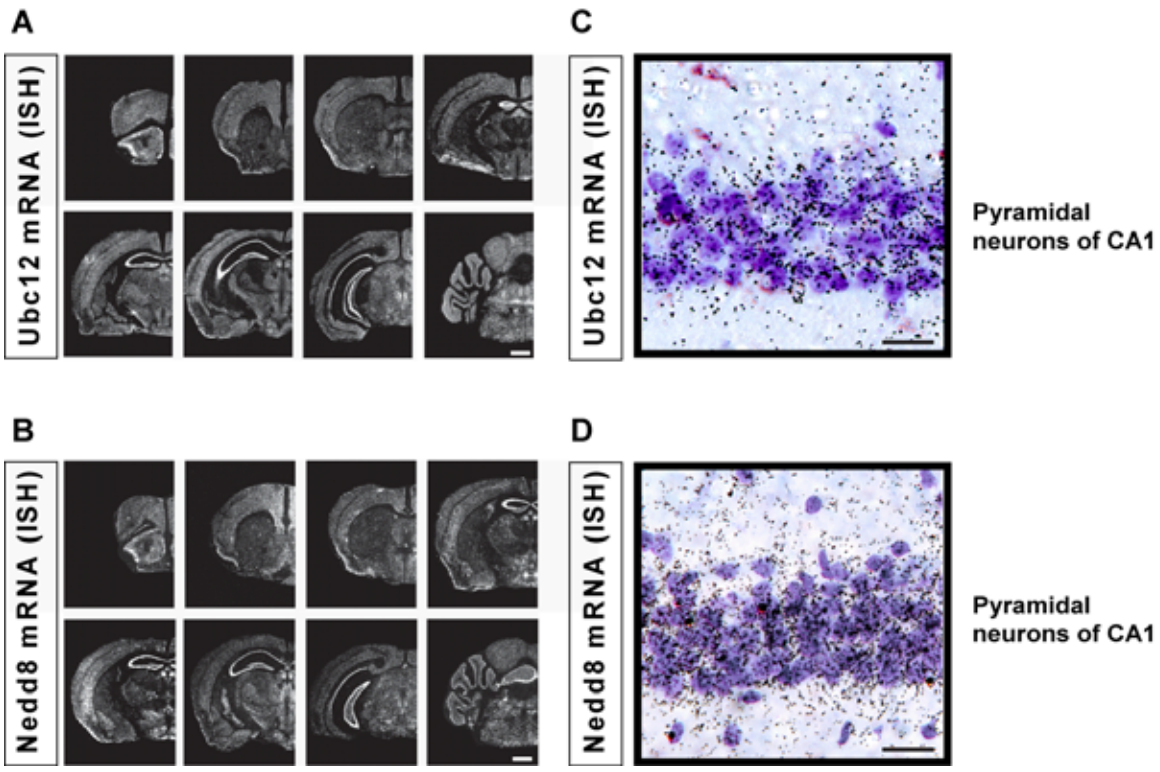


Figure 13: *In situ* hybridization in the adult mouse brain highlights the enrichment of Ubc12 and Nedd8 expression in neurons.

A) and **B)** Darkfield photomicrographs of radioactive ***in situ*** hybridizations showed high and ubiquitous mRNA expression of Ubc12 and Nedd8 throughout the adult mouse brain. **C)** and **D)** High magnification brightfield pictures, counterstained with cresyl-violet confirmed the enrichment of Ubc12 and Nedd8 expression in neurons, here shown for pyramidal neurons of CA1 of the hippocampus. Scale bars in (**A**) and (**B**) represent 1 mm; scale bars in (**C**) and (**D**) represent 25 µm.

4.1.2. Analysis of Ubc12 expression in a Ubc12-LacZ reporter mouse line by LacZ staining

To further analyze and confirm the expression pattern of Ubc12 in the mouse brain, LacZ stainings were performed on heterozygous Ubc12-LacZ reporter mice. For a detailed description of the generation of this mouse line see 4.9.1. Briefly, a gene trap cassette, containing the β-Galactosidase gene and a Neomycin-resistance gene, was randomly inserted into intron 1 of the Ubc12 gene, resulting in splicing of exon 1 of Ubc12 to the β-Galactosidase gene of the gene trap cassette. β-Galactosidase,

expressed under the control of the Ubc12 promoter, can therefore be used as a reporter of endogenous Ubc12 expression and can be visualized by LacZ staining. LacZ stainings on heterozygous Ubc12-LacZ reporter mice recapitulated the high, ubiquitous and widespread expression pattern of Ubc12 in the mouse brain obtained by *in situ* hybridizations (Figure 14.A). Furthermore combining LacZ staining with DAB-immunohistochemistry against the astrocyte marker glial fibrillary acidic protein (GFAP) on hippocampal brain sections confirmed the enrichment of Ubc12 expression in neurons shown in Figure 14.B (filled black arrow heads). An intense LacZ staining in the stratum pyramidale of CA1 of the hippocampus can be observed, whereas only a subset of GFAP-positive astrocytes in the stratum radiatum show a low to moderate LacZ staining (dashed black arrow heads). Of note, additional GFAP-negative cells in the stratum radiatum, presumably microglia or oligodendrocytes, also express Ubc12 (black arrow heads, Figure 14.B).

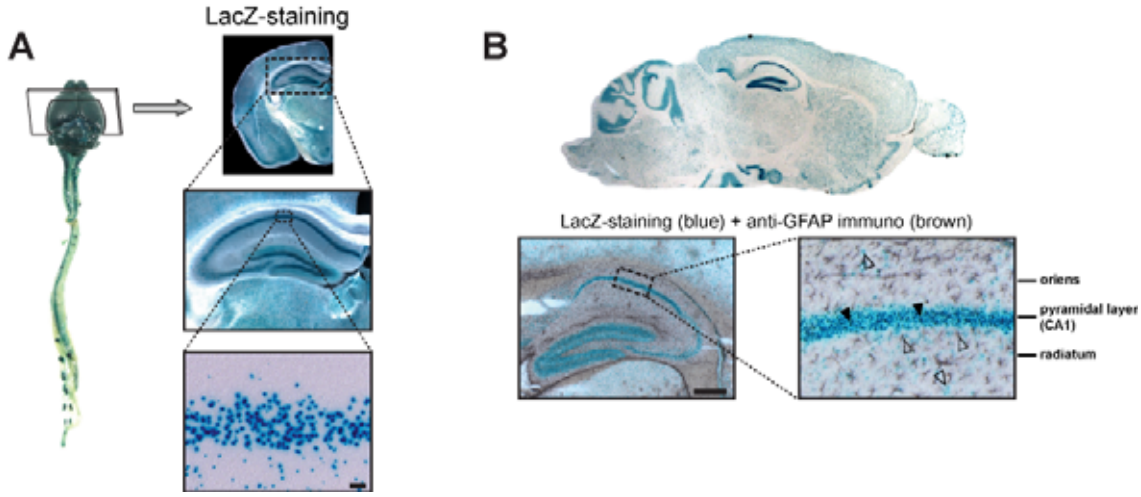


Figure 14: Ubc12 expression in a Ubc12-LacZ reporter mouse via LacZ staining.

A) LacZ staining on whole mount preparation of the brain and the spinal cord of a heterozygous Ubc12-LacZ reporter mouse as well as LacZ stainings on brain sections confirmed the ubiquitous and widespread expression of Ubc12 in the CNS of the adult mouse. **B)** LacZ staining (blue signal) combined with immunohistochemistry against the astrocyte-marker GFAP (brown DAB signal) highlighted the enrichement of Ubc12 expression in neurons. Filled black arrowheads show pyramidal neurons of CA1 in the hippocampus highly expressing Ubc12. Dashed black arrowheads show GFAP-positive astrocytes in the stratum oriens and stratum radiatum expressing Ubc12 at a lower level. Open black arrowheads show GFAP-negative cells in the stratum oriens and stratum radiatum expressing Ubc12. Scale bar in (**A**) represents 25 µm; scale bar in (**B**) represents 500 µm.

4.1.3. Subcellular localization of Ubc12 and Nedd8 in neurons

The identification of the subcellular localization of a particular protein is the first and mandatory step when searching for the unknown function of a specific candidate protein. To the best of our knowledge, the intracellular localization of Ubc12 and Nedd8 was never addressed systematically so far. To approach this question we first tested whether a set of different commercial antibodies raised against Nedd8 and Ubc12 might be suitable for immunohistochemical and immunocytochemical studies. Using tissue sections and cells either overexpressing or carrying shRNA constructs against Ubc12 and Nedd8 as positive and negative controls, respectively, we tested the sensitivity and specificity of selected antibodies under different immunohisto- and immunocytochemical conditions (different fixation methods, epitope unmasking protocols, permeabilization reagents and antibody incubation conditions). None of the analyzed antibodies proved to be good enough in terms of both sensitivity and specificity in immunohisto- or immunocytochemistry protocols. Thus, due to the lack of antibodies suitable to specifically detect Ubc12 and Nedd8 with immunofluorescence on a subcellular level in neurons, we cloned the mouse *Ubc12* and *Nedd8* cDNA in frame downstream to the eGFP sequence in the pEGFP-C1 vector (Clontech). We then transfected primary neurons with these constructs and analyzed the intracellular distribution of eGFP-Ubc12 and eGFP-Nedd8 fusion proteins at different developmental stages.

In young neurons (DIV 2-12) eGFP-Ubc12 and eGFP-Nedd8 were localized in the cytoplasm, minor neurites, dendrites as well as in the axon (Figure 15).

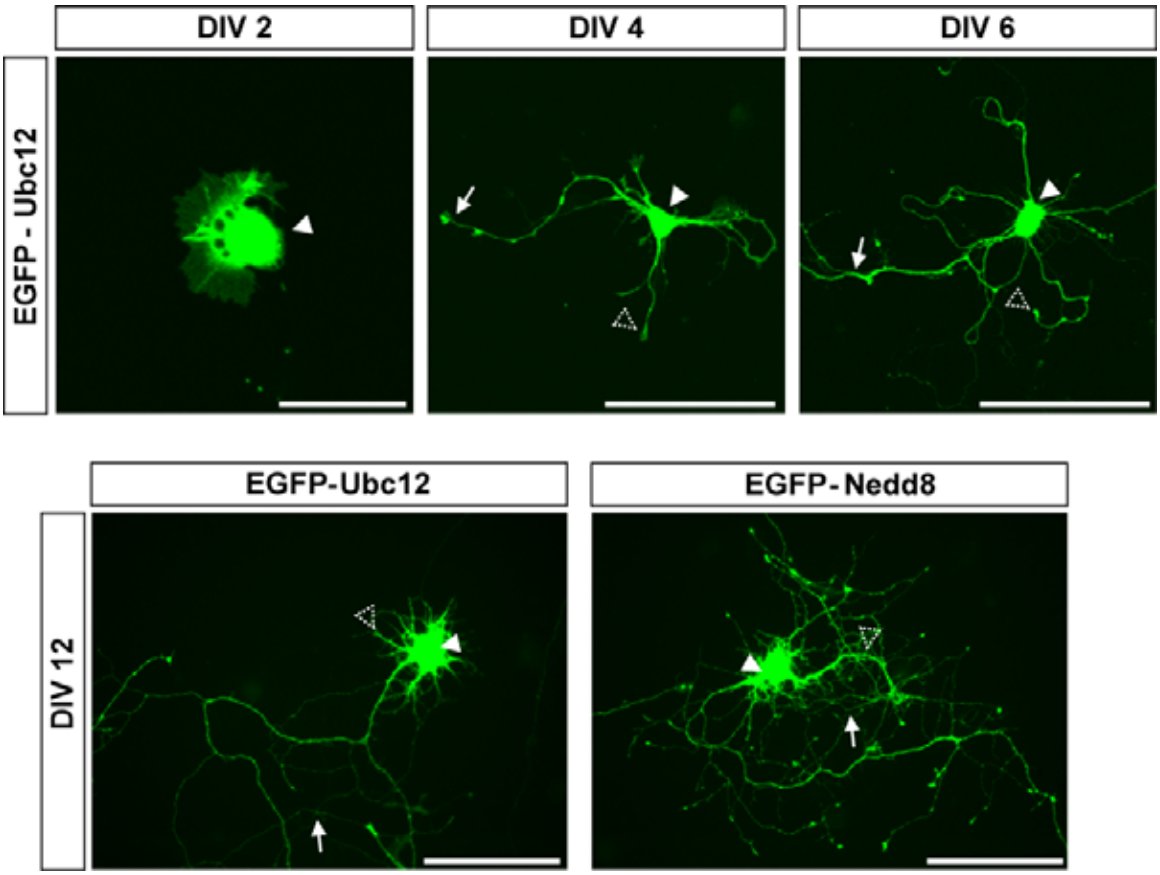


Figure 15: Intracellular localization of Ubc12 and Nedd8 in young neurons.

Primary hippocampal neurons were transfected with EGFP-Ubc12 and EGFP-Nedd8 constructs and fixed at the indicated DIVs. EGFP-Ubc12 and EGFP-Nedd8 signal was found in the soma of developing hippocampal neurons (marked by filled white arrowheads), localized in neurites at DIV 4 to 6 and later in dendrites at DIV 12 (marked by dashed white arrowheads). Furthermore, EGFP-Ubc12 and EGFP-Nedd8 were found in the axon at all time points studied (marked by white arrows). Scale bars represent 100 µm.

In mature neurons in culture (DIV 20), eGFP-Ubc12 and eGFP-Nedd8 still showed somatodendritic and axonal localization. Additionally, both are also found in dendritic protrusions such as filopodia and spines (Figure 16.A). To rule out that the 238 amino acids long eGFP tag disturbs the subcellular localization of Ubc12 or Nedd8, we generated FLAG-tagged Ubc12 and Nedd8 constructs. Immunofluorescence staining with anti-FLAG antibody revealed that FLAG-Ubc12 and FLAG-Nedd8 display identical intracellular localization as eGFP-tagged fusion proteins in neurons (Figure 16.B).

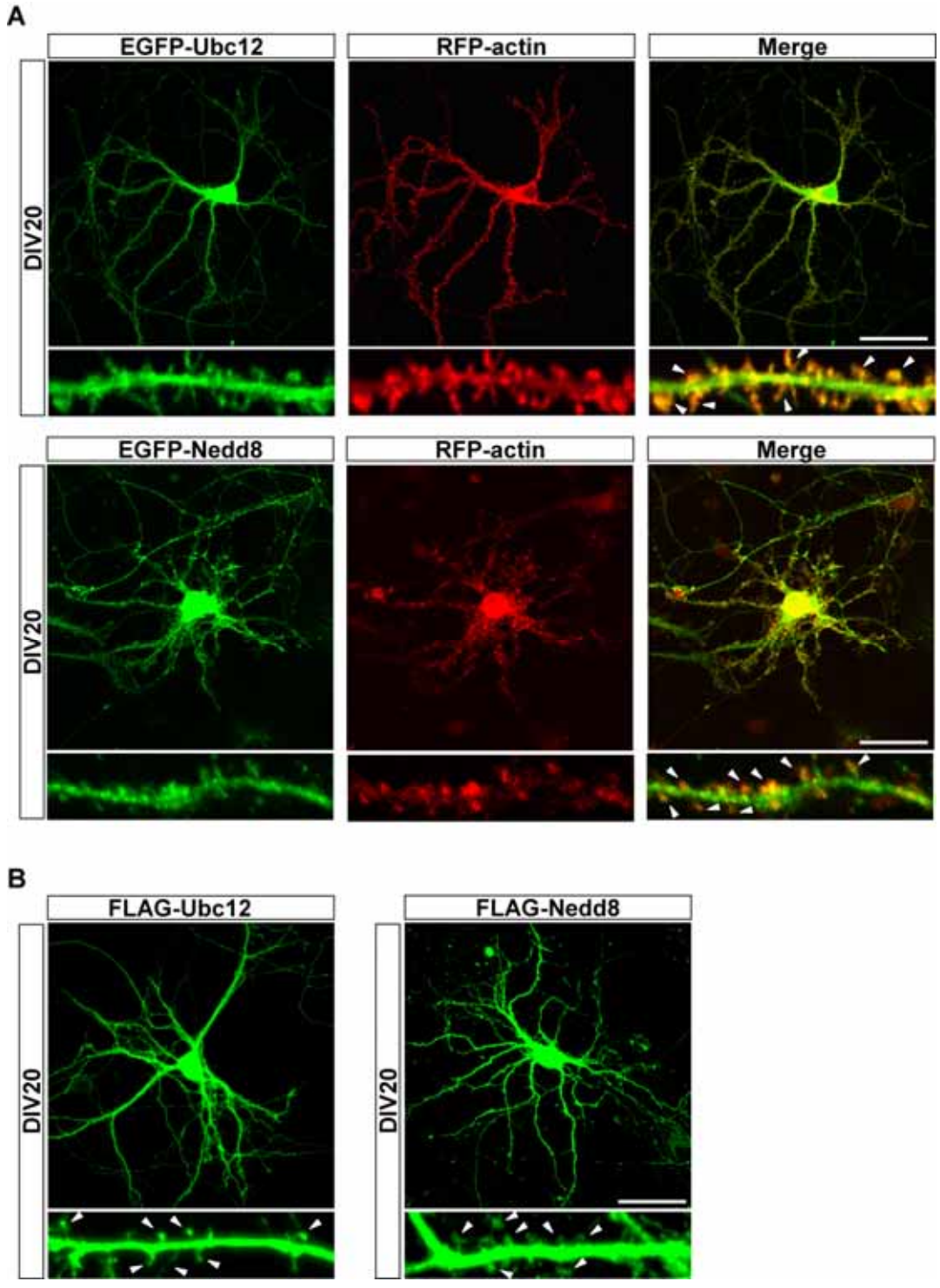


Figure 16: Subcellular localization of Ubc12 and Nedd8 in mature neurons.

Mature hippocampal neurons were transfected with (**A**) EGFP-Ubc12, EGFP-Nedd8 and (**B**) FLAG-Ubc12, FLAG-Nedd8 constructs at DIV 18 and analyzed at DIV 20. Ubc12 and Nedd8 showed somatodendritic and axonal localization and both were found in dendritic spines of mature neurons (highlighted by white arrowheads). Scale bars represent 50 µm.

4.1.4. Regulation of the Nedd8 pathway gene mRNA expression during neuronal development

To analyze the regulation of expression of the most important Nedd8 pathway genes, including *Appbp1, Uba3, Ubc12, Nedd8, Senp8, Cullin 1, 2, 3, 4a, 4b* and *5*, mRNA levels were measured by qRT-PCR during the development of primary rat hippcoampal neurons in culture, grown in the presence of Arabinofuranosyl Cytidine (Ara-C) to minimize the contribution of glial cells. Normalization to the house-keeping gene *Gapdh* revealed that all genes analyzed are constantly expressed throughout neuronal development (Figure 17).

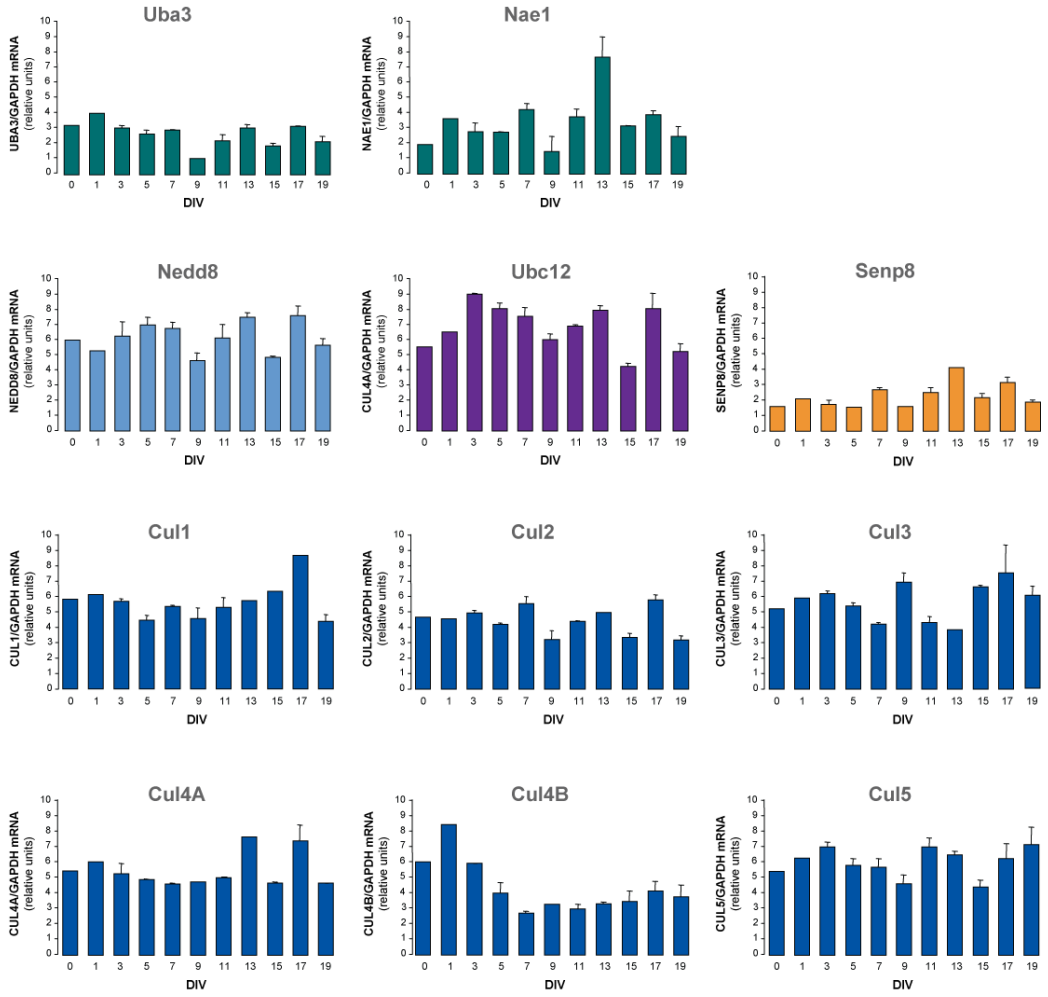


Figure 17: qRT-PCR analysis of the components of the neddylation pathway in developing neurons.

RNA was isolated of primary hippocampal neurons at the indicated DIVs and qRT-PCR for *Appbp1*, *Uba3*, *Ubc12*, *Nedd8*, *Senp8*, *Cullin 1*, *2*, *3*, *4a*, *4b* and *5* was performed. mRNA expression was normalized to the house-keeping gene *Gapdh*. All components of the neddylation pathway analyzed were continuously expressed during neuronal development.

4.1.5. Regulation of the Nedd8 pathway at the protein level during neuronal and brain development

To further investigate the regulation of expression of Nedd8 and Ubc12 and the abundance of neddylated target proteins during neuronal development, immunoblotting of primary rat cell culture samples and mouse brain lysates with anti-Nedd8 and anti-Ubc12 antibodies was performed. In primary hippocampal neurons, grown in the presence of Ara-C to suppress glial proliferation, Ubc12 and Ubc12-Nedd8 conjugates were detected throughout neuronal development from DIV 2 to 25 (Figure 18). Western blot with α-Nedd8 antibodies, from the company Epitomics as well as from Alexis Laboratories, revealed a smear of neddylated proteins ranging from 30 to 200 kDa, indicating that there are many additional Nedd8-targets in neurons apart from the cullin E3 ubiquitin ligases, the classical described Nedd8 targets, whose molecular weights range only from 98 to 110 kDa. During neuronal maturation we observed a peak in neddylation coinciding with dendritogenesis and synaptogenesis from DIV 7 to DIV 17. This increase was followed by a change in the pattern of differentially neddylated target proteins in mature neurons that established a stable synaptic network. In these experiments β-Actin served as the loading control, and the increasing levels of PSD-95, a scaffold protein in the PSD of spines, confirmed maturation and formation of synapses in the neuronal cell cultures.

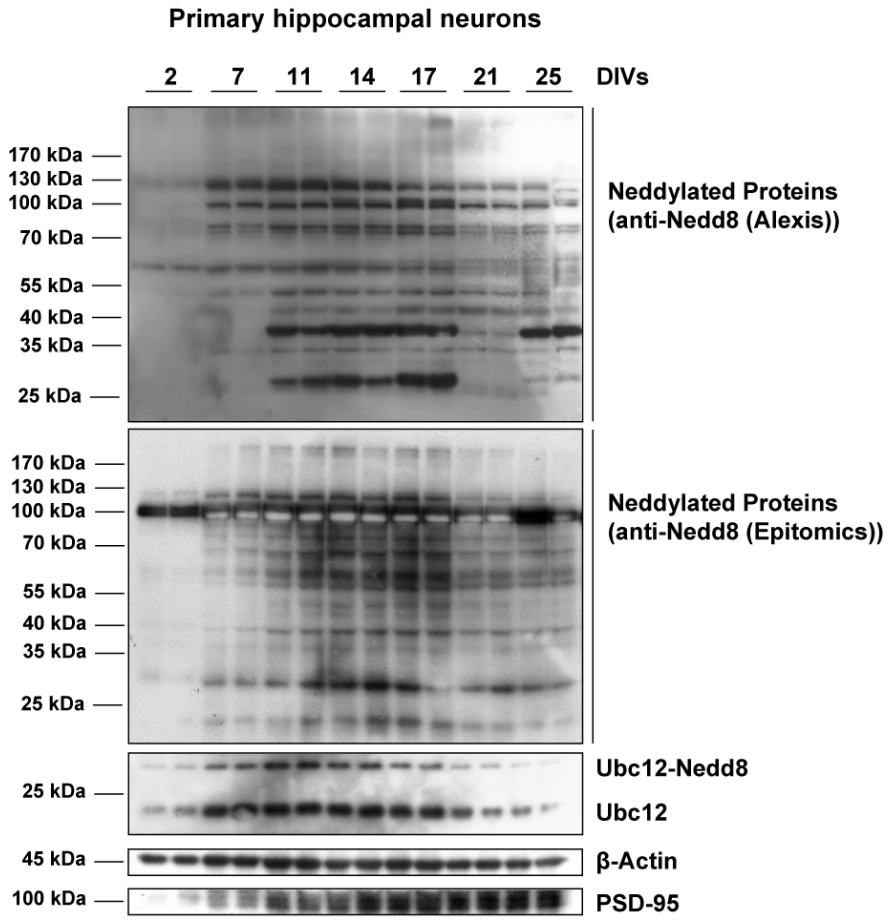


Figure 18: The Nedd8 pathway changes during the development of primary hippocampal neurons.

Protein extracts of primary hippocampal neurons at the indicated DIVs were subjected to immnublotting with α-Nedd8 and α-Ubc12 antibodies. β-Actin was used as a loading control and increasing PSD-95 levels served as a marker of neuronal maturation.

To validate the results from the cell culture experiments *in vivo* during the development of neurons in the central nervous system, we used immunoblotting of total brain extracts as well as cortical samples from mice at the indicated developmental stages (E12.5 to P35). α-Nedd8 Western blotting showed high abundance of neddylated target proteins during embryonic and postnatal development as well as in the adult brain samples (Figure 19). Interestingly, we observed changes in the pattern of Nedd8-modified targets. Especially during the first weeks of postnatal development (P6 to 21), the period which coincides with the time of synaptogenesis in the mouse/rodent brain, many proteins were found to be differentially conjugated to Nedd8 (Figure 19.A, B, highlighted

with red boxes). Ubc12 and Ubc12 conjugated to Nedd8 were present at all developmental time points studied in total brain extracts as well as in the cortical samples. In these experiments β-Actin served as the loading control and the increasing levels of the pre-synaptic protein Synaptophysin and PSD-95, a scaffold protein in the PSD of spines, confirmed maturation and formation of synapses in the developing brain.

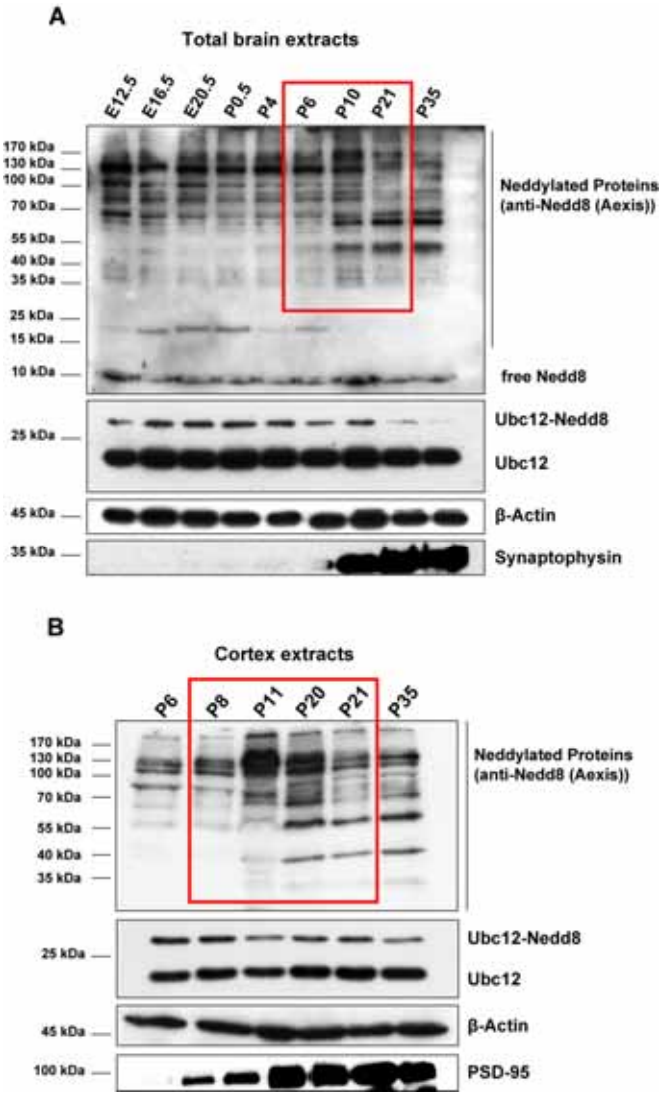


Figure 19: The Nedd8 pathway during brain development *in vivo*.

Protein extracts of (**A**) total mouse brain and (**B**) mouse cortical samples at the indicated developmental stages were subjected to immnublotting with α-Nedd8 and α-Ubc12 antibodies. β-Actin was used as a loading control and increasing Synaptophysin and PSD-95 levels served as markers of neuronal maturation. Red boxes highlight changes in neddylation patterns.

Altogether, these experiments demonstrate that the neddylation pathway is active in the CNS during development as well as in the adult mouse brain, and that Nedd8 is conjugated to many different, yet unidentified, proteins in the brain, apart from the classically described cullin proteins. Furthermore, our results show that Ubc12 and Nedd8 are enriched in neurons and that neddylation of target proteins is regulated during brain development.

Although Nedd8 has been previously reported to be developmentally downregulated (Kumar et al., 1993; Kamitani et al., 1997), we did not find any decrease in expression of the main members of the neddylation pathway, including Appbp1, Uba3, Ubc12, Nedd8, Senp8, Cullin 1, 2, 3, 4a, 4b and 5, in the central nervous system at any developmental stage or in the adult animal with any method applied (see also 4.8.2, Nae1 conditional KO mouse).

4.2. Validation of Nedd8 pathway inhibition via genetic and pharmacological tools

The neddylation enzyme cascade can be inhibited in a specific manner at different steps by either genetic or pharmacological tools that will be presented in the following section.

4.2.1. Dominant-negative Ubc12 – Ubc12-C111S

A dominant negative version of Ubc12, the E2 conjugating enzyme of the Nedd8 pathway, in which the amino acid residue cysteine 111 is mutated to serine, was kindly provided by Yosef Yarden (Wada et al., 2000; Oved et al., 2006). The dominant-negative Ubc12 (C111S) forms a stable heterodimeric complex with Nedd8. Hence, Nedd8 is sequestered by Ubc12-C111S and cannot be conjugated to target proteins anymore as shown in Figure 20 (Wada et al., 2000). Expression of Ubc12-C111S in the Neuro-2a hippocampal cell line resulted in a strong decrease of endogenously neddylated proteins (Figure 20.A) as well as in a strong reduction of the neddylation smear when co-expressed together with a 3xFLAG-Nedd8 construct (Figure 20.B).

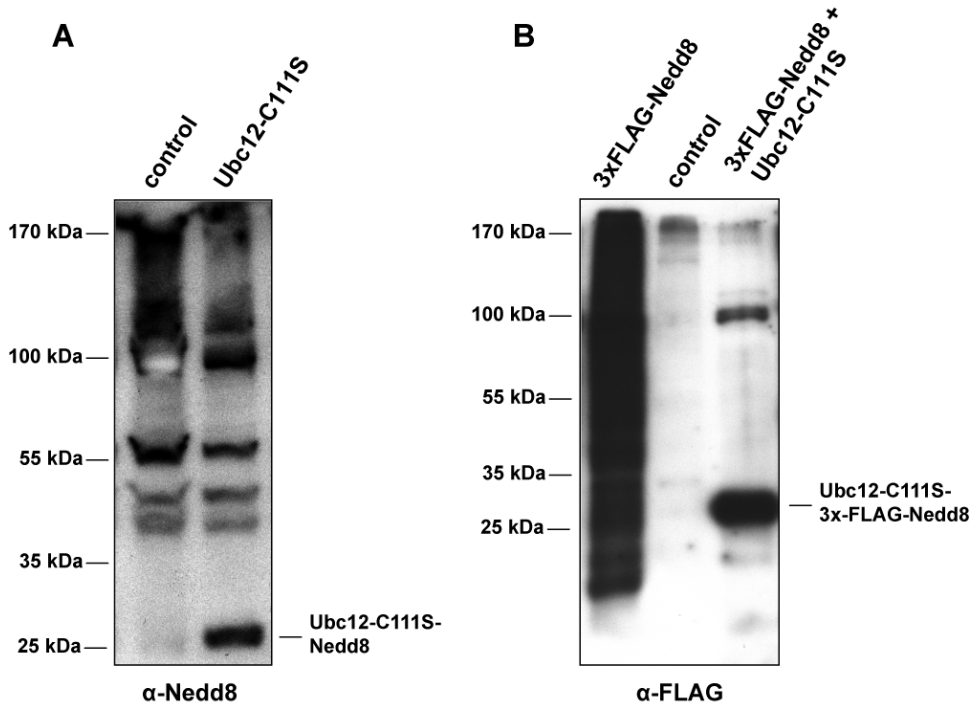


Figure 20: Ubc12-C111S inhibits neddylation.

Neuro-2a cells were transfected with the indicated plasmids for 36 h. Total cell extracts were subjected to immoblotting against (**A**) Nedd8 and (**B**) FLAG to analyze the abundance of neddylated target proteins. **A)** Ubc12-C111S proved effective in reducing endogeneous neddylation of targets in Neuro-2a cells evaluated by α-Nedd8 Western blot. **B)** Ubc12-C111S abolished neddylation in Neuro-2a cells co-transfected with a 3xFLAG-Nedd8 construct shown by α-FLAG Western blot.

4.2.2. shRNAs against Ubc12 and Nedd8

RNAi is a well established tool to knock-down the expression of specific genes *in vitro* and *in vivo*. Short hairpin RNA (shRNA) constructs targeting mouse Ubc12 and Nedd8 were cloned into the pSUPER vector (oligoengine), from which they are expressed under the control of the human polymerase III H1 promoter. For each gene, 4 to 5 different shRNA constructs were tested for their efficiency to downregulate the expression of the respective FLAG-tagged target gene transfected into Neuro-2a cells by Western blotting. A control shRNA sequence containing 5 basepair mismatches to any known human or mouse gene served as a control (Figure 21). The shRNA constructs with the highest efficacies were selected for further experiments – in the case of Ubc12 shRNA 2 and 3 and for Nedd8 shRNA 2 and 4.

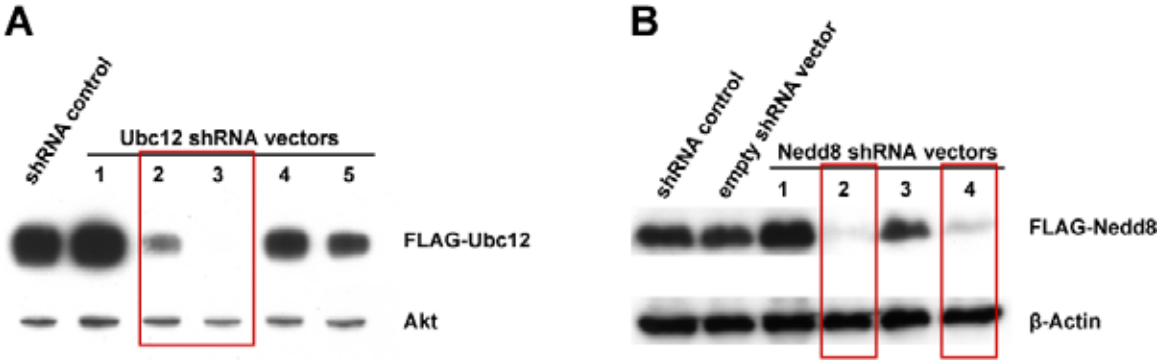


Figure 21: Validation of shRNA constructs targeting Ubc12 and Nedd8.

Neuro-2a cells were transfected with the indicated plasmids for 72 h. The efficiency of shRNA constructs to downregulate (**A**) Flag-Ubc12 and (**B**) FLAG-Nedd8 was assessed by Western blotting with α-FLAG antibodies. shRNA vectors 2 and 3 targeting the mouse Ubc12 sequence and shRNA vectors 2 and 4 targeting the mouse Nedd8 sequence displayed highest knock-down efficiencies. Akt or β-Actin served as loading controls.

4.2.3. Nedd8-activating enzyme (NAE)-inhibitor MLN4924

Recently, MLN4924 a selective small molecule inhibitor of the Nedd8-activating enzyme (NAE) was developed by Millenium Pharmaceuticals (Soucy et al., 2009). This compound showed anti-tumor activity in preclinical models of several tumor types and is currently in Phase I clinical trials as an anti cancer drug against solid and non-solid tumors.

For the current project, MLN4924 was either purchased at Active Biochem (A-1139) or kindly provided by Lak Shin Jeong from the Department of Bioinspired Science and Laboratory of Medicinal Chemistry, College of Pharmacy at the Ewha Womans University in Seoul, South Korea (Lee et al., 2011). The efficiency of both compounds to inhibit the neddylation pathway was tested in Neuro-2a cells by immunoblotting. Neuro-2a cells were treated with 1, 0.5, 0.1, 0.01 µM MLN4924 either from Active Biochem or synthesized by Lak Shin Jeong's Lab for 24 h under differentiation conditions (serum withdrawal). Immunoblots against Nedd8 and Cullin 1 were performed with total cell extracts. Both NAE-inhibitors inhibited neddylation to a similar degree in a dose-dependent manner shown for neddylated targets and for neddylated Cullin 1 (Figure 22).

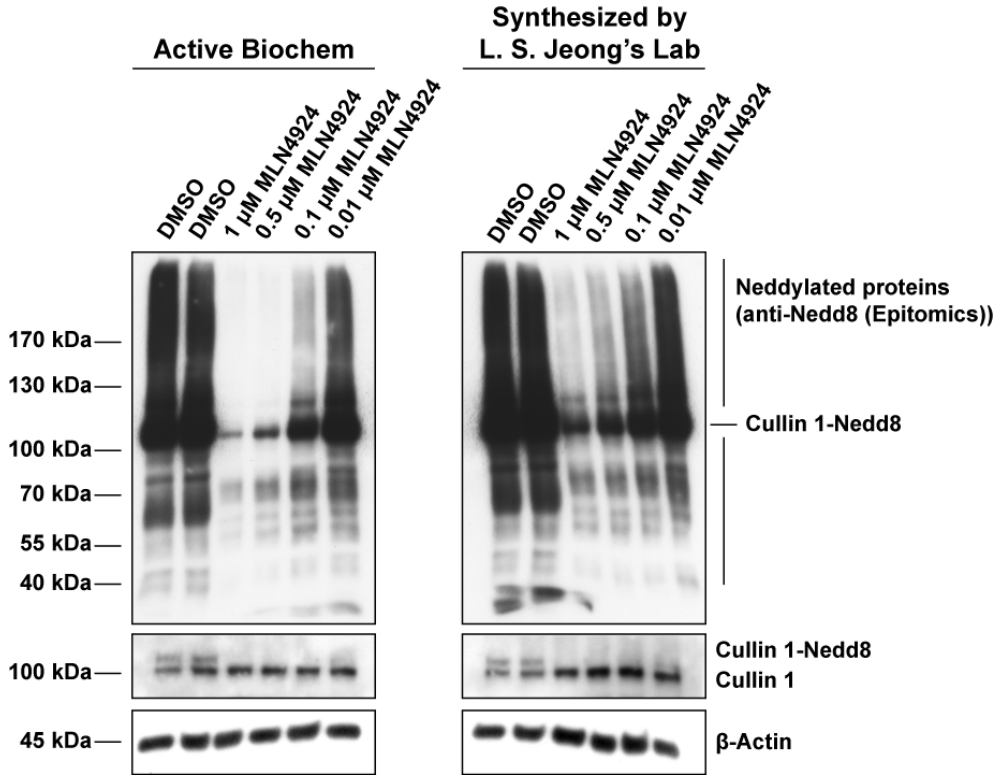


Figure 22: Comparison of NAE-inhibitors MLN4924.

Neuro-2a cells were treated with vehicle (DMSO) or 0.01, 0.1, 0.5 and 1 μM MLN4924 (Active Biochem) and MLN4924 (from Jeong's Lab) for 24h under differentiation conditions (serum-withdrawal). Total cell extracts were subjected to immunoblotting against Cullin 1 and Nedd8. MLN4924 abolished neddylation of cullins and efficiently reduced neddylation of target proteins. β-Actin was used as a loading control.

Furthermore, treatment with MLN4924 for 2 to 48 h efficiently inhibited neddylation in primary cortical and hippocampal neurons. In developing cortical (DIV 3) and hippocampal (DIV 14) neurons, treatment with 0.1 and 1 μM MLN4924 for 24 h abolished neddylation evaluated by Western blotting using antibodies against Cullin 1 and Nedd8 (Figure 23). In mature cortical neurons at DIV 22, treatment with 1 μM MLN4924 for 24 h also strongly reduced neddylation of target proteins, as shown by the Western blot against Cullin 1 and Nedd8 (Figure 24).

Of note, the pharmacological inhibitor of neddylation, MLN4924 was recently developed and published by Millenium Pharmaceuticals (Soucy et al., 2009). Unfortunately, this company refused to share the drug for any other experiments than studies focusing on cancer. Due to these unexpected difficulties and the fact that the synthesis is very complex and involves more than 10 synthesis steps, the inhibitor was available to us only during the last 7 months of this project. This accounts for the fact that some experiments performed do not include the pharmacological inhibitor but were performed exclusively with genetic tools.

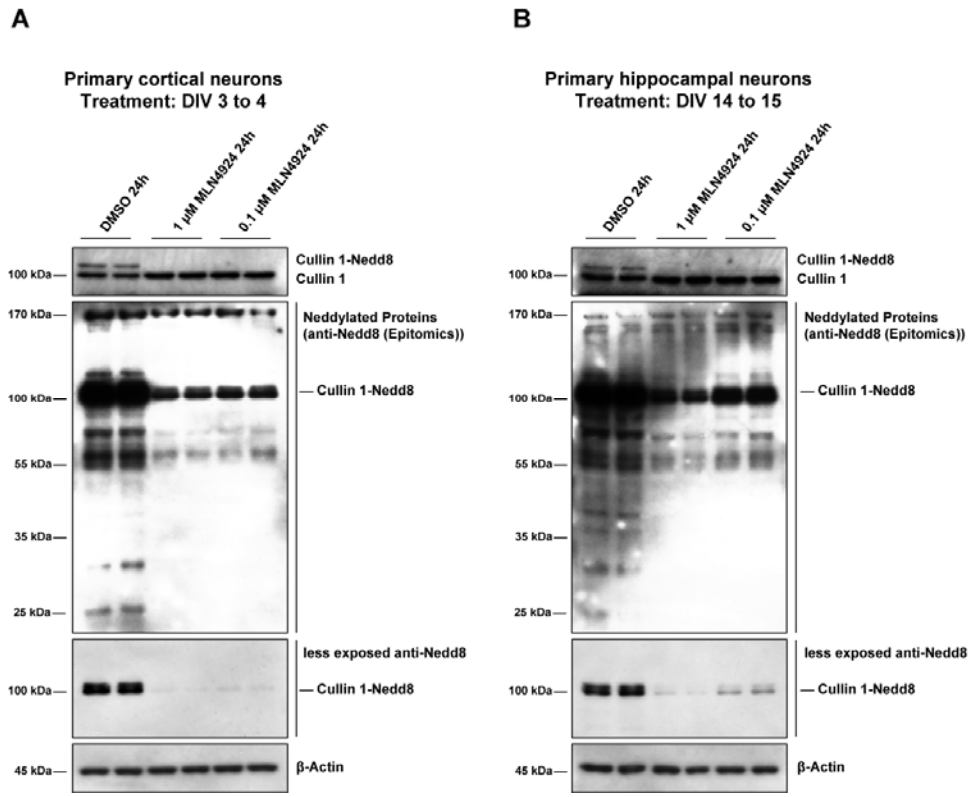


Figure 23: NAE-inhibitor MLN4924 inhibits neddylation in developing primary cortical and hippocampal neurons.

Rat cortical and hippocampal neurons were treated with vehicle (DMSO), 0.1 or 1 µM MLN4924 (Active Biochem) at DIV 3 or DIV 14 for 24 h. Total cell extracts were subjected to immunoblotting against Cullin 1, Nedd8, and β-Actin. MLN4924 abolished neddylation of cullins and efficiently reduced neddylation of target proteins, evaluated via α-Nedd8 Western blot. β-Actin was used as a loading control.

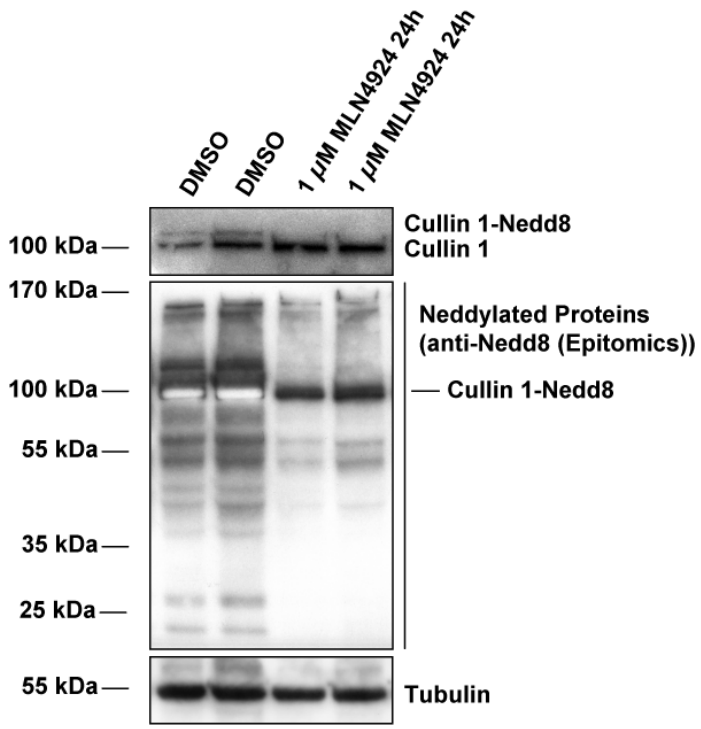


Figure 24: NAE-inhibitor MLN4924 inhibits neddylation in mature primary cortical neurons.

Rat cortical neurons were treated with vehicle (DMSO) or 1 µM MLN4924 (Active Biochem) at DIV 22 for 24 h. Total cell extracts were subjected to immunoblotting against Cullin 1, Nedd8, and Tubulin. MLN4924 abolished neddylation of cullins and efficiently reduced neddylation of target proteins, evaluated via α-Nedd8 Western blot. Tubulin was used as a loading control.

4.2.4. Dominant-negative Cullins, dominant-negative Rbx1, and shRNAs against Rbx1

To determine the contribution of cullin proteins – the best described Nedd8 targets – to the results observed in neurons, we used different approaches to inhibit neddylation of cullins. Conjugation of Nedd8 to a conserved lysine residue on the cullin scaffold is essential for the activity of CRLs. Therefore, we used dominant negative cullins, which lack the C-terminal region containing the conserved lysine residue, developed by the lab of Wade Harper (Shirogane et al., 2005). These mutant cullins sequester adaptors and substrate receptors but have no ubiquitin ligase activity, thus acting as dominant negatives. Another target to inhibit cullin 1 through 4 at the same time is the ring protein

Rbx1, which is necessary for the activity of every cullin. We used shRNA constructs to downregulate Rbx1 expression as well as the dominant-negative Rbx1 versions, Rbx1-C42S-C45S and Rbx1-C75S, that carry mutations in Zinc-binding cysteine residues of Rbx1 and therefore lack RING ligase activity, kindly provided by the lab of J. W. Conaway (Kamura et al., 1999a; Kamura et al., 1999b).

In case of Rbx1, different shRNA constructs directed against mouse Rbx1, purchased from Sigma Aldrich, expressed under the control of the human U6 promoter in the pLKO.1-puro vector, were analyzed for their efficiency to down-regulate the expression of FLAG-tagged Rbx1 expressed in Neuro-2a cells by Western blot (Figure 25). Rbx1 shRNA 3 and 4 displayed the highest knock-down efficiency and were therefore used in all subsequent experiments.

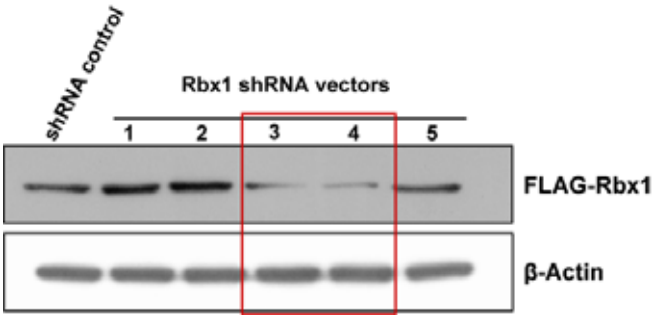


Figure 25: Validation of Rbx1 shRNAs.

Neuro-2a cells were transfected with the indicated plasmids for 72 h. Efficiency of shRNA constructs to downregulate Flag-Rbx1 was assessed by Western blotting with α-FLAG antibodies. shRNA vectors 3 and 4 targeting the mouse Rbx1 sequence displayed highest knock-down efficiencies. β-Actin served as a loading control.

Of note, recently it has been described by Huang *et al.* (Huang et al., 2009) that Ube2F, another E2 conjugating enzyme of Nedd8, displays a different target cullin specificity than Ubc12, which results in selective neddylation of cullin 5/Rbx2. We performed additional experiments in which we knocked down the expression of Ube2f via shRNA constructs. These experiments indicated that Ube2F and therefore its target cullin 5/Rbx2 is not involved in the effects we observe when we block neddylation in neurons and will not be further shown.

4.3. The Nedd8 pathway controls axonal development

To study the function of neddylation in the first steps of neuronal development we characterized its role during neuronal polarization, neurite initiation and axonal growth *in vitro* using dissociated primary hippocampal and cortical neurons, and *in vivo* using *in utero* intraventricular injection and electroporation of cortical and hippocampal neurons in the mouse embryo.

4.3.1. Neddylation controls polarization and axonal growth in primary neurons

In order to study the function of neddylation in neuronal polarization and axonal growth, the neddylation cascade has to be blocked quickly and efficiently directly after plating of neurons, since these processes take place within the first 72 hours after plating of primary neurons. To achieve this goal we inhibited the Nedd8 pathway via expression of the dominant-negative Ubc12-C111S using the Nucleofection System (Amaxa) in primary neurons at the time of plating. Mixing control neurons expressing RFP with neurons expressing an Ubc12-C111S-IRES-GFP construct in a 1:1 ratio, allowed us to simultaneously follow and record their development via confocal time lapse imaging. As shown in Figure 26.A, repeated imaging revealed that control neurons, in red, start to form neurites and polarize within 72 h after plating. 48 to 72 h after plating, one of the former neurites starts to grow out faster than all other neurites to become the future axon (marked by white arrows in Figure 26.A). In contrast, inhibition of neddylation in hippocampal neurons led to a severe defect in polarization. Neurons expressing Ubc12-C111S, in green, fail to develop neurites and are impaired in axon formation (marked by white arrowhead in Figure 26.A).

During neuronal development in cell culture systems, neuritogenesis and axon formation follow a well defined set of stages and accordingly neurons can be classified as stage 1, fully surrounded by lammellipodia and filopodia, stage 2, several minor neurites and stage 3, one defined axon and several shorter neurites (Dotti et al., 1988; Delima et al., 1997; Bradke and Dotti, 2000b; Barnes and Polleux, 2009; Polleux and Snider, 2010) (Figure 1). Quantification of stages 1 to 3 at DIV 1 (24 h after plating) revealed that in the

Ubc12-C111S group a larger proportion of neurons (94.4%) was still in stage 1 compared to control neurons (73.3%). At DIV 3 (72 h after plating) the majority of control neurons (80.6%) already progressed into stage 3 neurons extending an axon, whereas the majority of neurons expressing Ubc12-C111S (69.1%) was arrested in stage 1 and only 17.3% advanced to stage 3 (Figure 26.B).

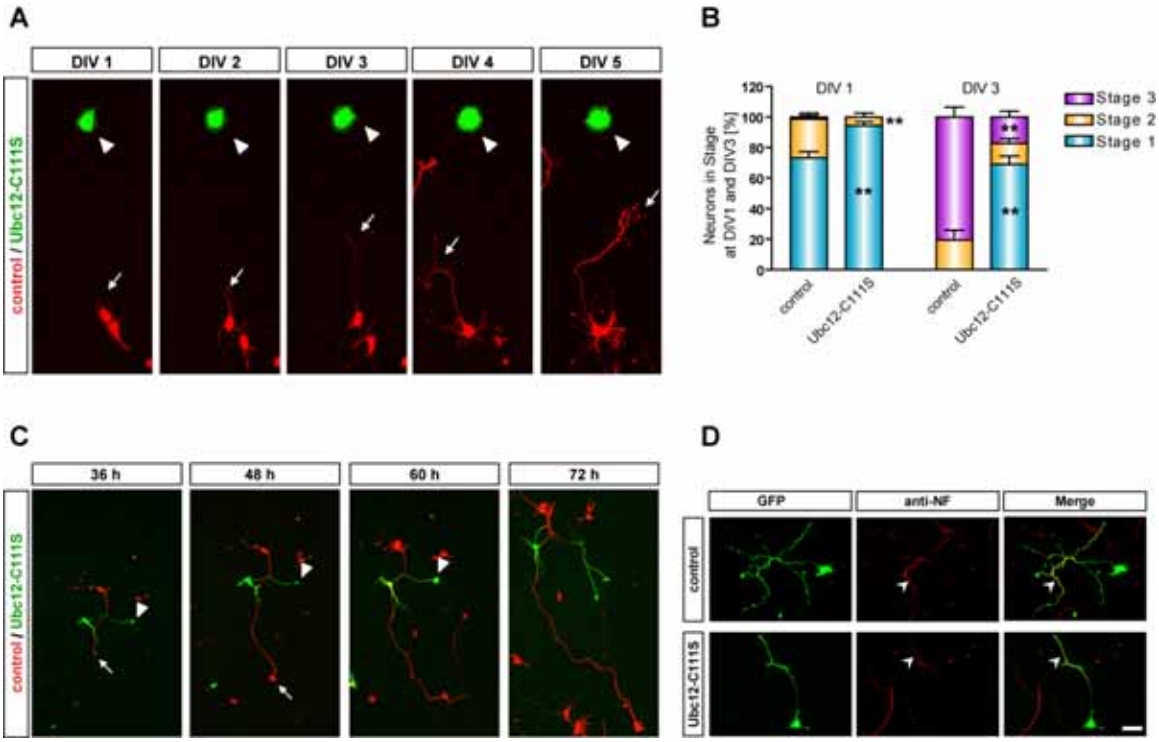


Figure 26: Neddylation controls polarization and axon development in primary neurons.

A) and **C)** Time lapse imaging of young (**A**) hippocampal and (**C**) cortical neurons transfected with control (red) and Ubc12-C111S (green) plasmids via nucleofection at the time of plating. **A)** Hippocampal neurons expressing Ubc12-C111S failed to polarize and to develop minor neurites and an axon. **B)** Hippocampal neurons transfected with control or Ubc12-C111S plasmids were classified into stages 1 to 3 at DIV 1 and 3. **C)** Cortical neurons expressing Ubc12-C111S showed strongly reduced axon growth. **D)** Immunocytochemistry with α-Neurofilament antibody (α-NF, SMI-312) confirmed axonal identity of processes of cortical neurons expressing Ubc12-C111S. Scale bar represents 50 µm. [Statistical analysis: DIV 1, 2 Way ANOVA, treatment x stage interaction, $F_{(2,78)}$=133.9, ***p<0.0001; Bonferroni post-hoc test, **p<0.01; DIV 3, 2 Way ANOVA, treatment x stage interaction, $F_{(2,137)}$=102.3, ***p<0.0001; Bonferroni post-hoc test, **p<0.01.]

Surprisingly, despite these severe impairments, neurons expressing Ubc12-C111S do not undergo apoptosis, instead they survive as stage 1 neurons for more than 5 DIVs.

A similar time lapse imaging experiment showed that in contrast to hippocampal neurons, cortical neurons expressing Ubc12-C111S were able to form neurites and to develop an axon, but axonal growth was impaired (Figure 26.C and Figure 27.A). Positive α-Neurofilament staining confirmed the axonal identity of the longest process of neurons expressing Ubc12-C111S (Figure 26.D). Analysis of axonal growth and branching via tracing measurements revealed a strong decrease in total axonal length and a reduced branching of the axon of cortical neurons expressing Ubc12-C111S from DIV 0 to 4 compared to control neurons (Figure 27.B, C).

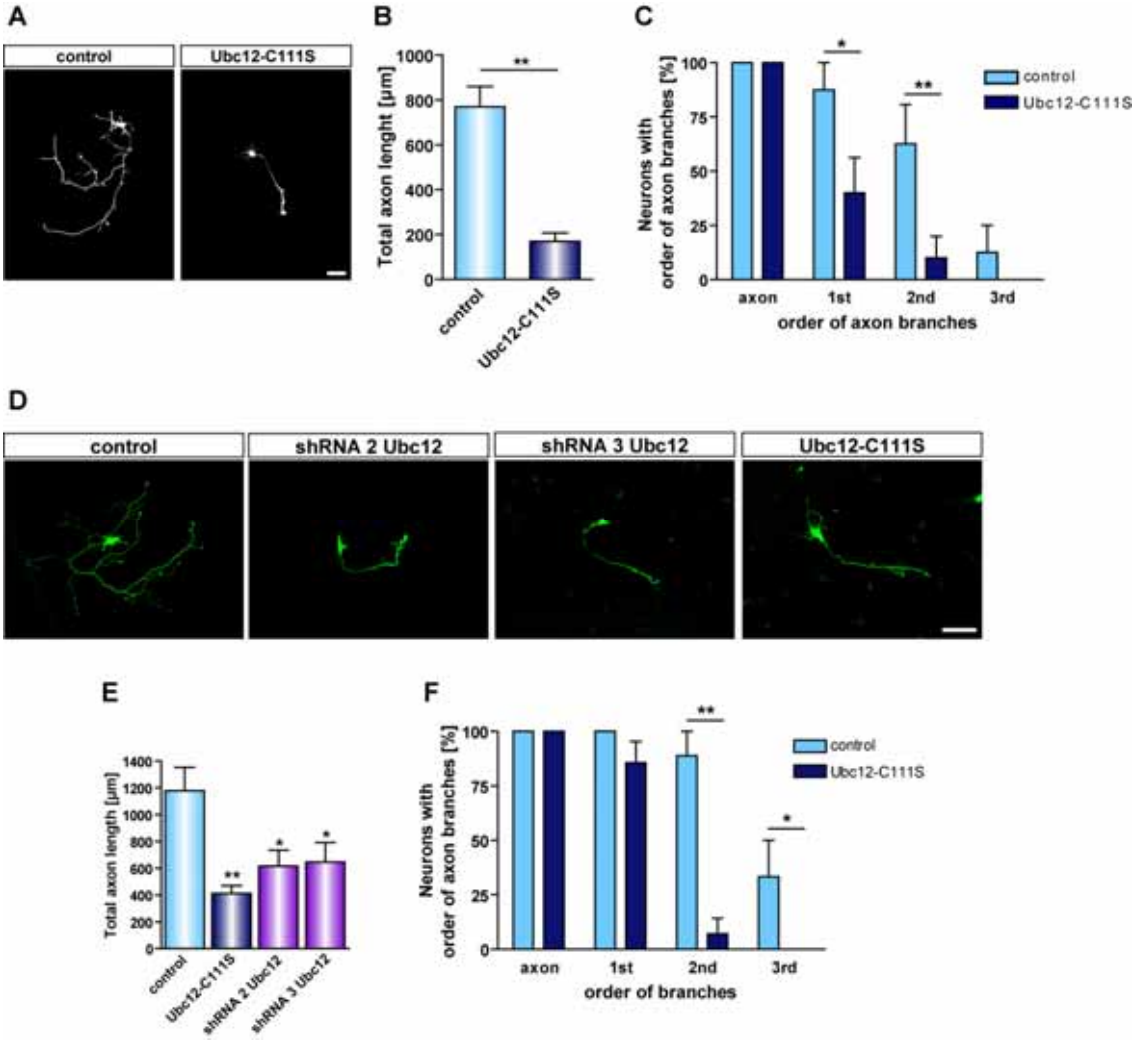


Figure 27: Neddylation controls axon growth in primary neurons.

A) Cortical neurons were transfected with control and Ubc12-C111S plasmids via nucleofection at the time of plating and fixed at DIV 4. **B)** Total axon length was reduced in cortical neurons expressing Ubc12-C111S. **C)** Axon branching was reduced in cortical neurons expressing Ubc12-

C111S. **D)** Hippocampal neurons transfected with shRNA constructs against Ubc12 and Ubc12-C111S from DIV 2 to 5 showed impairments in axon growth compared to control transfected neurons, shown in **E)** for total axon length and in **F)** for axon branching. Scale bars represent 50 µm. [Statistical analysis: (**C**) 2 Way ANOVA, treatment x branch order interaction, $F_{(3,64)}$=2.7, ***p<0.0001; Bonferroni post-hoc test, *p<0.05, **p<0.01; (**F**) 2 Way ANOVA, treatment x branch order interaction, $F_{(3,84)}$=10.6, ***p<0.0001; Bonferroni post-hoc test, *p<0.05, **p<0.01; (**B**) and (**E**) t-test, *p<0.05, **p<0.01.]

When neddylation was blocked in hippocampal neurons from DIV 2 to 5 using calcium-phosphate transfection of shRNAs against Ubc12 as well as Ubc12-C111S, a similar decrease in axonal growth and branching was observed as in cortical neurons (Figure 27.D). Tracing measurements confirmed a decrease in total axon length as well as in percentage of neurons having secondary and tertiary axonal branches (Figure 27.E, F).

4.3.2. Are the effects of Nedd8 on axonal growth dependent or independent of cullin-RING ligases?

To test experimentally if the cullins (Cul1, -2, -3, -4, or -5), the known targets of Nedd8, are involved in mediating the effects of neddylation on axonal growth, we used different approaches to interfere with the function of cullin RING ligases. When we transfected hippocampal neurons via calcium-phosphate transfection from DIV 2 to 5 (Figure 28.A) and cortical neurons using the nucleofection technique from DIV 0 to 4 (Figure 28.B) with dominant-negative versions of Cul1, -2, -3, -4A, -4B or -5, containing C-terminal deletions including the conserved neddylation site, none of them reproduced the impairment in axonal growth observed with Ubc12-C111S.

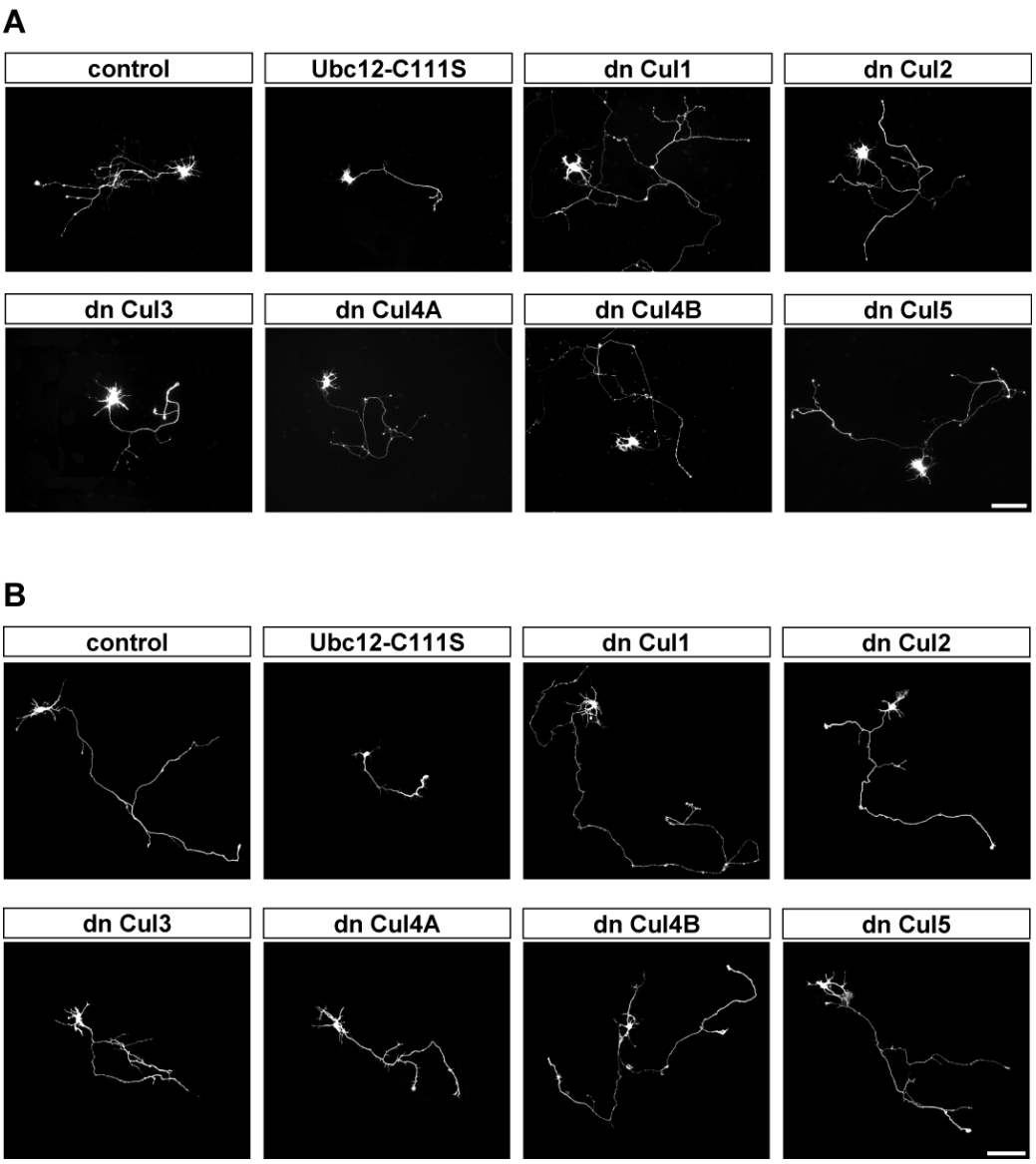


Figure 28: Nedd8 controls axon development independent of Cullins in primary neurons.

A) Primary hippocampal neurons were transfected from DIV 2 to 5 and **B)** primary cortical neurons were transfected from DIV 0 to 4 with control plasmids, Ubc12-C111S or dominant-negative (dn) Cullin 1, 2, 3, 4A, 4B, and 5. Scale bars represent 50 µm.

The same result was obtained when we performed co-transfection experiments in hippocampal neurons of all dominant-negative cullin constructs, in order to inhibit the function of all cullins at the same time (Figure 29.A).

Interestingly, expression of individual dominant-negative cullins showed different and opposite effects on axonal growth. Notably, expression of the dominant-negative Cul1 resulted in neurons with longer and more branched axons compared to control neurons,

whereas neurons expressing dominant-negative Cul3 and Cul4A developed slightly shorter axons compared to control neurons. Since we were interested in unraveling the mechanism by which neddylation impairs axon development and growth in neurons we did not investigate this in more detail.

Furthermore, inhibiting the function of cullin RING ligases (Cul1 through Cul4) via shRNA-mediated knockdown of the RING protein Rbx1 in cortical neurons did not result in defects in axonal growth as observed with shRNA constructs targeted against Ubc12 and Nedd8 (Figure 29.B).

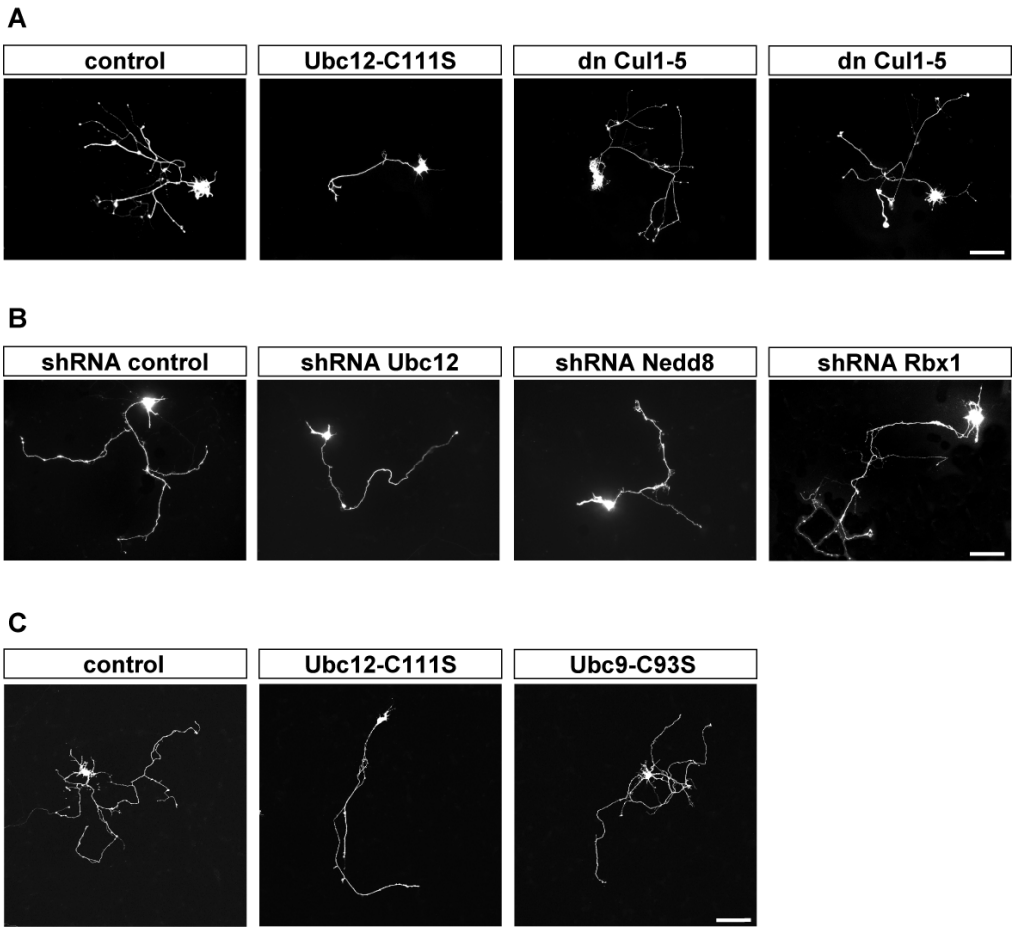


Figure 29: Nedd8 effects on axon development are independent of Cullins and specific for the Nedd8 pathway.

A) Primary hippocampal neurons were transfected from DIV 2 to 5 with control plasmids, Ubc12-C111S and all dominant-negative (dn) Cullins (1, 2, 3, 4A, 4B, and 5). **B)** Cortical neurons were transfected from DIV 2 to 5 with control shRNA or shRNAs against Ubc12, Nedd8 and Rbx1. **C)** Cortical neurons were transfected from DIV 2 to 5 with control, Ubc12-C111S or Ubc9-C93S. Scale bars represent 50 µm.

Inhibition of the sumoylation pathway, another ubiquitin-like pathway, via transfection of a dominant-negative version of the E2 enzyme of SUMO, Ubc9-C93S, did not result in any obvious impairment of axon development in cortical neurons from DIV 2 to 5 (Figure 29.C), further underlining the specific and unique role of neddylation during early neuronal development.

Taken together these experiments led to the conclusion that neddylation controls axonal growth in primary neurons independently of cullin RING ligases. Therefore, we started searching for new targets of Nedd8, other than the cullins, which could be involved in the observed axon phenotype.

4.3.3. Regulation of the cytoskeleton by the Nedd8 pathway in young neurons

Developing neurons possess a complex and highly dynamic cytoskeleton to accomplish the establishment of neuronal polarity. Initiation of neurites and formation of dendrites and axons underlies a coordinated and tightly regulated rearrangement of the cytoskeleton.
To study the mechanisms possibly underlying the impairment in axon formation, we analyzed the consequences of neddylation blockade on the microtubular and actin cytoskeleton by immunofluorescence stainings. Dynamic and stable microtubules can be discriminated using antibodies that detect different post-translational modifications of α-Tubulin; α-Tyrosinated-Tubulin to stain dynamic microtubules and α-Acetylated-Tubulin to stain stable microtubules after PFA-fixation and simultaneous extraction of unpolymerized tubulin monomers with Triton X-100. Filamentous actin (F-actin) can be visualized via binding of fluorescently-labeled Phalloidin.

Hippocampal neurons in stage 1, in which neddylation was blocked by expression of the dominant-negative Ubc12-C111S exhibited severe distortions of their microtubular and actin cytoskeleton at 24 and 48 h after plating (Figure 30.A, B). In control neurons, numerous long and thin dynamic microtubules extend from the center and invade the

discontinous F-actin patches on the cell periphery (highlighted by white arrows in Figure 30.A, B). In contrast, in neurons expressing Ubc12-C111S the extension of dynamic microtubules towards the actin cytoskeleton at the periphery is abolished. Immunostaining for Tyrosinated-Tubulin is concentrated in a ring-like condensed structure around the center of the neuron (highlighted by white arrowheads in Figure 30.A, B). Furthermore suppression of neddylation results in a higher degree of continous F-actin accumulated at the periphery of the neuron (Figure 30.A, B). Accordingly to the observed alterations in the cytoskeleton induced by inhibition of the Nedd8 pathway by expressing Ubc12-C111S, treatment with Taxol at low concentration (10 nM Taxol), which promotes microtubule stabilization, and treatment with 1 µM Cytochalasin D, which disrupts actin filaments (Witte et al., 2008), proved effective in rescuing the morphological impairments in axon growth induced by Ubc12-C111S in hippocampal and cortical neurons (DIV 0 to 4) (Figure 30.C, D).

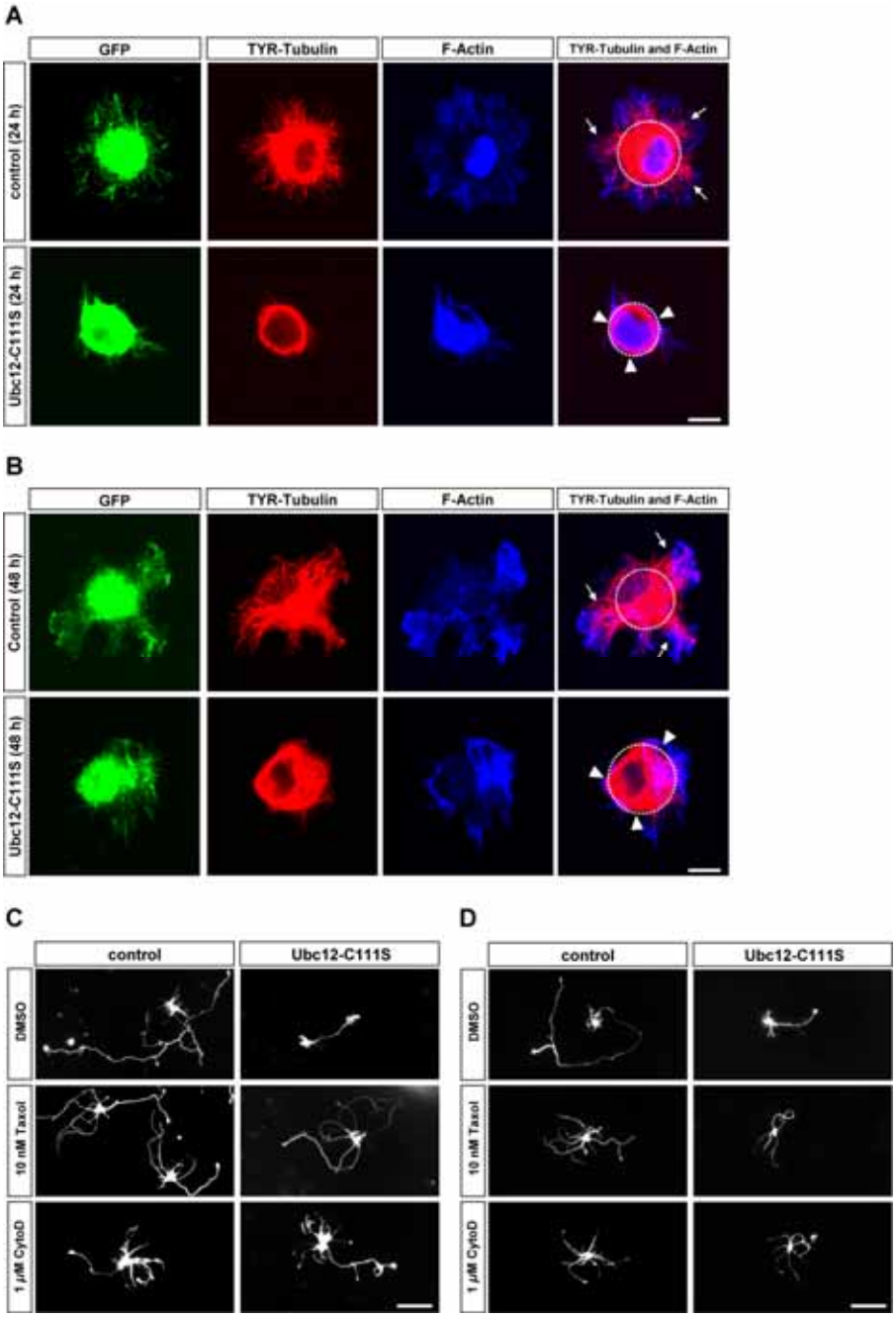


Figure 30: Regulation of the cytoskeleton by the Nedd8 pathway.

Hippocampal neurons transfected with control or Ubc12-C111S plasmids were fixed **A)** 24 h after plating and **B)** 48 h after plating and the microtubular and actin cytoskeleton was stained via immunofluorescence. Dynamic microtubules (in red) were stained with α-Tyrosinated-Tubulin antibody and F-Actin (in blue) was stained using fluorescent Phalloidin. **C)** Hippocampal and **D)** cortical neurons were transfected with control or Ubc12-C111S plasmids from DIV 0 to 4. Treatment with 10 nM Taxol and 1 µM Cytochalasin D induced formation of multiple axons and thereby rescued the defects in axon growth produced by Ubc12-C111S. Scale bars in (**A**) and (**B**) represent 10 µm; scale bars in (**C**) and (**D**) represent 50 µm.

Primary hippocampal neurons treated with the pharmacological NAE-inhibitor MLN4924 for 48 h after plating showed a dose-dependent polarization defect and impairment in axonal growth, resembling the effects observed with the genetic tools (Ubc12-C111S and shRNAs against Ubc12 and Nedd8).

Treatment with high concentrations (1 μM) of MLN4924 lead to a failure in polarization (Figure 31.A). Analysis of stages 1 to 3 revealed that 69.3% and 28.8% of neurons treated with 1μM MLN4924 for 48 h after plating were arrested in stage 1 and 2, respectively, whereas the majority of vehicle-treated neurons developed into polarized stage 3 neurons possessing an axon (69.2%) (Figure 31.B). Low (0.1 μM) and moderate (0.5 μM) concentrations of MLN4924 displayed dose-dependent alleviated defects in neuronal polarization, and severely affected axonal growth (Figure 31.A and Figure 32.C). 49.7% of neurons treated with 0.1 μM MLN4924 and 29.6% of neurons treated with 0.5 μM MLN4924 for 48 h after plating developed morphologically into stage 3 neurons (Figure 31.B and Figure 32.C), but total axonal length was strongly decreased compared to vehicle-treated neurons (Figure 31.C and Figure 32.B).

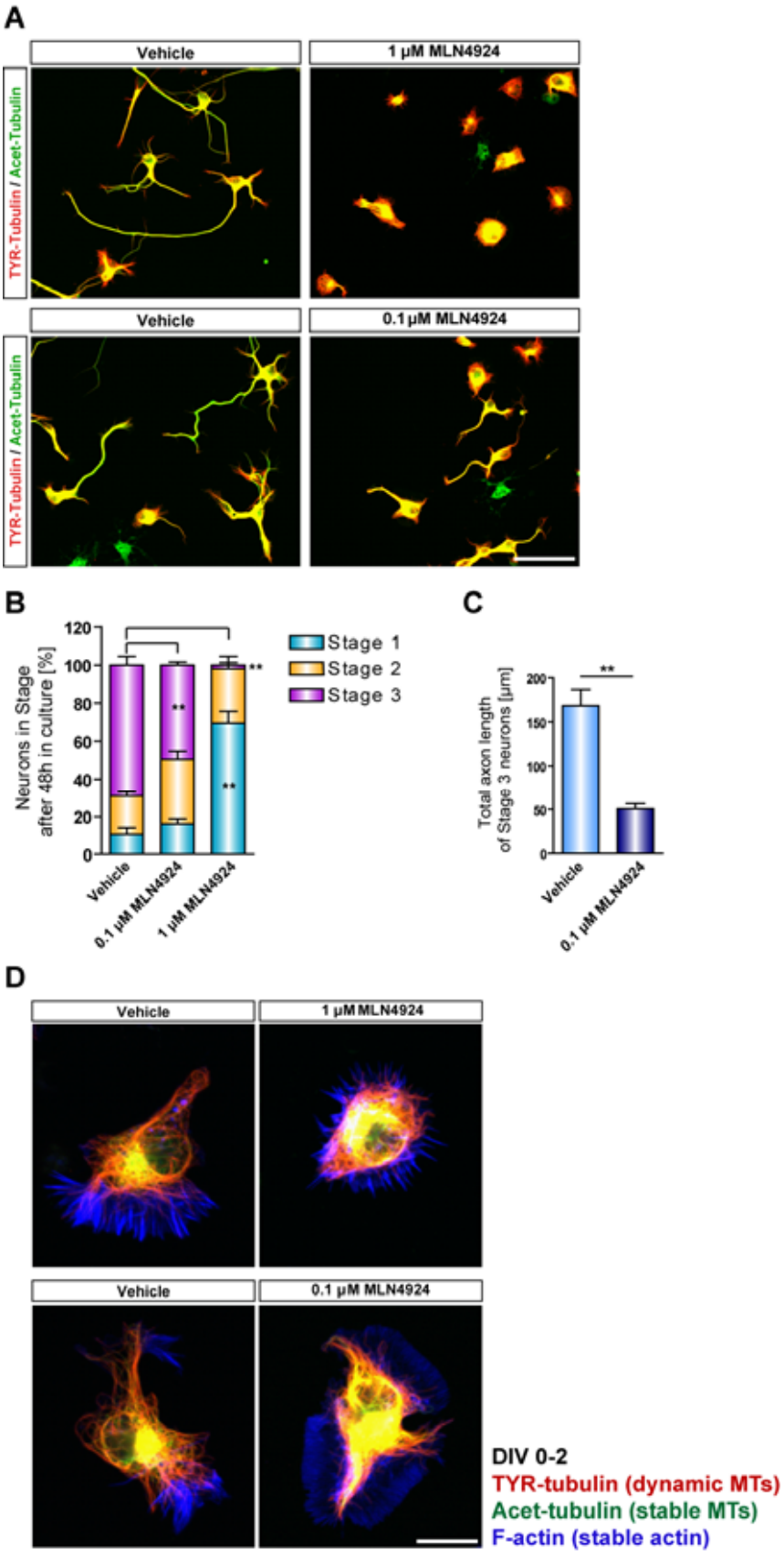

A
Vehicle
1 µM MLN4924
TYR-Tubulin / Acet-Tubulin
Vehicle
0.1 µM MLN4924
TYR-Tubulin / Acet-Tubulin
B
Neurons in Stage after 48h in culture [%]
Stage 1
Stage 2
Stage 3
Vehicle
0.1 µM MLN4924
1 µM MLN4924
C
Total axon length of Stage 3 neurons [µm]
Vehicle
0.1 µM MLN4924
D
Vehicle
1 µM MLN4924
Vehicle
0.1 µM MLN4924
DIV 0-2
TYR-tubulin (dynamic MTs)
Acet-tubulin (stable MTs)
F-actin (stable actin)

Figure 31: Regulation of polarization and axon growth by the NAE-inhibitor MLN4924.

A) Primary hippocampal neurons, treated with vehicle (DMSO), 0.1 or 1 µM MLN4924 for 48 h and stained with α-Tyrosinated- (dynamic MTs) and α-Acetylated-Tubulin (stable MTs), showed a dose-dependent impairment in polarization and axon formation. **B)** Vehicle (DMSO)- and MLN4924-treated neurons were classified into stages 1 to 3 and **C)** total axon length of stage 3 neurons (vehicle (DMSO) and 0.1 µM MLN4924 treatment) was measured. **D)** Hippocampal neurons, treated with vehicle (DMSO), 0.1 or 1 µM MLN4924 from DIV 0 to 2, were fixed and the microtubular and actin cytoskeleton was stained via immunofluorescence. Distortions in dynamic microtubules (in red), stained with α-Tyrosinated-Tubulin antibody, stable microtubules (in green), stained with α-Acetylated-Tubulin, and F-Actin (in blue), stained via fluorescent Phalloidin, were observed in neurons treated with MLN4924 compared to vehicle (DMSO)-treated neurons. Scale bar in (**A**) represents 50 µm; scale bar in (**D**) represents 20 µm. [Statistical analysis: (**B**) 2 Way ANOVA, treatment x stage interaction, $F_{(4,57)}$=66.1, ***p<0.0001; Bonferroni post-hoc test, **p<0.01; (**C**) t-test, **p<0.01.]

Immunofluorescence stainings of the cytoskeleton showed distortions in the microtubular and actin networks of neurons treated with the NAE-inhibitor MLN4924 compared to vehicle-treated control neurons, recapitulating the defects in dynamic microtubules, stained by α-Tyrosinated-Tubulin, as well as F-actin, stained by fluorescently-labeled Phalloidin, observed in neurons expressing the dominant-negative Ubc12-C111S (Figure 31.D). Furthermore, analysis of stable microtubules, using antibodies to specifically detect Acetylated-Tubulin, revealed that neddylation blockade via MLN4924 leads to a decrease in stable microtubules in axons of stage 3 neurons compared to vehicle-treated stage 3 neurons (Figure 31.A).

In summary, from the analysis of the cytoskeleton via immunofluorescence stainings of microtubules and actin we concluded that inhibition of neddylation results I) in a decrease of stable microtubules (α-Acetylated-Tubulin), and therefore, in a higher ratio of dynamic/stable microtubules in the axon; II) in a distorted distribution of dynamic microtubules (α-Tyrosinated-Tubulin); and III) in a higher amount of F-actin surrounding the neuronal periphery. Accordingly to these morphological changes, treatment with 1 µM Cytochalasin D and low concentration (10 nM) of Taxol proved effective to rescue polarization, axon formation and growth in MLN4924-treated neurons (DIV 0 to 2) analyzed by classification of neurons into stage 1 to 3 (Figure 32.A, C to E).
Furthermore, immunofluoresence stainings also showed that Taxol and Cytochalasin D treatment increased the amount of stable microtubules resulting in a higher ratio of stable/dynamic MTs in the axons of MLN-treated neurons comparable to vehicle-treated neurons (Figure 32.C to E) and in the induction of formation of multiple axons in vehicle-treated as well as in MLN4924-treated neurons (Figure 32.F).

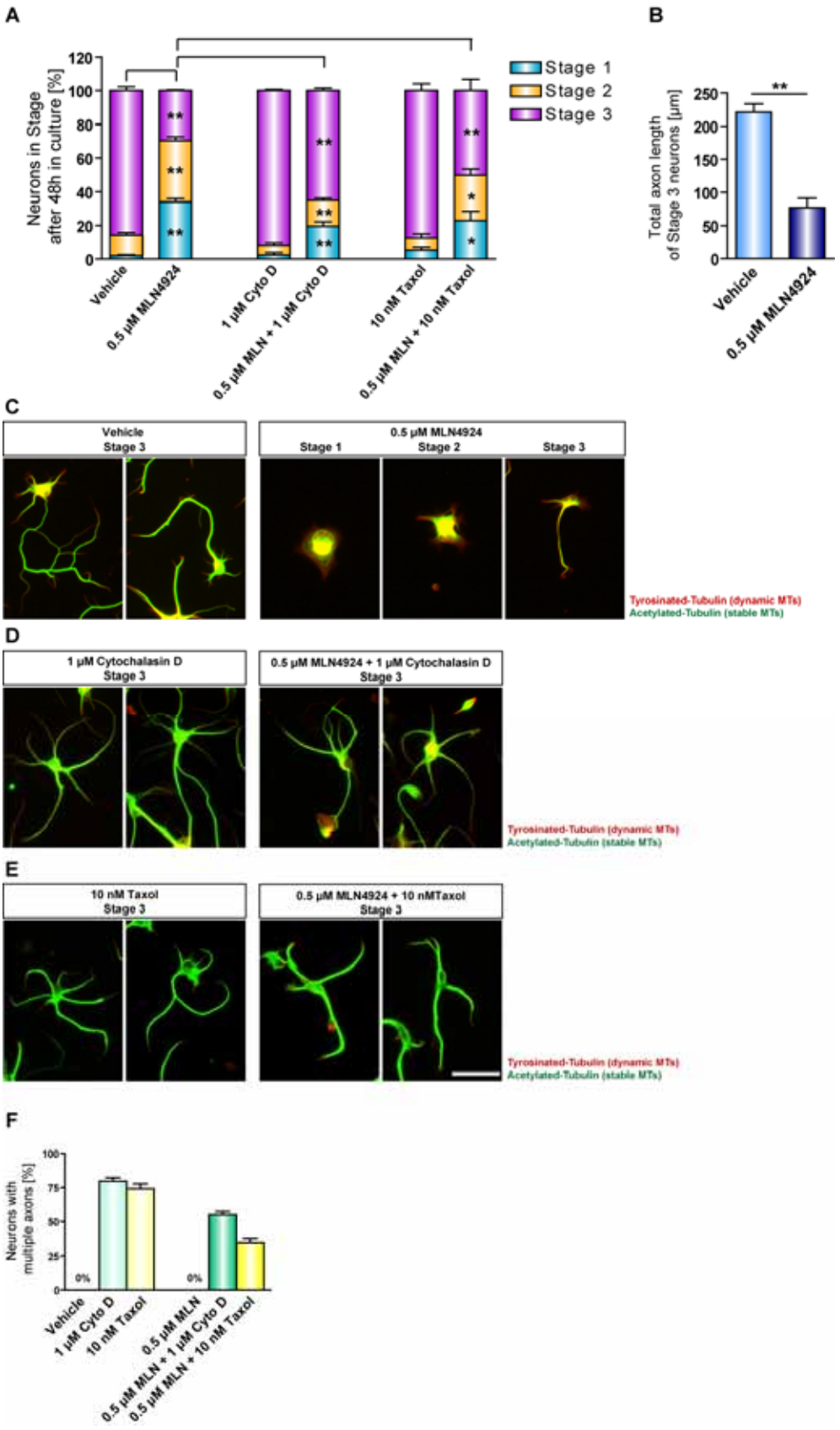

A
Neurons in Stage after 48h in culture [%]
Stage 1
Stage 2
Stage 3
Vehicle
0.5 µM MLN4924
1 µM Cyto D
0.5 µM MLN + 1 µM Cyto D
10 nM Taxol
0.5 µM MLN + 10 nM Taxol
B
Total axon length of Stage 3 neurons [µm]
Vehicle
0.5 µM MLN4924
C
Vehicle
Stage 3
0.5 µM MLN4924
Stage 1
Stage 2
Stage 3
Tyrosinated-Tubulin (dynamic MTs)
Acetylated-Tubulin (stable MTs)
D
1 µM Cytochalasin D
Stage 3
0.5 µM MLN4924 + 1 µM Cytochalasin D
Stage 3
Tyrosinated-Tubulin (dynamic MTs)
Acetylated-Tubulin (stable MTs)
E
10 nM Taxol
Stage 3
0.5 µM MLN4924 + 10 nMTaxol
Stage 3
Tyrosinated-Tubulin (dynamic MTs)
Acetylated-Tubulin (stable MTs)
F
Neurons with multiple axons [%]
Vehicle
1 µM Cyto D
10 nM Taxol
0.5 µM MLN
0.5 µM MLN + 1 µM Cyto D
0.5 µM MLN + 10 nM Taxol

Figure 32: Taxol and Cytochalasin D rescue of axon defects induced by MLN4924.

A) to **E)** Primary hippocampal neurons were treated directly after plating with vehicle (DMSO) or 0.5 µM MLN4924 to block the neddylation pathway. Neurons were co-treated with vehicle (DMSO), 10 nM Taxol or 1 µM Cytochalasin D. 48 h after plating neurons were fixed and dynamic and stable microtubules were stained with α-Tyrosinated-Tubulin (red) antibodies and α-Acetylated-Tubulin (green) antibodies respectively. **A)** Neurons of all treatments were classified into stages 1 to 3 and **B)** total axon length was measured of vehicle (DMSO)- and 0.5 µM MLN4924-treated stage 3 neurons. **C)**, **D)** and **E)** Co-treatment of neurons with 10 nM and 1 µM Cytochalasin D rescued MLN4924-induced polarization and axonal growth defects. **F)** Analysis of percentage of neurons having multiple axons induced by Taxol and Cytochalasin D treatment. Scale bar represents 50 µm. [Statistical analysis: (**A**) 2 Way ANOVA, treatment x stage interaction, $F_{(6,42)}$=96.4, ***p<0.0001; Bonferroni post-hoc test, *p<0.05, **p<0.01; (**C**) t-test, **p<0.01.]

To further investigate the dynamic regulation of the actin cytoskeleton, we used Lifeact-meGFP to fluorescently label F-actin in living cells without altering actin expression and without disturbing actin dynamics (Riedl et al., 2008). In contrast to other actin-binding proteins, which have to be overexpressed to track actin dynamics and to visualize actin-related protein complexes, Lifeact does not interfere at all with actin dynamics, which makes it especially suitable for live imaging experiments. We transfected HT22 cells and primary hippocampal neurons (at DIV 0) using nucleofection with either control plasmid or Ubc12-C111S to block neddylation and Lifeact-meGFP to visualize actin dynamics. Fast confocal time lapse movies were recorded 24 h after transfection of HT22 cells and 12 h after nucleofection of primary neurons (Figure 33.A, B). When neddylation was inhibited via expression of Ubc12-C111S, filopodia structures labeled by Lifeact-meGFP displayed a very high motility and turnover compared to control cells suggesting that neuronal cells cannot form stable actin structures under Nedd8-conjugation blockade conditions. Addition of 1 µM Cytochalasin D and 10 nM Taxol immediately attenuated actin dynamics and triggered outgrowth of axons in neurons expressing Ubc12-C111S within 12 h (Figure 33.C).

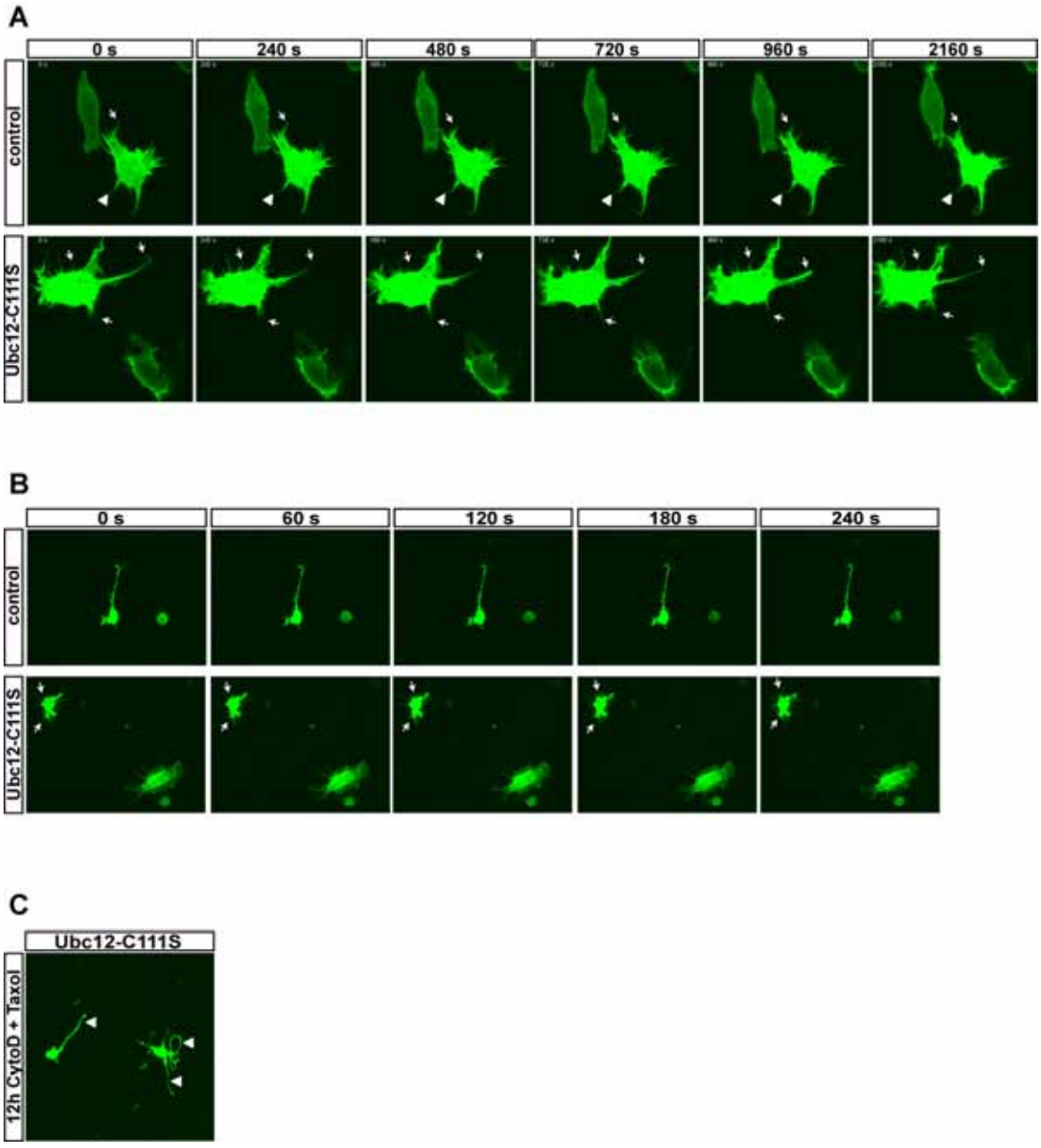


Figure 33: Time lapse imaging of hippocampal neurons transfected with Lifeact-meGFP.

A) HT22 cells were transfected with control or Ubc12-C111S plasmids and with Lifeact-meGFP to visualize actin dynamics. 24 h after transfection, fast time lapse movies were recorded. Z-stacks were taken during 36 min, with a time interval of 60 seconds. In (**A**) collapsed Z-stacks are shown from timepoints 0, 240, 480, 720, 960 and 2160 seconds. Ubc12-C111S-expressing cells displayed a higher dynamic behavior of actin-rich filopdia structures compared to control cells. (White arrowheads highlight stable filopdia, white arrows highlight dynamic filopdia.) **B)** Primary hippocampal neurons were transfected via nucleofection with control or Ubc12-C111S plasmids and with Lifeact-meGFP at the time of plating (DIV 0) and were imaged 12 h later. Time lapse movies were recorded during 5 min, with a time interval of 60 seconds. Filopdia of Ubc12-C111S-expressing neurons were highly dynamic compared to control neurons. (White arrows highlight

dynamic filopdia.) Notably, already 12 h after transfection control neurons acquired axonal polarization, whereas Ubc12-C111S-expressing cells were still stage 1 neurons. **C)** Treatment of Ubc12-C111S-expressing neurons with 10 nM Taxol and 1 µM Cytochalasin D immediately attenuated actin-dynamics and triggered axonal outgrowth within 12 h (Axons are highlighted by white arrowheads).

In summary, the presented experiments in primary neurons established the Nedd8 pathway as a critical cellular regulator of axon polarization and growth and further demonstrated the direct involvement of the actin and microtubular cytoskeleton in this mechanism.

4.3.4. Neddylation controls axon growth *in vivo*

In a next step, we attempted to validate the previously observed effects of neddylation on axon growth in *in vivo* models. Therefore, we applied *in utero* intraventricular injection and electroporation of plasmid constructs in mouse embryos to express Ubc12-C111S in neurons of the retrosplenial cortex and in pyramidal neurons of CA1 of the hippocampus. As a read-out of axon growth, we analyzed the axon projections to and terminals in the contralateral brain hemispheres via co-expression of fluorescent proteins, e.g. GFP, RFP and myr-Venus (membrane-bound Venus). Electroporation of neurons of the retrosplenial cortex, which is part of the cingulate cortex, at E13.5 with Ubc12-C111S resulted in decreased axon fasciculation and lower terminal numbers in the contralateral cortex compared to control electroporated mice at P10 as well as on P21 (Figure 34).

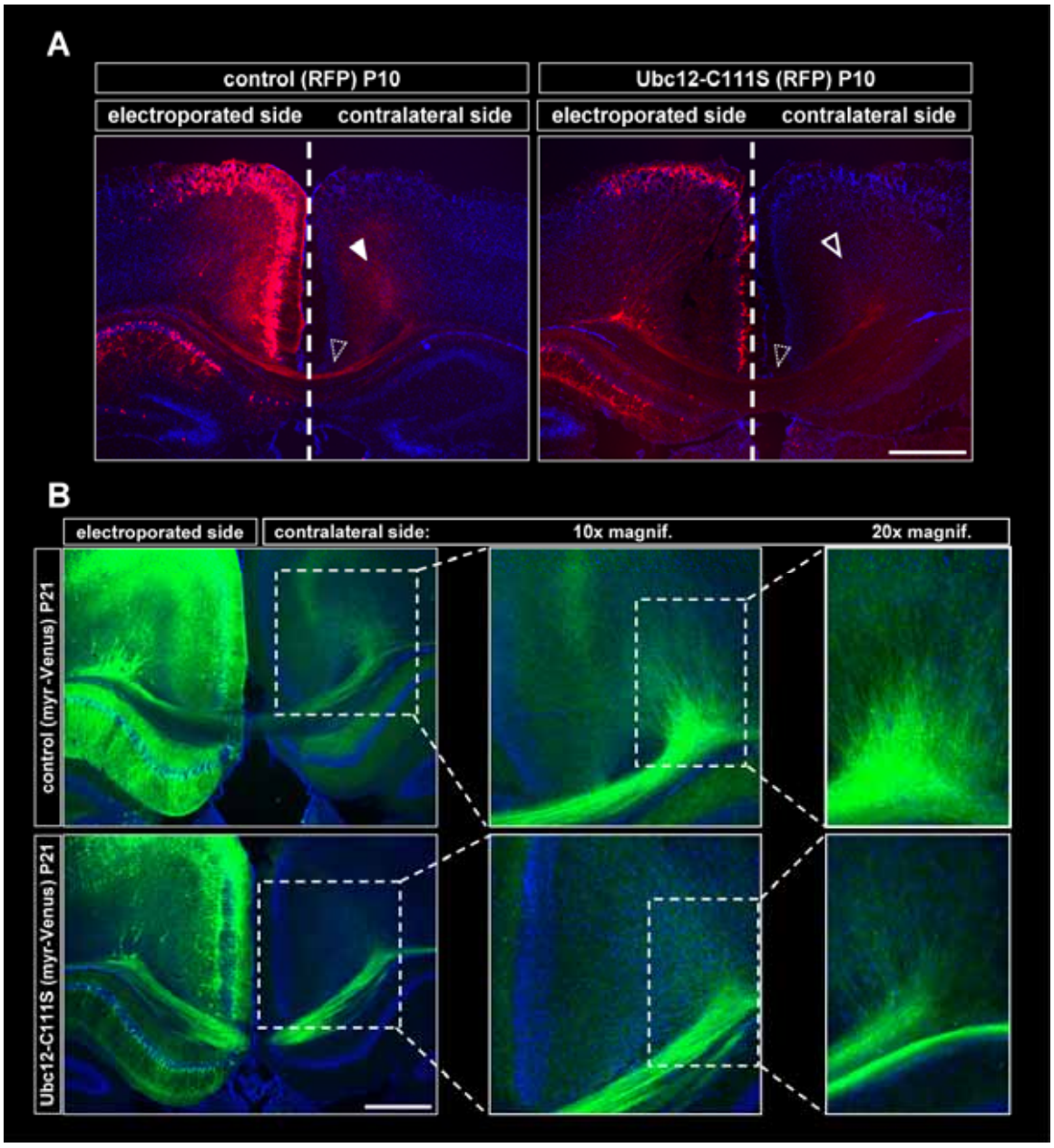


Figure 34: The Nedd8 pathway regulates axon growth during development of the cortex *in vivo*.

At E13.5 mouse embryos were microinjected with control or Ubc12-C111S and RFP or myr-Venus plasmid DNA into the lateral ventricles and electroporated to target neurons in the inner retrosplinial cortex and the hippocampus. Brains were fixed and axonal projections from neurons of the inner retrosplinial cortex through the corpus callosum to the contralateral brain side were analyzed at **A)** P10 and **B)** P21. In **A)** the filled white arrowhead marks axon terminals of control neurons targeting postsynaptic neurons in the cortex of the contralateral side. Open white arrowhead shows absence of axon terminals of Ubc12-C111S-expressing neurons in the contralateral side. Dashed white arrowheads highlight callosal fibers (axons projecting through the corpus callosum to the contralateral brain half). Sale bars represent 500 µm.

A similar axonal growth pattern was observed at the hippocampal level. Pyramidal neurons of CA1 in the hippocampus were electroporated at E14.5 and their projections to hippocampal CA1 pyramidal neurons in the contralateral side were evaluated at P21 and P60. As a result of neddylation blockade induced by the expression of Ubc12-C111S, axons and their terminals were completely absent in the contralateral stratum oriens, where they establish synaptic contacts with the basal dendrites of pyramidal neurons of CA1 in control electroporated brains. Besides, in Ubc12-C111S-electroporated brains, significantly less axon trajectories and terminals were observed in the stratum oriens and stratum radiatum in the contralateral hemispheres, where synapses are usually established with apical dendrites of CA1 pyramidal neurons (Figure 35).

The inhibition of the Nedd8 pathway *in vivo*, using *in utero* intraventricular injection and electroporation in mouse embryos, resulted in substantial impairments in axonal growth and terminal targeting of axons, which further confirmed the results obtained in dissociated primary hippocampal and cortical neurons. Noteworthy, there are clear limitations of the *in utero* electroporation technique for studying axon formation *in vivo*. The variability in the targeting procedure is one of the main drawbacks of this approach. After the electroporation, every brain carries a unique set of transfected neurons. Since we evaluated axon projections to the contralateral brain side it is important to be able to compare similar populations of transfected neurons which express the fluorescent protein at equal levels. In order to avoid false interpretations of the presence or absence of axon projections, we only chose highly similar electroporated brains in terms of region and expression levels. This in turn limits the number of brains available for analysis. The generation of conditional Nae1 knockout and conditional Ubc12-C111S overexpressing mice will allow a more detailed analysis of axon growth, guidance and synaptic targeting during brain development *in vivo* (see later in 4.8).

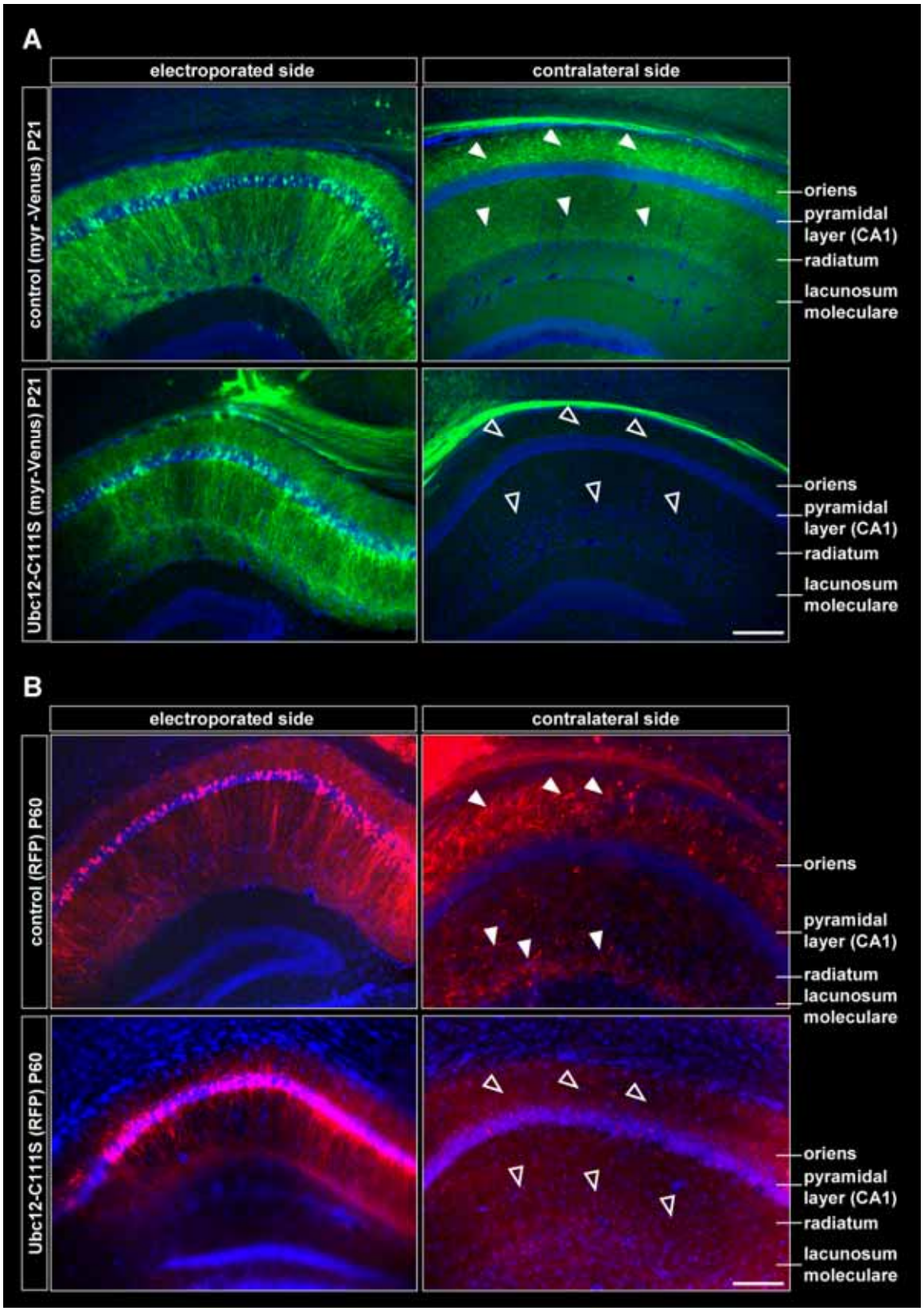

A
electroporated side
contralateral side
control (myr -Venus) P21
Ubc12-C111S (myr-Venus) P21
oriens
pyramidal layer (CA1)
radiatum
lacunosum moleculare
B
electroporated side
contralateral side
control (RFP) P60
Ubc12-C111S (RFP) P60
oriens
pyramidal layer (CA1)
radiatum
lacunosum moleculare

Figure 35: The Nedd8 pathway regulates axon growth during development of the hippocampus *in vivo*.

At E14.5 mouse embryos were microinjected with control or Ubc12-C111S and RFP or myr-Venus plasmid DNA into the lateral ventricles and electroporated to target pyramidal neurons in CA1 of the hippocampus. Brains were fixed and axons of pyramidal neurons of CA1 in the hippocampus projecting to pyramidal neurons of CA1 in the contralateral side were analyzed at **A)** P21 and **B)** P60. Filled white arrowheads mark axon terminals of control neurons targeting postsynaptic neurons in CA1 at basal dendrites in stratum oriens and apical dendrites in stratum radiatum and stratum lacunosum moleculare. Open white arrowheads show absence of axon terminals of Ubc12-C111S-expressing neurons in the contralateral brain half. Scale bars represent 200 µm.

4.3.5. EB3, a MT-tip binding protein, is a target of Nedd8

So far it has been demonstrated that neddylation can regulate cytoskeletal dynamics and structures through cullin E3 ubiquitin ligase complexes in non-neuronal cells (Kurz et al., 2002; Pintard et al., 2003). However, our experiments in hippocampal and cortical neurons show that neddylation regulates the actin and microtubular cytoskeleton during polarization and axon formation in a direct, cullin E3 ubiquitin ligase-independent manner. But how does Nedd8 control cytoskeletal behavior on a molecular level in neurons in the CNS?

The dynamic properties of MTs are regulated by microtubule-destabilizing proteins, such as the Kinesin-13 or the KIF2 family, and by microtubule-stabilizing proteins, a heterogenous and large group of proteins called microtubule-associated proteins (MAPs), including the plus-end tracking proteins, +TIPs. +TIPs specifically accumulate at the growing microtubule plus ends, and were shown to contribute to the regulation of microtubule dynamics, mediate the cross-talk between microtubule ends and the actin cytoskeleton, and to participate in transport and positioning of structural and regulatory factors and membrane organelles (Tirnauer and Bierer, 2000; Galjart, 2005; Komarova et al., 2005; Akhmanova and Hoogenraad, 2005; Geraldo et al., 2008; Akhmanova and Steinmetz, 2008; Komarova et al., 2009). The EB family is a member of the +TIPs family and consists of three homologs in mammals, EB1, EB2/RP1 and EB3, from which EB3 is expressed preferentially in the nervous system (Nakagawa et al., 2000). Recently, EB3 and EB1 have been shown to promote persistent microtubule growth by inhibiting MT catastrophes (Komarova et al., 2005; Komarova et al., 2009). Furthermore, inhibition

of EB3-function, specifically loss of the interaction of the F-actin binding protein drebrin with EB3, results in a severe impairment of neuritogenesis in cortical neurons (Geraldo et al., 2008).

To test if EB3 might be neddylated, co-immunoprecipitation experiments were performed. Only when EB3-GFP and 3xFLAG-Nedd8 were expressed together in hippocampal HT22 cells, immunoprecipitation with α-GFP antibody and immunoblotting with α-FLAG antibody, and *vice versa*, revealed a band in the Western blot with the expected shift in size corresponding to EB3-Nedd8 conjugates (Figure 36.A, highlighted by dashed blue boxes). Neddylation of EB3 was strongly reduced by co-expression of Ubc12-C111S (Figure 36.A). To analyze the functional consequences of neddylation of EB3, we transfected Neuro-2a cells with EB3-GFP and control or Ubc12-C111S plasmids for 48 h. When neddylation was blocked via expression of Ubc12-C111S, EB3 levels were decreased compared to control cells. The decrease was partially compensated by co-treatment for the last 6 h of the experiment with the proteasome inhibitor MG132 suggesting that the decrease in EB3 levels is due to increased proteasome-mediated degradation of EB3 under neddylation blockade (Figure 36.B). Furthermore, blocking neddylation by Ubc12-C111S resulted in decreased endogenous EB3 levels in Neuro-2a cells analyzed by Western blotting 48 h after transfection (Figure 36.C). Neddylation blockade did not alter endogenous EB3 RNA transcript levels measured by qRT-PCR at 18, 24 and 48 h after transfection, confirming that the effect of neddylation is directly exerted on protein stability of EB3 (Figure 36.D). Therefore, we concluded that under normal physiological conditions neddylation of EB3 might protect against ubiquitination and subsequent proteasome-mediated degradation. Moreover, treatment of primary neurons with the Nedd8-inhibitor MLN4924 reduced endogenous EB3 levels, further supporting a physiological role of EB3 neddylation in neurons (Figure 36.E, F).

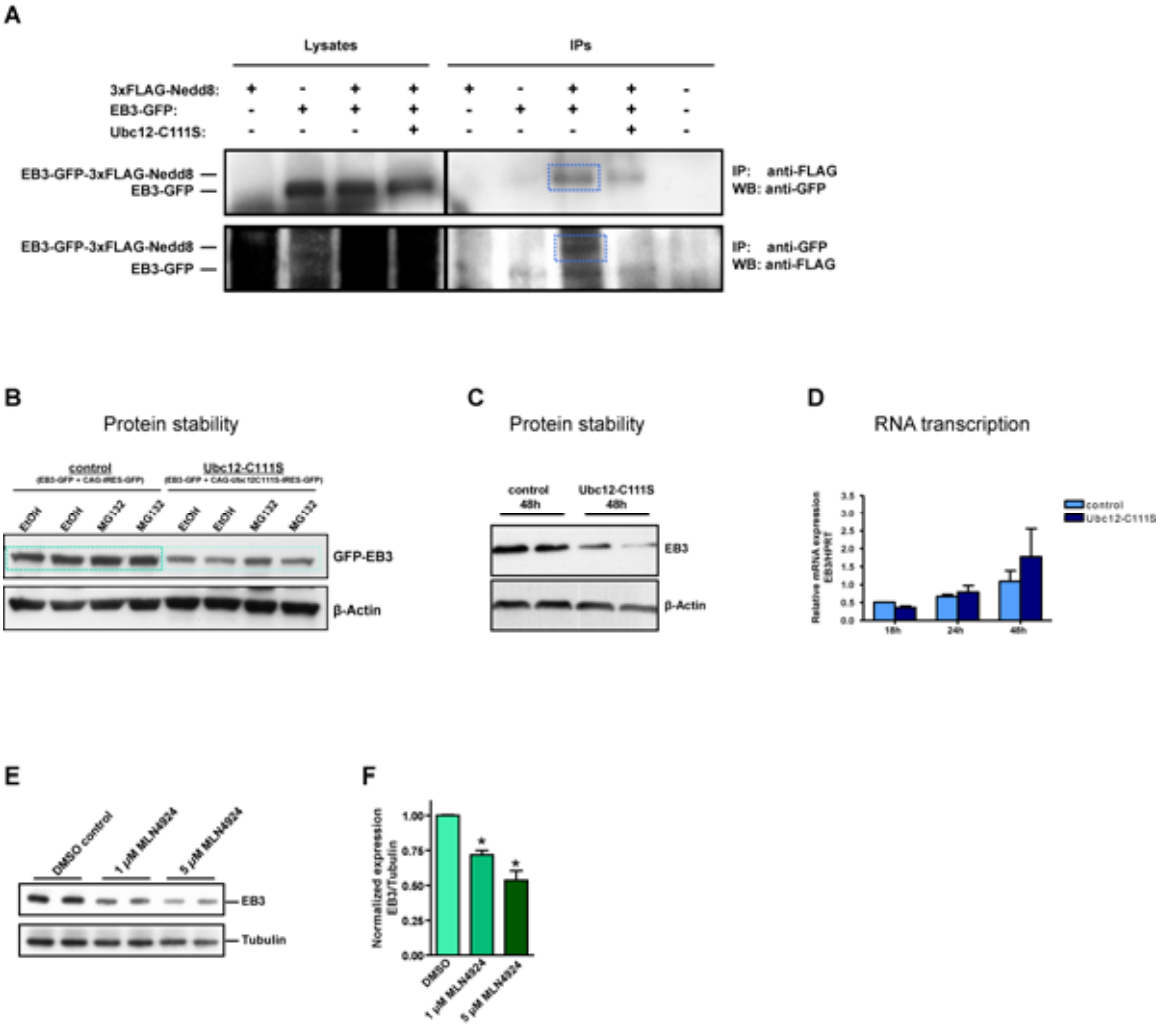


Figure 36: EB3 is neddylated.

A) EB3 is neddylated in immunoprecipitation experiments. HT22 cells were transfected with the indicated combinations of plasmids (EB3-GFP, 3xFLAG-Nedd8, Ubc12-C111S), and extracts were subjected to immunoprecipitation with anti-FLAG or anti-GFP antibodies, followed by immunoblotting. Only when EB3-GFP and 3xFLAG-Nedd8 were co-expressed, immunoblotting revealed a band corresponding to EB3-Nedd8 conjugates (marked with blue boxes). Co-expression of Ubc12-C111S strongly reduced neddylation of EB3. **B)** Neddylation controls protein stability of EB3-GFP. Neuro-2a cells were transfected with EB3-GFP and control or Ubc12-C111S plasmids as indicated and were differentiated by serum-withdrawal for 48 h. 6 h before protein extraction, Neuro-2a cells were treated with 20 μM MG132, a proteasome inhibitor, or vehicle. Cell extracts were subjected to immunoblotting with anti-GFP and anti-β-Actin antibodies. Blockade of neddylation resulted in reduced EB3-GFP levels, and this decrease could be partially rescued by proteasome inhibition via MG132 treatment. β-Actin served as the loading control **C)** Neddylation controls protein stability of endogenous EB3. Neuro-2a cells were transfected with control or Ubc12-C111S plasmids as indicated and were differentiated by serum-withdrawal for 48 h. Immunoblotting against EB3 showed a decrease of endogenous EB3 levels when neddylation was blocked. β-Actin served as the loading control. **D)** Neddylation does not regulate EB3 mRNA. mRNA was isolated from Neuro-2a cells transfected for 18, 24 and 48 h with control or Ubc12-C111S plasmids as indicated. Inhibition of neddylation does not alter EB3 mRNA

transcript levels shown by qRT-PCRs. EB3 were normalized to the house-keeping gene *Hprt*. **E)** EB3 protein stability is controlled by neddylation in primary neurons. Cortical neurons were treated with 1 and 5 µM MLN4924 or vehicle for 48h and protein extracts were immunoblotted with anti-EB3 antibody. MLN4924 treatment decreased EB3 levels. Tubulin served as a loading control. **F)** Quantification of Western blot signals revealed a decrease of EB3 levels in response to neddylation blockade.

4.3.6. Partial rescue of axon growth by EB3 overexpression

In line with our previous finding that neddylation controls the stability of EB3 and with the well known role of EB3 to increase the stability of microtubules in neurons (Komarova et al., 2005; Komarova et al., 2009), overexpression of EB3 rescued the impairment in axon growth induced by blockade of the Nedd8 pathway in hippocampal and cortical neurons expressing Ubc12-C111S (Figure 37.A, B).

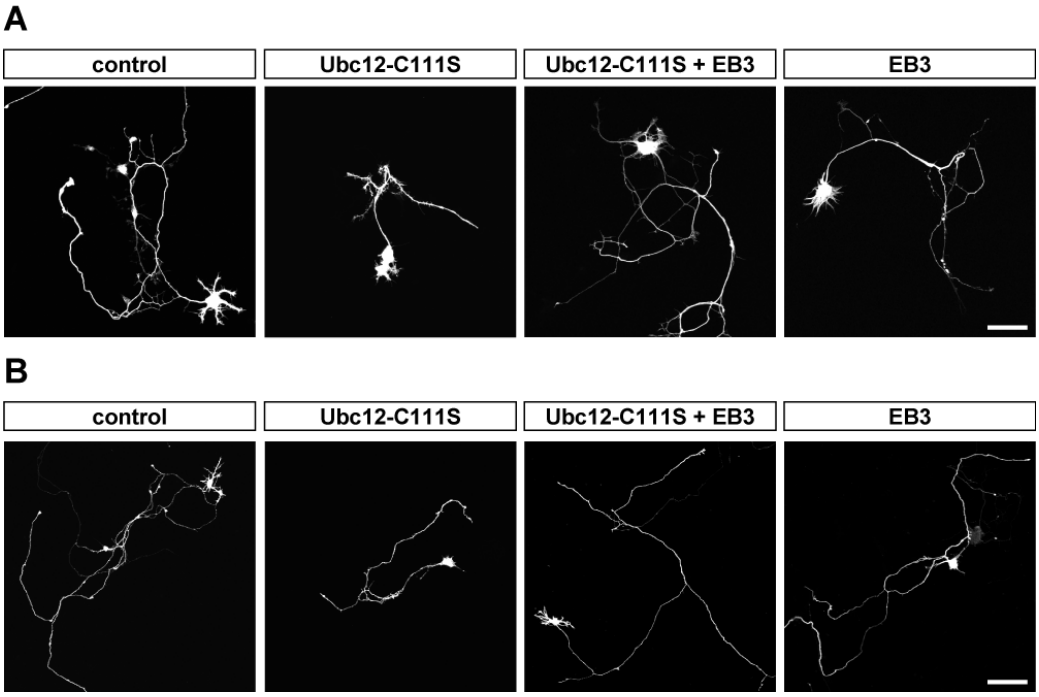


Figure 37: Rescue of axon growth by EB3 overexpression in primary neurons.

A) Hippocampal neurons and **B)** cortical neurons were transfected from DIV 2 to 5 with the indicated plasmids. Overexpression of EB3 rescued axon growth in neurons expressing Ubc12-C111S. Scale bar in (**A**) represents 25 µm and scale bar in (**B**) represents 50 µm.

The rescue by EB3 and Taxol strongly suggests that microtubule stability is affected by blockade of neddylation and that the microtubular cytoskeleton is involved in mediating the effects of neddylation on axon formation and growth. Although EB3 overexpression was sufficient to restore axon growth during Nedd8-blockade in primary neurons, *in vivo* EB3 overexpression did not rescue axon terminals in the cortex and hippocampus in *in utero* intraventricular injection and electroporation experiments in mouse embryos (data not shown). This result suggests that additional yet unidentified Nedd8 targets affect the regulation of axon formation and growth during neuronal development in the brain.

4.4. Neddylation controls dendrite development

In primary dissociated neurons, polarization and axon elongation is followed by growth and arborization of dendrites. *In vivo*, cortical and hippocampal dendrite formation starts during embryogenesis and continues during postnatal development. We first analyzed the role of the Nedd8-conjugation pathway in dendrite growth and arborization in primary neurons using the pharmacological NAE-inhibitor MLN4924 or expressing Ubc12-C111S. In the next step we studied the function of neddylation in dendrite formation *in vivo*, (a) during development of the cortex and hippocampus using *in utero* gene delivery and (b) during adult neurogenesis of granule neurons in the dentate gyrus of the hippocampus using stereotactic injection of retroviruses into the brain of adult mice.

4.4.1. The role of Nedd8 in dendrite growth and arborization in primary neurons

To examine the function of the Nedd8 pathway during growth and arborization of dendrites, we first blocked Nedd8-conjugation in hippocampal neurons and assessed dendrite morphology via measurement of total dendritic length and complexity of dendritic trees using Sholl analysis (Sholl, 1953; Meijering et al., 2004). Dendrites were identified based on morphological criteria and selective immuno-staining with α-MAP2, a dendrite marker. Neurons treated with MLN4924 from DIV 8 to 12 displayed a strong decrease in total dendrite length and a reduction of dendrite arborization (Figure 38).

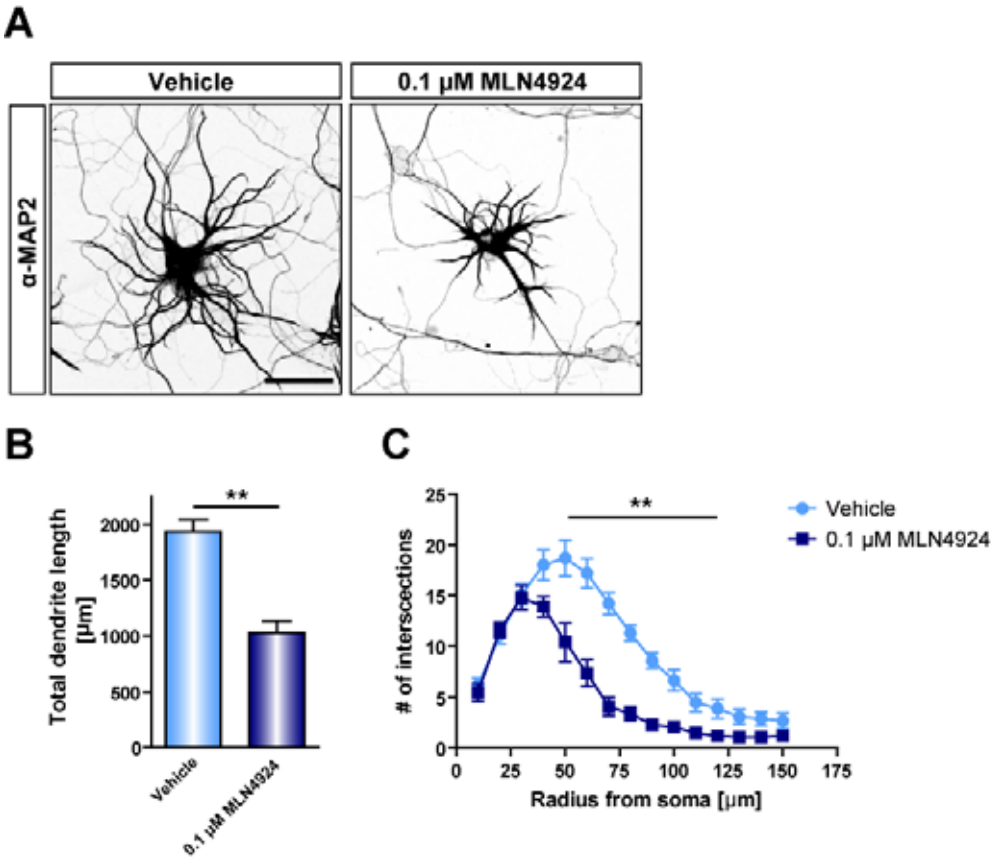


Figure 38: Dendrite growth is inhibited by MLN4924.

A) Primary hippocampal neurons at DIV 8 were treated repeatedly every 48 h with vehicle (DMSO) or 0.1 µM MLN4924. At DIV 12 neurons were fixed and immunofluorescence staining with α-MAP2 antibodies was performed to label dendrites. Scale bar represents 50 µm. **B)** Total dendritic length and **C)** dendritic arborization, measured by Sholl analysis, were severly decreased in neurons treated with MLN4924 compared to vehicle-treated neurons. [Statistical analysis: (**B**) t-test, **p<0.01; (**C**) 2 Way ANOVA, treatment x radius interaction, $F_{(14,270)}$=6.8, ***p<0.0001; Bonferroni post-hoc test, **p<0.01.]

Inhibition of neddylation in hippocampal neurons by Ubc12-C111S transfection from DIV 6 to 10, resulted in similar defects in dendritic growth, shown for total dendrite length as well as arborization, compared to those observed with pharmacological inhibition of the Nedd8 pathway by MLN4924 treatment (Figure 39).

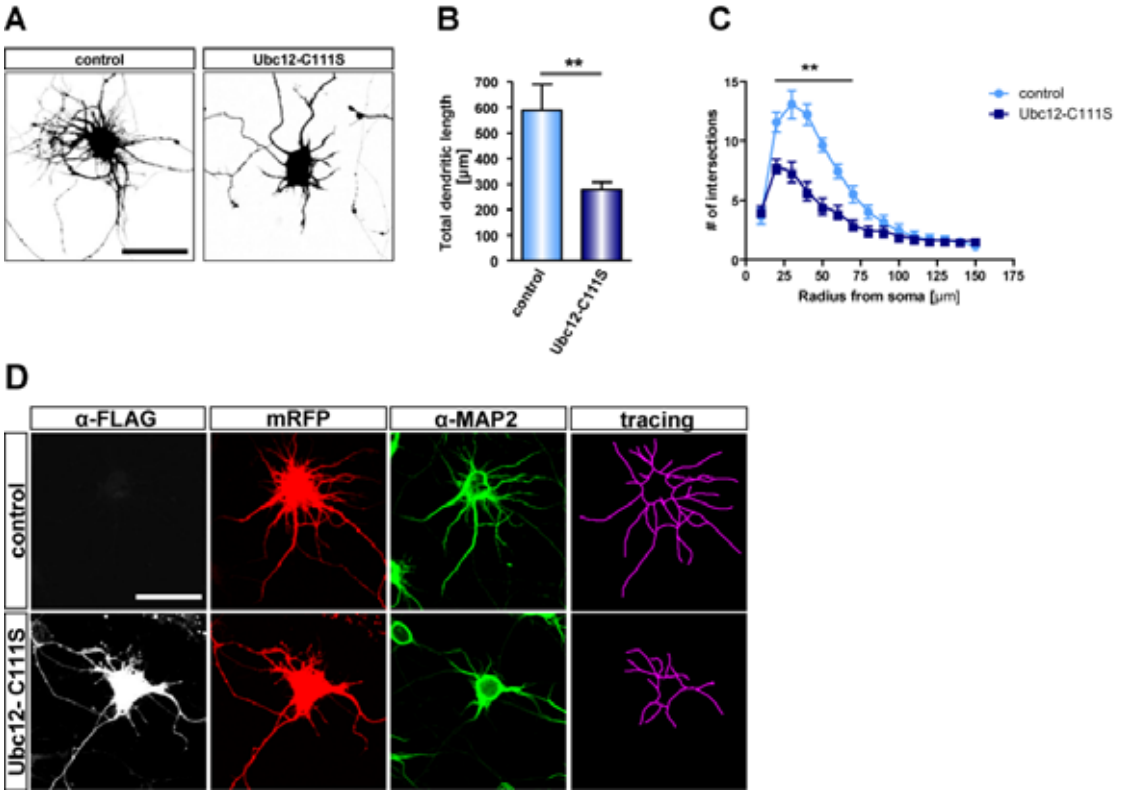


Figure 39: Blocking neddylation via Ubc12-C111S decreases dendrite growth.

A) Primary hippocampal neurons were transfected at DIV 6 with control or Ubc12-C111S plasmids and fixed at DIV 10. **B)** Total dendritic length and **C)** dendritic arborization were measured by tracing of MAP2 positive processes. Total dendritic length and dendritic complexity assessed by Sholl analysis were strongly reduced in Ubc12-C111S-expressing neurons compared to control neurons. [Statistical analysis: (**B**) t-test, **p<0.01; (**C**) 2 Way ANOVA, treatment x radius interaction, $F_{(14,465)}$=9.3, ***p<0.0001; Bonferroni post-hoc test, **p<0.01.] **D)** Immunocytochemistry was performed to detect expression of FLAG-tagged Ubc12-C111S and to label dendrites using α-MAP2 antibodies. Examples of dendrite tracings are shown for control and Ubc12-C111S-expressing neurons. Scale bars represent 50 µm.

These experiments led to the conclusion that neddylation is essential for dendrite development in primary neurons. The next aim was to analyze the contribution of cullin E3 ubiquitin ligases, known targets of Nedd8, to the observed dendrite effects. First, hippocampal neurons transfected with dominant-negative versions of cullin 1 to 5 did not show the strong impairment in dendritic growth and branching as observed when neddylation was blocked via expression of Ubc12-C111S (Figure 40.A). To block cullin 1 to 4 at the same time we transfected hippocampal neurons with dominant-negative Rbx1 constructs (Rbx1-C42S-C45S and Rbx1-C75S). Both, neurons expressing Rbx1-C42S-C45S and Rbx1-C75S developed dendrites similar in length and complexity to dendrites

of control neurons, whereas blockade of neddylation via Ubc12-C111S expression led to impairments in dendrite formation as observed before (Figure 40.B). Therefore, we concluded that neddylation might regulate dendrite development independently of cullin RING E3 ubiquitin ligases in primary neurons.

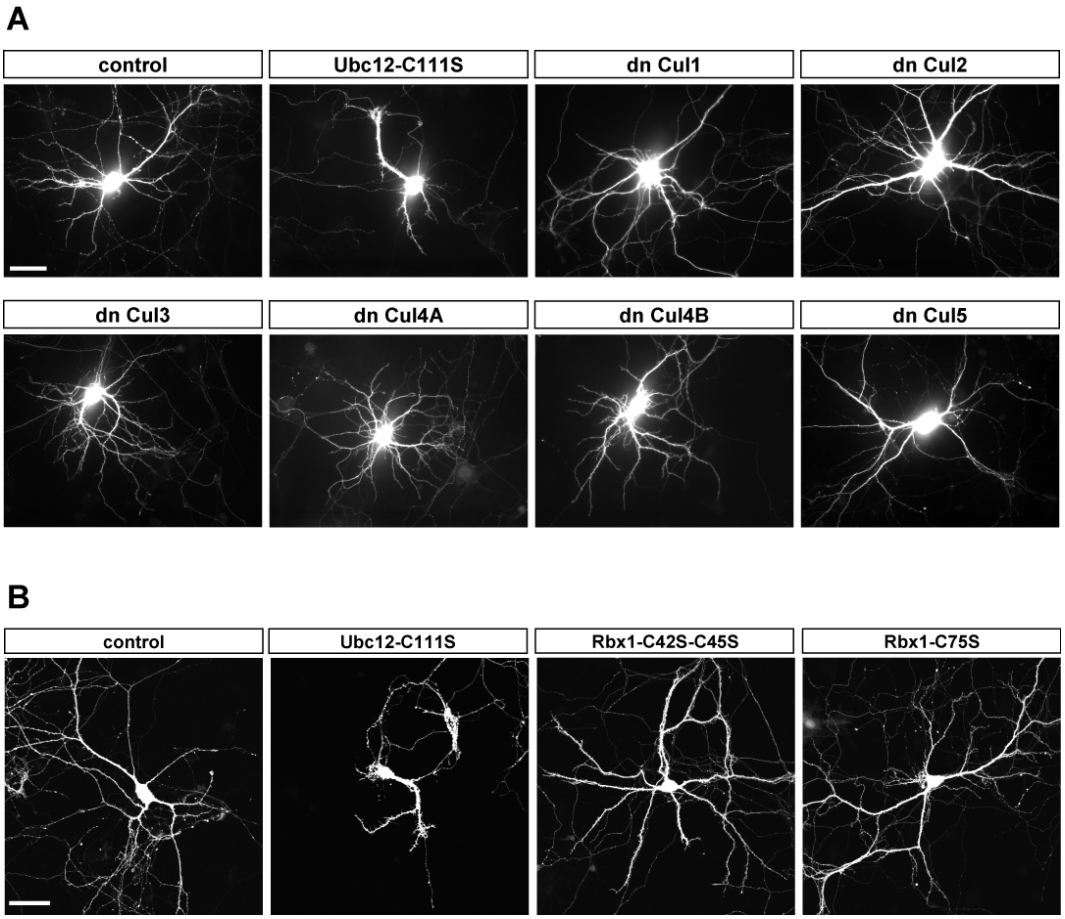


Figure 40: Effects of neddylation on dendrite development are independent of cullin E3 RING ligases.

A) Primary hippocampal neurons were transfected with the indicated plasmids, control, Ubc12-C111S or dominant-negative cullins 1 to 5 at DIV 11 and fixed at DIV 18. **B)** Primary hippocampal neurons were transfected with the indicated plasmids, control, Ubc12-C111S or dominant-negative Rbx1 constructs (Rbx1-C42S-C45S or -C75S) at DIV 11 and fixed at DIV 18. Inhibition of cullin RING ligases didn't result in impaired dendrite development as observed during neddylation blockade via Ubc12-C111S expression. Scale bars represent 50 µm.

4.4.2. Neddylation regulates dendrite formation of pyramidal neurons during development of the cortex and hippocampus *in vivo*

In order to validate *in vivo* the results obtained in primary cell cultures described above, we employed different models to address the function of Nedd8 in dendritic growth and arborization in the entire animal.

First we analyzed dendrite formation *in vivo* during development of the cortex using i*n utero* intraventricular injection and electroporation of mouse embryos. To study the development of upper layer cortical neurons, we injected and electroporated control-GFP or Ubc12-C111S-IRES-GFP constructs at E13.5 and fixed the brains at P2 (Figure 41). Establishment of the apical primary dendrite of Ubc12-C111S-expressing cortical neurons was severly impaired compared to control neurons (Figure 41.A). Moreover, inhibition of neddylation led to a strong defect in radial migration of pyramidal neurons, evident in an overmigration phenotype (Figure 41.A). Control neurons electroporated at E13.5 migrated to the upper cortical layers and were distributed throughout layer II and III of the cortex, whereas neurons expressing Ubc12-C111S migrated too far compared to control neurons and were lined up at the distal border of layer II, directly below the marginal zone (Figure 41.A). Interestingly, upper layer cortical neuron identity was preserved in Ubc12-C111S-expressing neurons, shown by positive Cux1 staining (Figure 41.A, highlighted by white arrowheads). Furthermore, we observed two additional effects in Ubc12-C111S-electroprated brains. At P7, we detected few cells expressing Ubc12-C111S in the corpus callosum, and in the cortex below layers II and III (Figure 41.B). These different developmental defects might be due to different expression levels of Ubc12-C111S in individual cells, and hence different degrees of inhibition of the Nedd8 pathway.

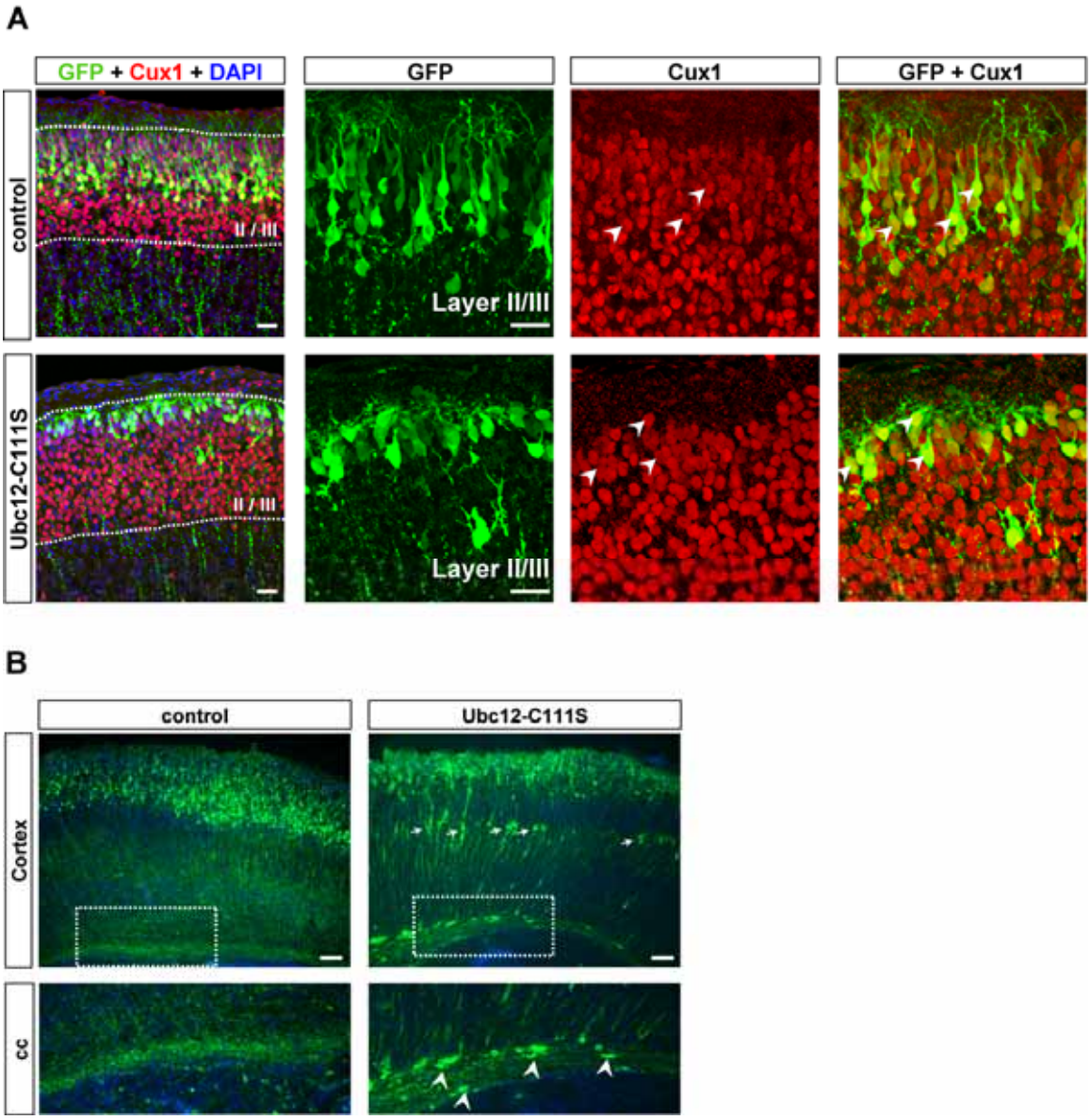


Figure 41: Neddylation regulates dendrite formation and neuronal migration in the upper layers of the developing cortex *in vivo*.

A) At E13.5 mouse embryos were microinjected with plasmid DNA (control-GFP and Ubc12-C111S-IRES-GFP) and electroporated to target the upper layers (II/III) in the developing neocortex. At P2 brains were fixed, and immunofluorescence staining against Cux1 (in red), a marker of upper layer cortical neurons, was performed on mounted cryosections. Ubc12-C111S-expressing cells showed defects in migration and strong impairments in dendrite formation compared to control neurons, yet they were Cux1-positive (white arrowheads highlight Cux1-positive neurons) **B)** At P7, cell bodies, marked by white arrowheads in the magnification of the boxed region, were observed in the fiber tracts of the corpus callosum, and cells, marked by white arrows, were observed below layers II and III in the cortex, in brains electroporated with Ubc12-C111S compared to control brains. Scale bars represent 100 µm.

In addition, analysis of the development of lower layer (V/VI) pyramidal neurons in the cortex also revealed a general requirement of the neddylation pathway during neuronal development of lower cortical layers. To block neddylation in developing neurons of cortical layer V/VI, embryos were microinjected and electroporated with Ubc12-C111S or control plasmids at E12.5 and brains were analyzed at P2 and P21. Neurons expressing control plasmids showed the typical morphology of layer V pyramidal neurons, extending one primary apical dendrite towards the upper cortical layers, where it is then extensively branched. Furthermore, during this specific developmental time window all electroporated control neurons stopped migration in the lower cortical layers as expected. In contrast, neurons expressing Ubc12-C111S were scattered throughout all cortical layers recapitulating the defect in radial migration observed during development of the upper cortical layers (Figure 42.A). In addition to the migration phenotype, blocking neddylation in layer V cortical neurons resulted in a strongly distorted dendrite development as observed previously in upper layer cortical neurons (Figure 42.B).

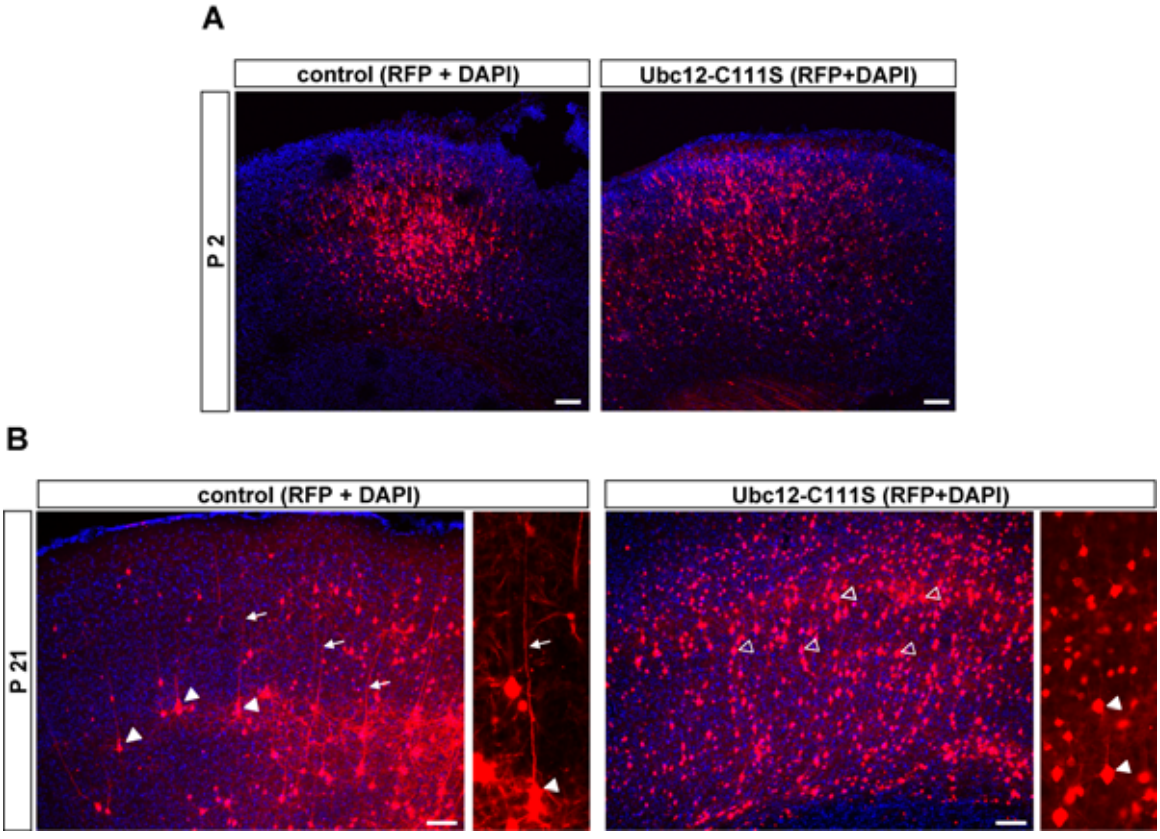


Figure 42: Neddylation controls development of lower layer cortical neurons.

Mouse embryos were *in utero* electroporated with control or Ubc12-C111S constructs at E12.5 to target the lower layers (V/VI) of the developing neocortex. Brains were fixed on **(A)** P2 and **(B)** P21. **A)** At P2 neurons expressing Ubc12-C111S were found in all cortical layers whereas control-electroporated neurons were restricted to the lower cortical layers. **B)** Ubc12-C111S-expressing neurons were severly impaired in dendrite formation and growth compared to control neurons. Scale bars represent 100 µm. Filled white arrowheads mark cell bodies, white arrows mark apical dendrites of layer V control neurons and open white arrowheads mark Ubc12-C111S-expressing neurons that failed to develop apical dendrites.

To gain more insight into the regulation of dendritic growth by the Nedd8 pathway, we then targeted pyramidal neurons of CA1 in the hippocampus. During brain development CA1 pyramidal neurons establish their characteristic bipolar morphology by extending basal and apical dendrites towards the stratum oriens and stratum radiatum, respectively. During early postnatal development basal and apical dendrites undergo extensive growth and branching. Intraventricular injection and electroporation of the hippocampus in mouse embryos at E14.5 and subsequent analysis of electroporated brains at P21 revealed a strong reduction of dendritic growth of CA1 pyramidal neurons expressing Ubc12-C111S (Figure 43.A). Inhibition of neddylation resulted in decreased total dendritic length and reduced dendritic arborization compared to control neurons, measured via tracing of neurons using the Neurolucida software (mbf Bioscience) and Sholl analysis (Figure 43.B, C), whereas control parameters such as the area of somas was not altered (Figure 43.D). In addition to this general impairment in dendritic growth, CA1 pyramidal neurons expressing Ubc12-C111S displayed a characteristic distortion in their morphology compared to control neurons. The distal ends of their apical dendrites didn’t grow into the stratum lacunosum moleculare, where they would receive synaptic inputs, mainly from the entorhinal cortex (layer III) via the perforant pathway as well as from thalamic nuclei (Figure 43.A, highlighted by the white dashed lines).

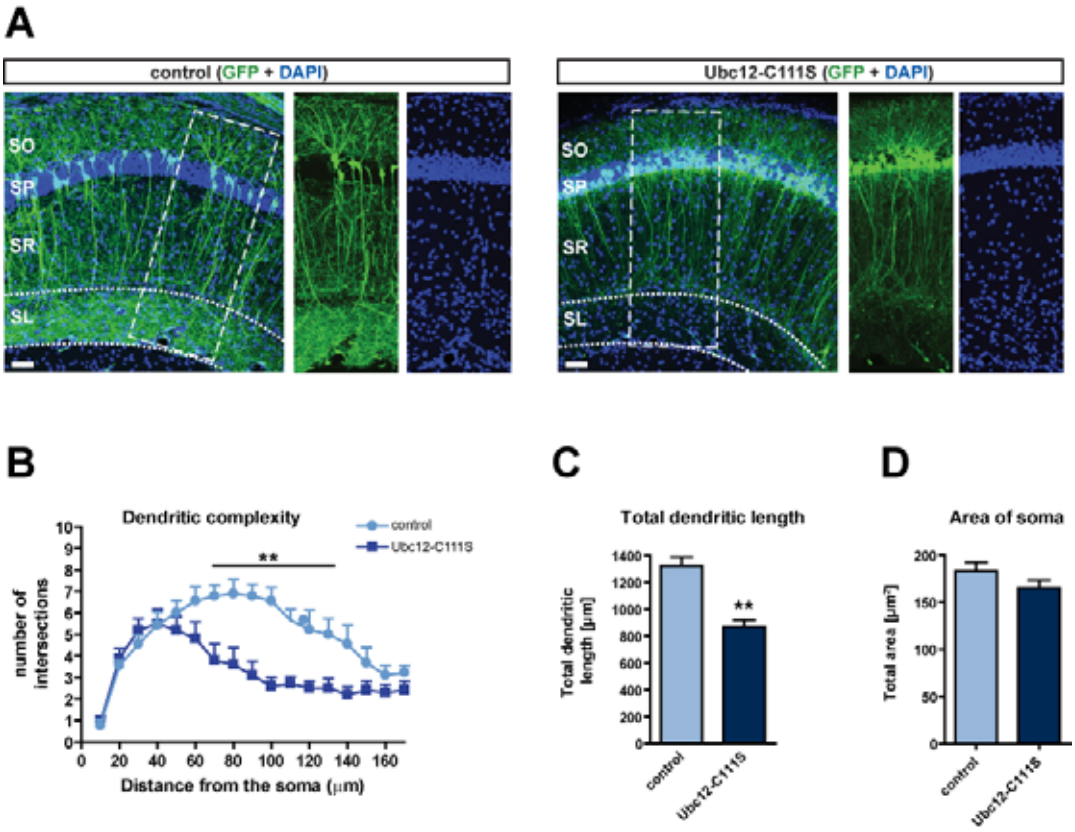


Figure 43: Dendrite formation in the developing hippocampus is impaired by Nedd8 blockade.

A) Mouse embryos were *in utero* electroporated with control or Ubc12-C111S constructs at E14.5 to target the developing hippocampus. Brains were fixed and analyzed on P21. Ubc12-C111S-expressing CA1 pyramidal neurons in the hippocampus showed defects in dendrite development compared to control neurons. Their apical dendrites failed to grow into stratum lacunosum moleculare, denoted by white dashed lines. Scale bars represent 50 µm. SO, stratum oriens, SP, stratum pyramidale, SR, stratum radiatum, SL, stratum lacunosum moleculare. **B)** Dendritic complexity, measured by Sholl analysis, and **C)** total dendritic length (sum of basal and apical dendrites) was decreased in Ubc12-C111S-expressing CA1 pyramidal neurons compared to control neurons, whereas the control measurement of **D)** the soma area was not changed. [Statistical analysis: (**B**) 2 Way ANOVA, treatment x radius interaction, $F_{(16,289)}$=3.5, ***p<0.0001; Bonferroni post-hoc test, **p<0.01; (**C**) and (**D**) t-test, **p<0.01.]

Interestingly, as observed before during cortex development, blocking the neddylation pathway in the hippocampus also produced a slight defect in neuronal migration during development. As expected, in control brains the somas of all electroporated neurons were found in stratum pyramidale, whereas, a few cell bodies of CA1 pyramidal neurons expressing Ubc12-C111S migrated too far into stratum oriens and initiated dendritic development (Figure 44, marked by white arrowheads).

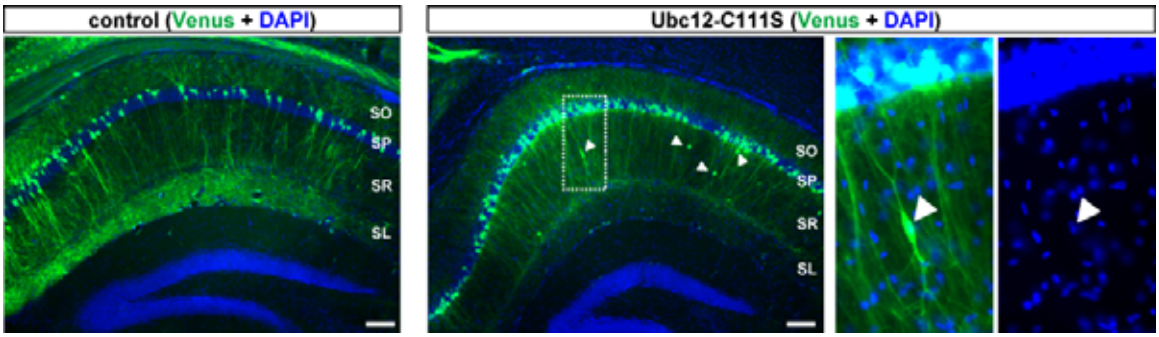


Figure 44: Mismigration of hippocampal neurons upon neddylation blockade.

Mouse embryos were *in utero* electroporated with control or Ubc12-C111S constructs at E14.5 to target the developing hippocampus. Brains were fixed and analyzed on P28. Ubc12-C111S-expressing CA1 pyramidal neurons in the hippocampus showed defects in dendrite development compared to control neurons. The magnification of the boxed region shows a mismigrated Ubc12-C111S-expressing cell. White arrowheads are marking the cell bodies of Ubc12-C111S-expressing neurons, that mismigrated into stratum radiatum. SO, stratum oriens, SP, stratum pyramidale, SR, stratum radiatum, SL, stratum lacunosum moleculare. Scale bars represent 100 µm.

These experiments clearly indicate that the neddylation pathway is necessary for normal migration and dendritic growth and arborization of pyramidal neurons during cortical and hippocampal development.

4.4.3. Neddylation is required for dendrite formation of granule neurons during adult neurogenesis in the dentate gyrus

To establish the Nedd8 pathway as a key-regulator of dendrite development *in vivo*, we further analyzed its role in dendrite formation targeting another brain region, with another type of neuron in a different (micro-) environment, using the process of adult neurogenesis as a model to study neuronal development.

Adult neurogenesis refers to the production of new neurons in an adult brain. The two neurogenic niches in the rodent brain are the subgranular zone (SGZ) of the dentate gyrus (DG) and the subventricular zone (SVZ) of the lateral ventricle. In the DG of young adult rodents, thousands of new neurons are generated everyday and this process is dynamically regulated by various internal and environmental factors (Cameron and Mc Kay, 2001; Ming and Song, 2005; Balu and Lucki, 2009). The process of adult

neurogenesis represents an interesting model to study the role of neddylation in basic aspects of neuronal development and neuroplasticity *in vivo*, because it is controlled by well established developmental programs which are different from those controlling embryonic neuronal development. Besides, this approach allows us to characterize in parallel the function of neddylation in different cellular processes during adult neurogenesis: proliferation, migration, maturation and terminal differentiation of the newborn neurons. To achieve this, we stereotactically injected mixtures of two retroviruses into the DG: CAG-Ubc12-C111S-IRES-GFP or CAG-GFP control viruses together with another control virus encoding RFP (CAG-RFP), in collaboration with Dr. Chichung Lie's group at the Helmholtz-Zentrum München. In the adult animal, retroviruses offer a unique advantage by selectively transducing only dividing progenitor cells in the SGZ, thereby keeping local mature glial and neuronal cells unaffected.

Via asymmetric cell division these progenitors give rise to fluorescently labeled newborn neurons migrating into the granule cell layer of the DG, establishing the characteristic unipolar dendrite morphology of granule neurons and eventually integrating into the existing hippocampal circuit. Different parameters of neuron development were analyzed 14 and 96 days post injection (dpi) of retroviruses. At 14 dpi, newborn neurons infected with control viruses were found in the granule cell layer developing a unipolar dendritic tree as expected. Whereas neurons infected with the Ubc12-C111S virus displayed strong distortions and impairments in dendrite formation. Analysis of dendrite tracings revealed a severe decrease in total dendritic length as well as a reduction of dendritic complexity measured by the number of branch nodes per dendrite (Figure 45.A). Further morphological analysis showed that inhibition of Nedd8 resulted in the development of abnormal bi- or multi-polar dendrites and/or axonal processes of granule neurons compared to control neurons (Figure 45.B). However, at 14 dpi, Ubc12-C111S-GFP-transduced cells were positively stained with the immature neuron marker doublecortin (DCX), confirming their neuronal identity, suggesting that neuronal differentiation remained unaffected (Figure 45.C).

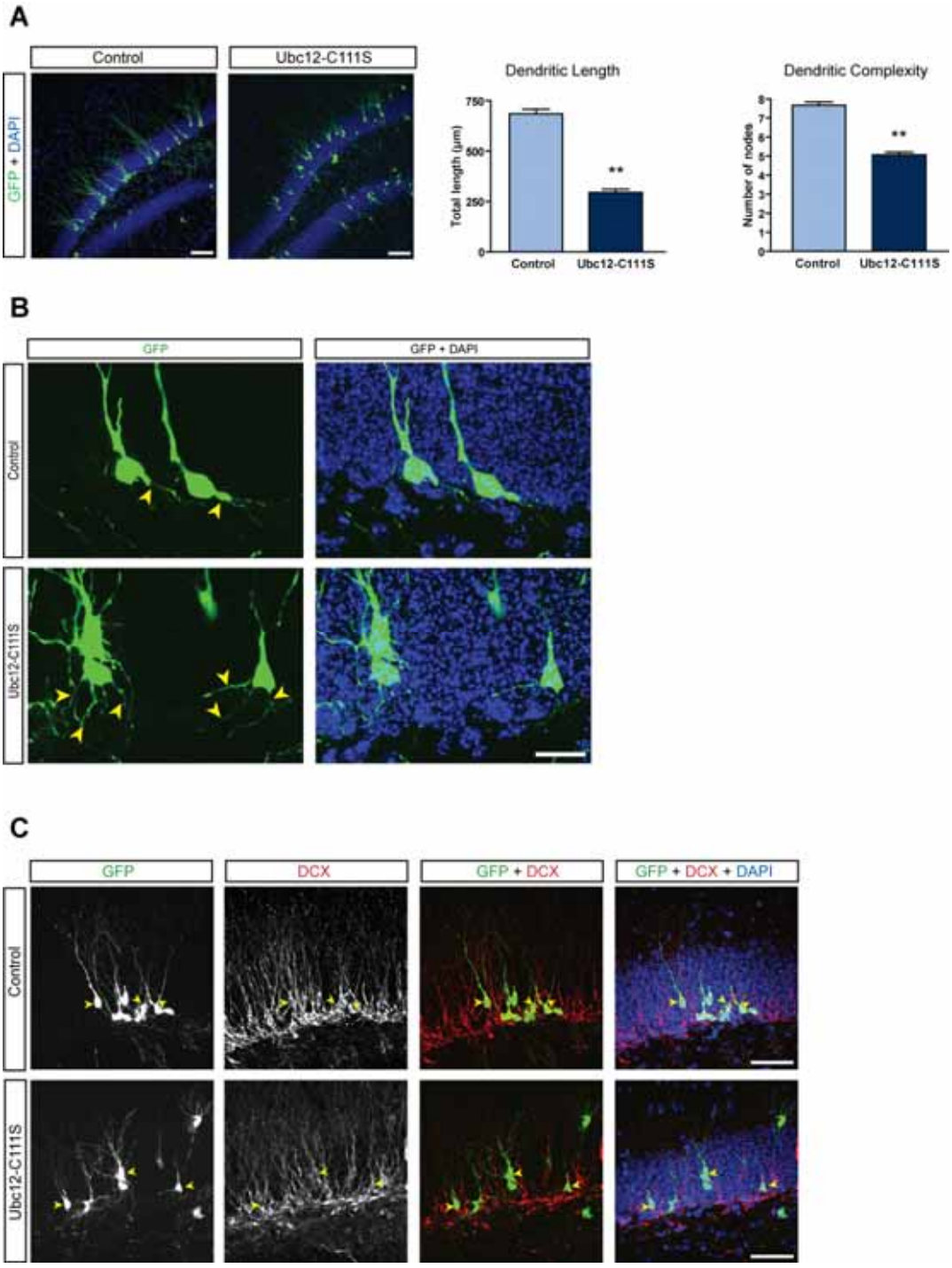

A
Control
Ubc12-C111S
GFP + DAPI
Dendritic Length
Total length (µm)
750
500
250
0
**
Control
Ubc12-C111S
Dendritic Complexity
Number of nodes
**
Control
Ubc12-C111S
B
GFP
GFP + DAPI
Control
Ubc12-C111S
C
GFP
DCX
GFP + DCX
GFP + DCX + DAPI
Control
Ubc12-C111S

Figure 45: Nedd8 controls dendrite formation of newborn granule neurons in the dentate gyrus of adult mice.

Adult mice were stereotactically injected with CAG-GFP or CAG-Ubc12-C111S-IRES-GFP retroviruses to label newborn neurons in the dentate gyrus. (The retroviruses were produced in collaboration with Dr. Chichung Lie at the Helmholtz-Zentrum München.) **A)** At 14 dpi (days post injection), newborn granule neurons expressing Ubc12-C111S displayed strong morphological defects. Neurons were traced and total dendritic length and dendritic complexity (number of nodes) were anaylzed using Neurolucida software (mbf Bioscience). **B)** Higher magnification images of control and Ubc12-C111S-expressing newborn neurons at 14 dpi. Blocking neddylation resulted in the formation of bi- and multipolar dendrites and misprojecting axons (marked by yellow arrowheads). **C)** Immunofluorescence staining against DCX, a marker of immature neurons, confirmed the neuronal identity of GFP-labeled control and Ubc12-C111S-expressing cells (marked by yellow arrowheads). Scale bars represent: in (**A**) 50 µm; in (**B**) 20 µm; in (**C**) 50 µm. [Statistical analysis: t-test, **p<0.01.]

During neuronal maturation, newborn granule neurons have to migrate through the granule cell layer of the DG, from the SGZ towards the more apical regions of the granule cell layer. The granule cell layer can be artificially divided into an inner, middle and outer zone as shown in Figure 46.A. Counting of neurons at 14 dpi revealed that the majority of cell bodies of control neurons migrated a short distance and were found in the inner zone as expected. In contrast, inhibition of neddylation led to a defect in migration, manifested in a shifted distribution of newborn neurons in the three zones. A significant fewer number of neurons expressing Ubc12-C111S stopped the migration in the inner zone, instead they continued migrating into the middle and outer zone of the granule cell layer (Figure 46.A). Interestingly, some newborn neurons expressing Ubc12-C111S even migrated out of the granule cell layer as shown in Figure 46.B at 96 dpi.

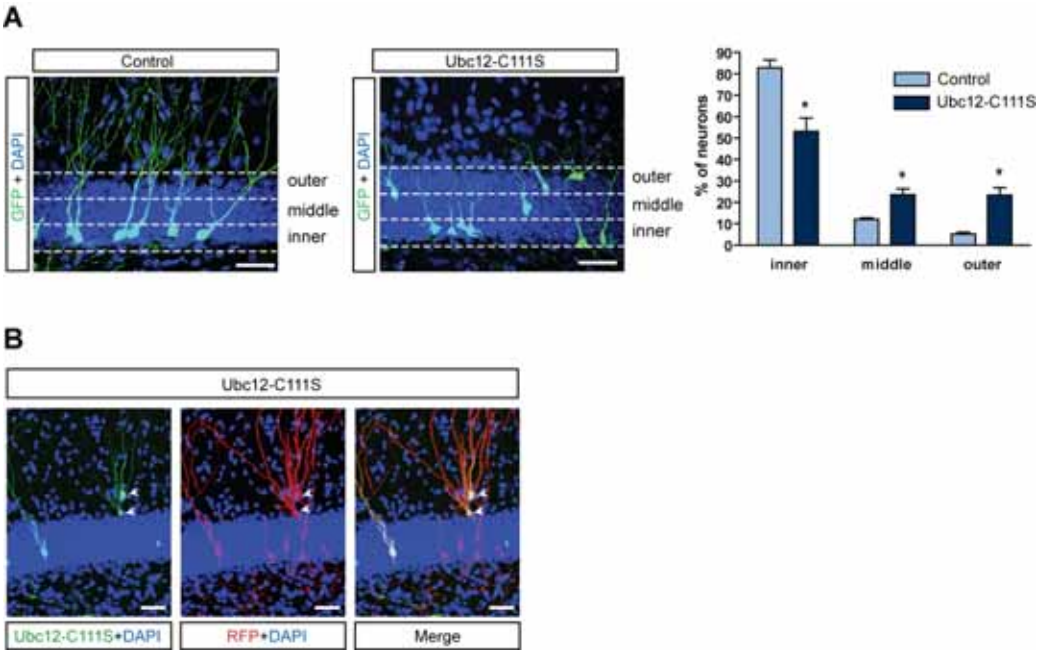


Figure 46: Inhibition of Nedd8 disturbs migration of newborn granule neurons.

A) The number of cell bodies of newborn neurons in three zones of the granule cell layer of the DG (inner, middle and outer zone), were counted in brains injected with control and Ubc12-C111S-expressing retroviruses at 14 dpi. Analysis revealed a distorted migration of Ubc12-C111S-expressing newborn neurons compared to control neurons. **B)** At 96 dpi, newborn neurons transduced with Ubc12-C111S-expressing retroviruses migrated out of the granule cell layer of the DG (marked by white arrowheads). Scale bars in (**A**) represent 35 µm; scale bars in (**B**) represent 50 µm. [Statistical analysis: t-test, *p<0.05.] (The retroviruses were produced in collaboration with Dr. Chichung Lie at the Helmholtz-Zentrum München.)

Due to the infection properties of the retroviruses used, co-injection of GFP and RFP coding viruses did not result in 100% co-infection rates. Instead similar proportions of neurons expressed either GFP, RFP or both fluorescent proteins, that allowed the quantification of neuron survival over time by means of analyzing the ratio of the number of GFP- and RFP-expressing neurons (“yellow”) divided by the sum of “yellow” and RFP-expressing neurons (“red”). The evaluation of neuronal survival over time would not be possible if only GFP-coding viruses were injected because of the variations in efficiency of infection and accuracy of stereotactic injection between different animals. Analysis over time revealed a decrease in survival of Ubc12-C111S-expressing newborn neurons at 14 and 96 dpi compared to control neurons (Figure 47). The observed reduction in neuronal survival upon neddylation blockade might represent a consequence of the strong morphological distortions that impair proper integration into the existing hippocampal circuit, eventually leading to the elimination of these neurons.

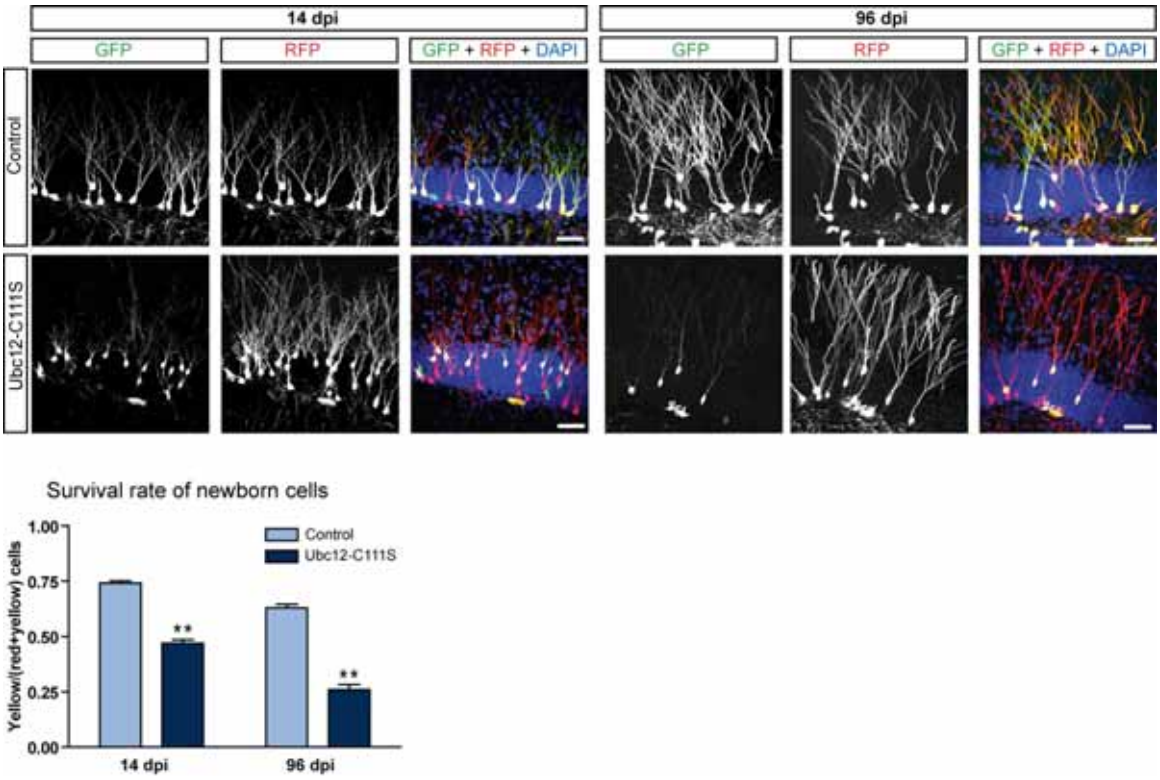


Figure 47: Blocking the Nedd8 pathway decreases survival of newborn neurons in the dentate gyrus.

Survival of newborn neurons in the DG was analyzed at 14 and 96 dpi by the ratio of the number of GFP- and RFP-expressing neurons ("yellow") divided by the sum of "yellow" and RFP-expressing neurons ("red"). Blocking neddylation resulted in decreased survival of newborn neurons compared to control neurons. Scale bars represent 50 µm. [Statistical analysis: t-test, **p<0.01.] (The retroviruses were produced in collaboration with Dr. Chichung Lie at the Helmholtz-Zentrum München.)

By analyzing the function of neddylation in the development of granule neurons during adult neurogenesis in the dentate gyrus, we fully recapitulated the effects on dendrite formation and neuronal migration observed in *in utero* electroporation experiments during the development of pyramidal neurons in the cortex and hippocampus.

In summary, *in utero* electroporation and adult neurogenesis experiments demonstrated that an intact neddylation system is indispensable for the establishment of dendrite patterning of different types of neurons during different developmental time periods. This suggests, that the neddylation pathway participates in cell intrinsic developmental programs and orchestrates core machineries, thereby controlling critical and basic (but not cell type-specific) processes of dendritic development.

4.5. Neddylation controls spine and synapse formation during development

Once axon polarity is established and growth of dendritic trees and axons is advanced, neurons start to establish synaptic contacts among each other. The most accepted hypothesis of synapse formation in principal neurons of neo- and allocortical structures states that during development, long, thin and motile structures called filopodia emerge from dendrites and explore the neuropil seeking for presynaptic contacts in order to establish new synapses (Yuste and Bonhoeffer, 2004). Thus, during the 2nd and 3rd week of development of hippocampal neurons in culture, extensive filopodia formation precedes the time of mature mushroom-like spine development (Dailey and Smith, 1996; Ziv and Smith, 1996; Fiala et al., 1998; Maletic-Savatic et al., 1999; Marrs et al., 2001; Portera-Cailliau et al., 2003; de Roo et al., 2008). *In vivo,* in the mouse brain the peak of massive synaptogenesis and spine formation occur during the first three weeks of postnatal development (Galofre and Ferrer, 1987; Petit et al., 1988; Miller, 1988; Lubke and Albus, 1989; Zecevic et al., 1989; Zecevic and Rakic, 1991; Eckenhoff and Rakic, 1991; Bourgeois and Rakic, 1993; Anderson et al., 1995; Zhang and Benson, 2000). To analyze the function of Nedd8-conjugation in spine development we combined different *in vitro* and *in vivo* approaches. We used genetic and pharmacological inhibition of neddylation in primary neurons to first establish and characterize the role of Nedd8 during spine development, followed by *in vivo* experiments to confirm and extend the function of neddylation during spine formation in the cortex and hippocampus in the developing mouse brain, applying the *in utero* electroporation technique of mouse embryos.

4.5.1. Inhibition of Nedd8 conjugation in primary neurons results in impaired spine formation

Different genetic and pharmacological approaches used to inhibit neddylation during the critical time window of spinogenesis and synaptogenesis in primary hippocampal and cortical neurons (between DIV 12 to 20) resulted in severely impaired spine formation. Hippocampal neurons expressing either Ubc12-C111S or shRNA constructs against Nedd8 and Ubc12 displayed an absence of mature mushroom-like spines and an increased number of long dendritic filopodia. A similar filopodia-phenotype was observed when neddylation was blocked pharmacologically in hippocampal neurons by MLN4924 treatment (Figure 48).

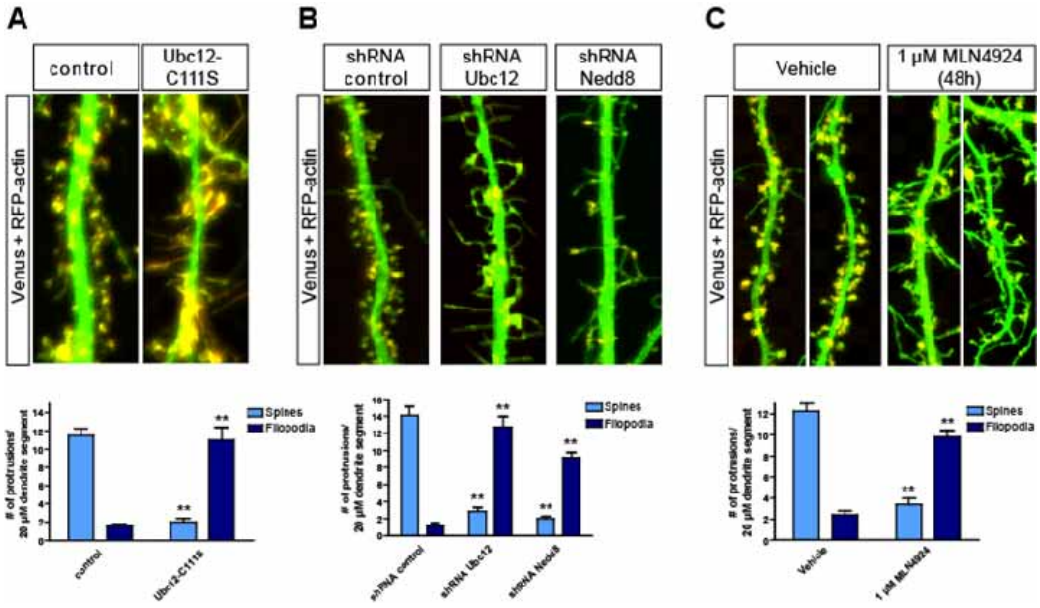


Figure 48: Neddylation critically controls spine development in primary neurons.

Neddylation was blocked in primary hippocampal neurons by different techniques between DIV 12 and 20. The number of dendritic protrusions (number of spines and filopodia) was counted per 20 µm dendrite segments. **A)** Neurons expressing Ubc12-C111S, **B)** neurons expressing shRNAs to knockdown Ubc12 and Nedd8 **C)** as well as neurons treated with 1 µM MLN4924 for 48 h showed impaired spinogenesis compared to their respective controls. Neddylation blockade resulted in a decreased number of spines and an increased number of filopodia. [Statistical analysis: (**A**) 2 Way ANOVA, treatment x dendritic protrusion interaction, $F_{(1,34)}$=133.9, ***p<0.0001; Bonferroni post-hoc test, **p<0.01; (**B**) 2 Way ANOVA, treatment x dendritic protrusion interaction, $F_{(2,50)}$=150.6, ***p<0.0001; Bonferroni post-hoc test, **p<0.01; (**C**) 2 Way ANOVA, treatment x dendritic protrusion interaction, $F_{(1,44)}$=198.3, ***p<0.0001; Bonferroni post-hoc test, **p<0.01.]

Furthemore, the effects of neddylation blockade on spine formation were also verified in cortical neurons, indicating that first neddylation is required in general for proper spine development in neurons and second that the function of Nedd8 during spinogenesis is unique among the UBLs, since blockade of the sumoylation pathway via expression of a dominant-negative version of the SUMO-conjugating enzyme Ubc9 (Ubc9-C93S) did not disturb spine formation (Figure 49).

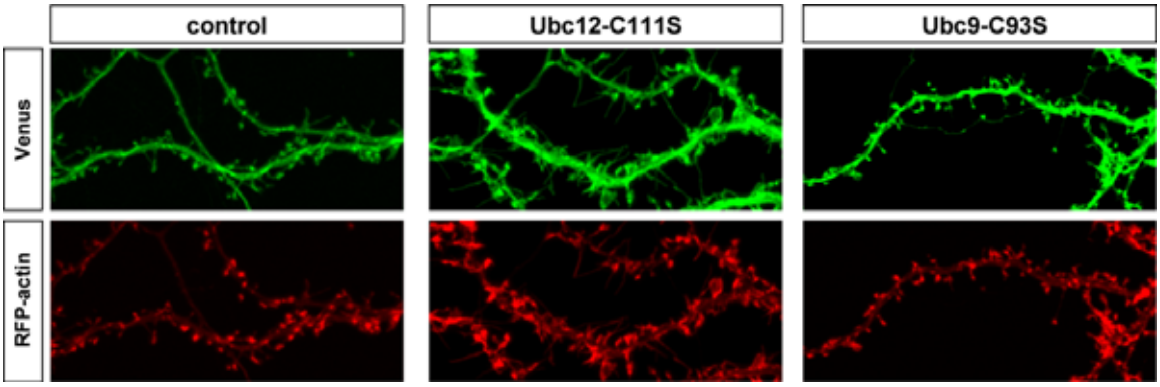


Figure 49: Inhibition of neddylation impairs spine formation in cortical neurons.

Primary cortical neurons were transfected with the indicated plasmids at DIV 13. Neurons were fixed at DIV 19 and spine development was analyzed. Inhibition of the Nedd8 pathway via expression of Ubc12-C111S resulted in increased filopodia formation, whereas blockade of the SUMO pathway via transfection of Ubc9-C93S didn't impair the formation of mature mushroom-shaped spines.

4.5.2. Synaptogenesis and neurotransmission are affected by neddylation blockade

Spine formation is a process tightly coupled to synaptogenesis. To assess the contribution of neddylation in synapse formation we performed immunofluorescence stainings to label pre-synapses with specific markers, e.g. Synapsin. In control neurons almost every spine established a synaptic contact, shown by the close proximity of post-synaptic RFP-actin puncta and pre-synaptic Synapsin clusters. In contrast, the long filopodia of Ubc12-C111S-expressing neurons failed to establish any synaptic contacts, shown by the absence of Synapsin clusters on the tips of filopodia, leading to a general decrease in number of synaptic contacts. However, a number of synapses that formed directly on the dendritic shaft of Ubc12-C111S-transfected neurons was observed, indicating that neddylation is specifically relevant for the establsihment of new synapses on dendritic spines (Figure 50.A). The observed morphological impairments in spine and synapse development evoked by neddylation blockade were functionally correlated with altered synaptic neurotransmission. To characterize electrophysiologically the neurotransmission properties of these neurons, we recorded miniature Excitatory Postsynaptic Potentials (mEPSPs) in collaboration with Prof. Dr. Gerhard Rammes at the MPI of Psychiatry. We found a strong reduction in the frequency of spiking in Ubc12-C111S-transfected neurons compared to control cells. This functional impairment in spiking frequency would reflect the decreased number of synapses. The mean amplitude of spikes was not significantly changed, suggesting that the synapses on the dendritic shafts are not only present, as demonstrated by immunostaining experiments, but also electrophysiologically functional (Figure 50.B).

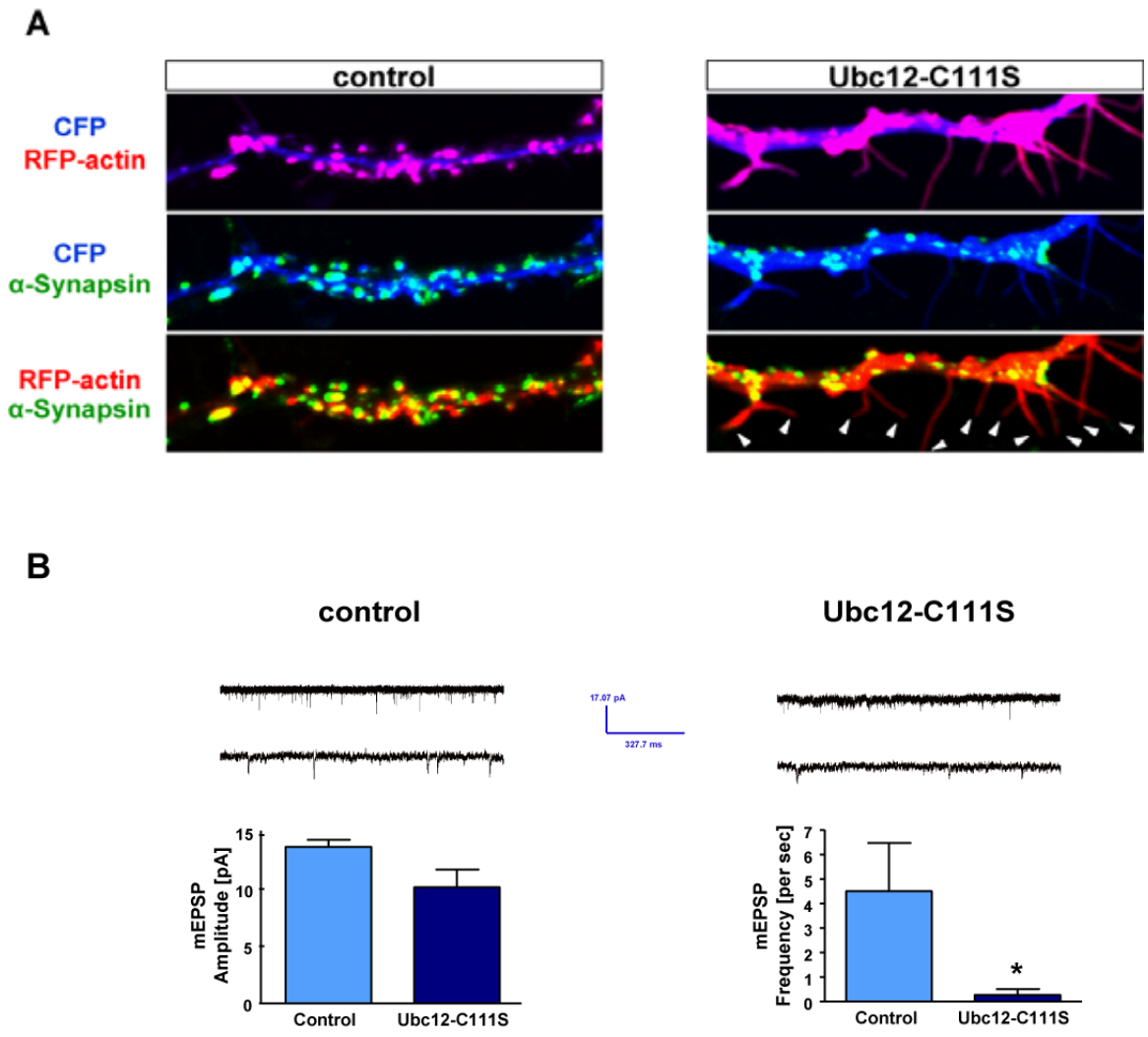


Figure 50: The Nedd8 pathway controls synaptogenesis and neurotransmission in primary neurons.

A) Blocking neddylation impaired synaptogenesis. Primary hippocampal neurons were transfected at DIV 13 with the indicated plasmids. At DIV19, neurons were fixed and pre-synapses were immunofluorescently stained with α-Synapsin antibodies. The long and thin filopodia of neurons expressing Ubc12-C111S were not able to achieve synaptogenesis; pre-synaptic boutons marked by α-Synapsin (green) didn't show the classical apposition with the post-synapsis, marked by RFP-actin (red) as clearly observed in control-transfected neurons. Nevertheless, a reduced number of synaptic contacts were established directly on the dendritic shafts of Ubc12-C111S-expressing neurons. **B)** Inhibition of neddylation affects neurotransmission. Electrophysiological patch-clamp recordings of mEPSPs, done in collaboration with Prof. Dr. Gerhard Rammes, showed a decrease in spiking frequency accompanied by normal mean amplitudes in Ubc12-C111S expressing neurons compared to control neurons. The reduction in frequency of mEPSPs reflects the reduced number of synapses in Ubc12-C111S-expressing neurons, while the normal amplitude of mEPSPs indicates that the dendritic shaft synapses are functional. [Statistical analysis: t-test, *$p<0.05$.]

In summary these experiments demonstrated a specific role for neddylation in spine development and synaptic function.

4.5.3. Filopodia of Ubc12-C111S-expressing neurons display dynamic behavior

Fast dynamics and high motility are characteristics of filopdia *in vitro* and *in vivo* supporting the hypothesis that filopdia are needed during development to explore the neuropil and search for presynaptic partners (Dailey and Smith, 1996; Portera-Cailliau et al., 2003). To analyze the dynamic behavior and motility of fliopodia formed during neddylation blockade we repeatedly imaged living neurons to record fast confocal time lapse movies. Fast time-lapse movies of neddylation-deficient neurons showed that the filopodia exhibited their characteristic, highly motile dynamics, forming and retracting within minutes, implying that the lack of spines is not due to a reduction in the motility that filopodia need in order to encounter synaptic partners (Figure 51). On the contrary, these filopodia seem to be unable to form stable structures that are required to procede in synaptogenesis. As expected, spines of control neurons were less dynamic and showed relatively little motility in this brief time intervals (90 s) compared to filopodia structures (Figure 51).

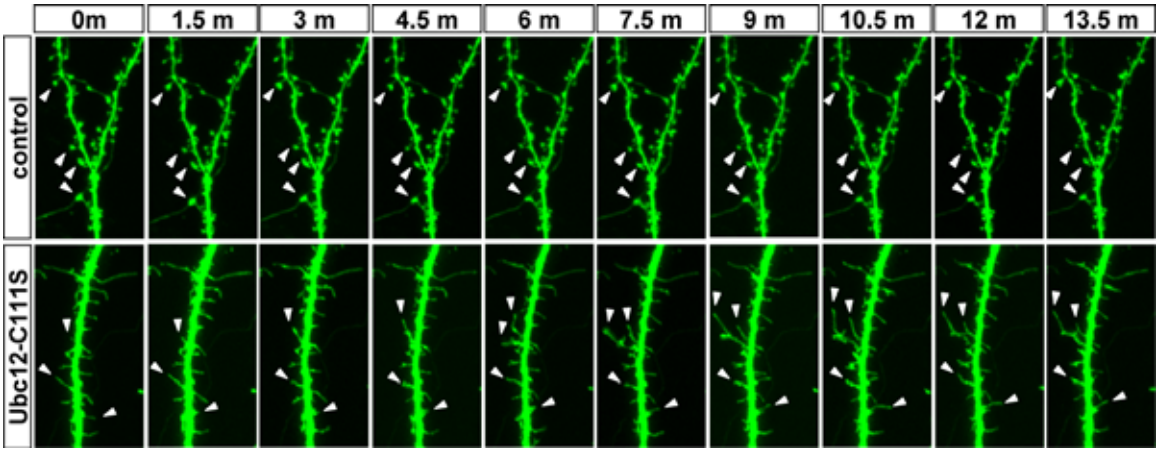


Figure 51: Dynamic behavior of filopodia of neddylation-deficient neurons.

Primary hippocampal neurons were transfected at DIV 14 with control or Ubc12-C111S and myr-Venus (membrane-bound Venus) constructs. Fast confocal time lapse movies, recorded at DIV 18 with a time interval of 90 s, revealed a highly dynamic behavior of filopodia of Ubc12-C111S-expressing neurons, and rather stable mature spines of control neurons.

These results led to the hypothesis that filopdia-like structures of neddylation-deficient neurons remain highly dynamic and motile and that they are probably impaired in stabilizing synaptic contacts.

4.5.4. Nedd8 effects on spine development are independent of cullin E3 ligases

To analyze the contribution of cullin E3 ligases, as known targets of Nedd8, in mediating the effects of neddylation on spine formation, we transfected primary hippocampal neurons with dominant-negative versions of cullin 1 to 5, shRNAs against Rbx1 as well as dominant-negative Rbx1 constructs (Rbx1-C42S-C45S and Rbx1-C75S) (Figure 52). None of these transfections, that inhibit cullin E3 ligase function, could recapitulate the filopdia-like phenotype induced by inhibition of the Nedd8 pathway. Therefore, we proposed that novel, yet unidentified, neuronal targets of Nedd8 are mediating the effects of neddylation during spine and synapse formation.

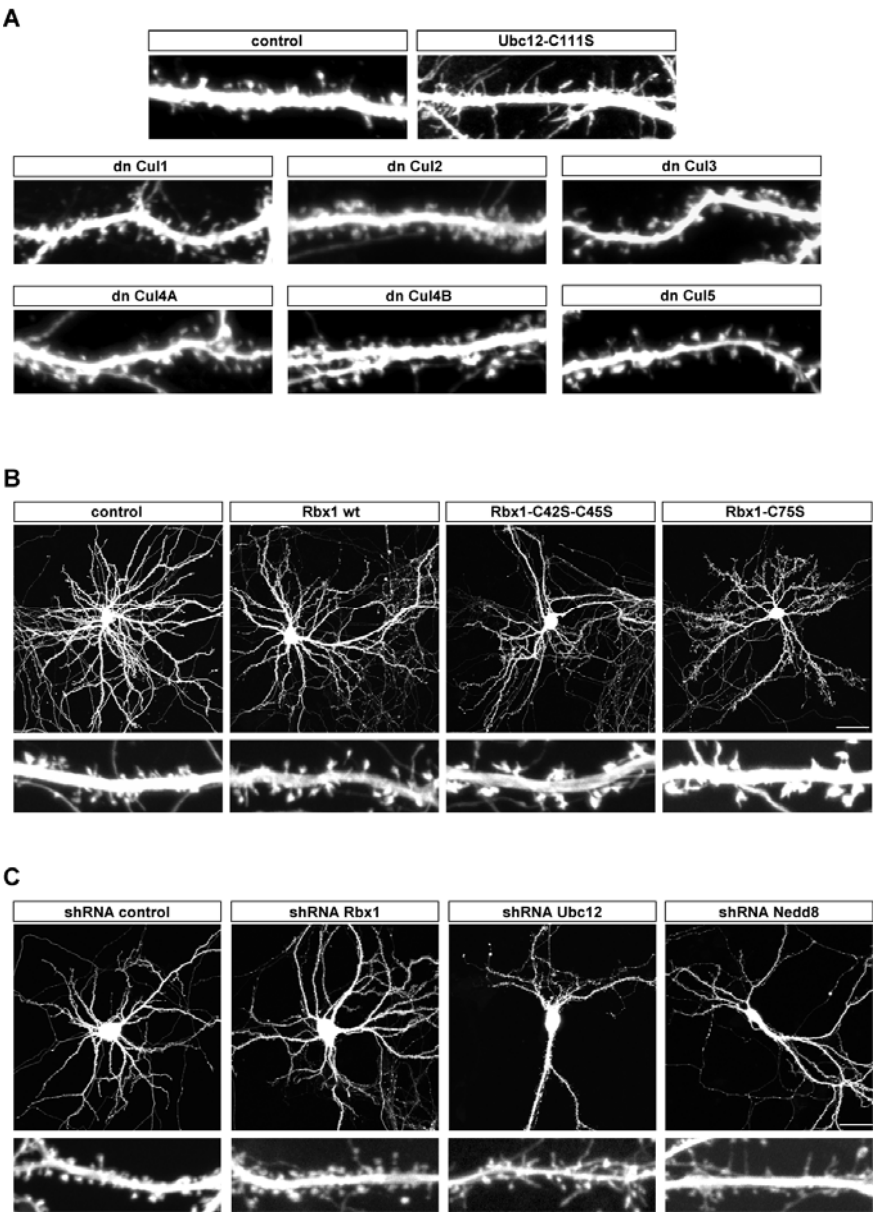


Figure 52: Effects of neddylation on spine formation are independent of cullin E3 ligases.

A) Primary hippocampal neurons were transfected with the indicated plasmids and eGFP constructs at DIV 13. Neurons were fixed at DIV 19 and spine development was analyzed. Expression of (**A**) dominant-negative cullins and (**B**) shRNA constructs to down-regulate Rbx1 or (**C**) dominant-negative Rbx1 constructs didn't arrest spine formation at the filopdia stage as observed by blocking neddylation via Ubc12-C111S or shRNA constructs targeting Ubc12 and Nedd8, indicating that Nedd8 affects spine development independently of cullin RING ligases. Scale bars represent 50 µm.

4.5.5. Neddylation controls spine development *in vivo*

To further confirm the role of the Nedd8 pathway as a new regulator of spine development *in vivo*, we studied spine formation in the hippocampus and cortex during postnatal brain development of *in utero* electroporated mouse embryos. At E14.5 mouse embryos were *in utero* microinjected with plasmid DNA and electroporated to target pyramidal neurons of the hippocampus and cortex, and brains were fixed and analyzed at different postnatal stages.

Analysis of early postnatal time points, at P5 for cortical neurons and P21 for hippocampal neurons, revealed a higher abundance of long filopodia on apical dendrites of cortical layer II/III pyramidal neurons electroporated with Ubc12-C111S compared to controls (Figure 53.A) as well as on apical dendrites of hippocampal CA1 pyramidal neurons expressing Ubc12-C111S compared to control-electroporated neurons (Figure 53.B). However due to methodological and technical limitations the preservation of dendritic filopdia during fixation of brain tissue is limited and challenging. Therefore, filopodia are much less abundant in fixed brain slices than in primary neurons or in living organotypic slice cultures (Yuste, 2010). Since inhibition of neddylation led to severe migratory and dendritic defects in the cortex as was shown in 4.4.2, we decreased the amount of Ubc12-C111S electroporated in this experiment to diminish and weaken impairments in migration and dendritic growth in order to be able to evaluate generation of filopodia and spines.

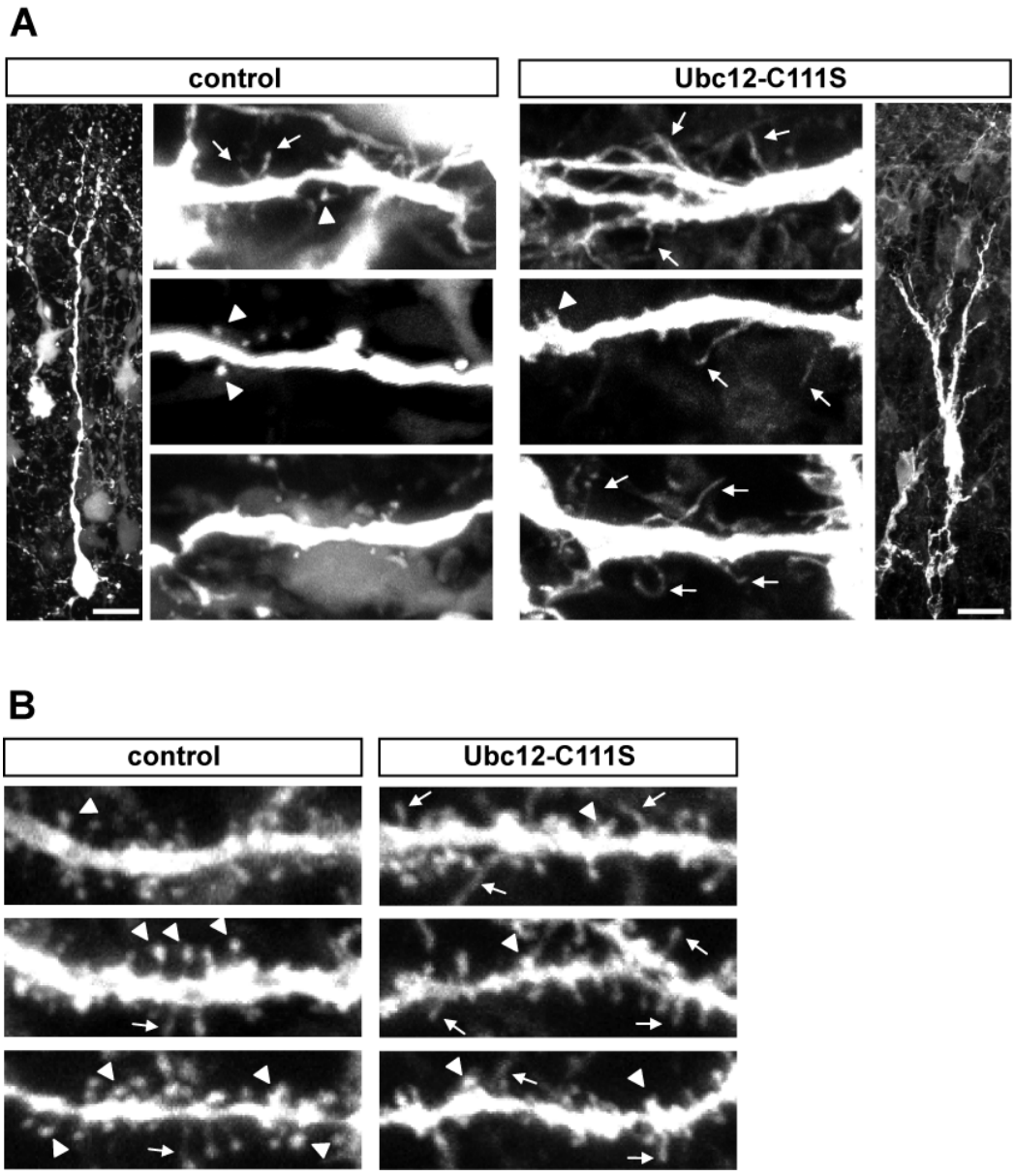


Figure 53: The Nedd8 pathway regulates early spine development *in vivo.*

A) At E13.5 mouse embryos were microinjected with control or Ubc12-C111S plasmids and myr-Venus (membrane-bound Venus) plasmid and electroporated to target the upper layers (II/III) in the developing neocortex. At P5, brains were fixed and analyzed. Long and thin filopodia were more abundant on apical dendrites of Ubc12-C111S-expressing cortical neurons (marked by white arrows), whereas control pyramidal neurons possessed less and shorter filopodia and already developed spines (marked by white arrowheads). Scale bars represent 20 µm. **B)** At E14.5 mouse embryos were microinjected with control or Ubc12-C111S plasmids and myr-Venus (membrane-bound Venus) plasmid and electroporated to target pyramidal neurons of the CA1 region in the developing hippocampus. At P21, brains were fixed and analyzed. Filopodia were more abundant on apical dendrites of hippocampal CA1 pyramidal Ubc12-C111S-expressing neurons (marked by white arrows) compared to control electroporated neurons, which developed mushroom-like spines (marked by white arrowheads) and only rarely showed filopodia.

Four weeks after birth the wave of massive spinogenesis and synaptogenesis during early postnatal development already declines and synaptic circuits are being established and refined. At P28, we observed that spine formation, represented by the number of spines, on both basal and apical dendrites of hippocampal CA1 pyramidal neurons expressing Ubc12-C111S was severly reduced compared to control-electroporated neurons (Figure 54).

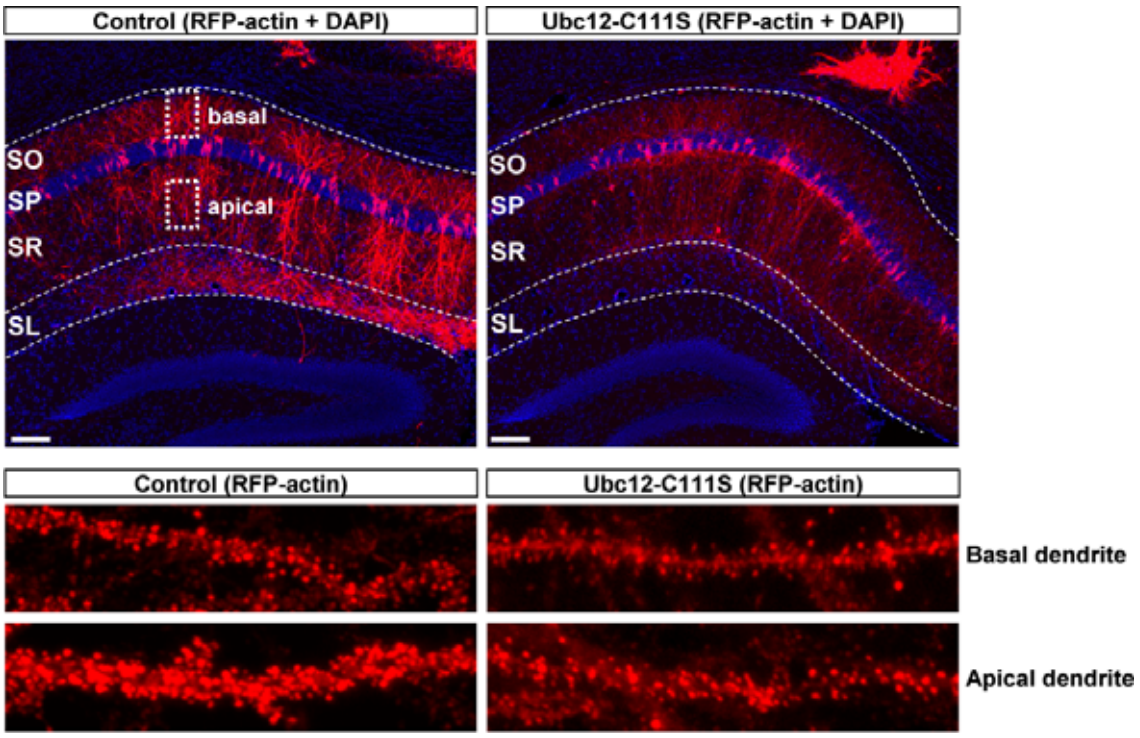


Figure 54: Neddylation controls spine formation *in vivo* in adolescent mice.

At E14.5 mouse embryos were microinjected with control or Ubc12-C111S plasmids and RFP-actin plasmid and electroporated to target pyramidal neurons of the CA1 region in the developing hippocampus. At P28, brains were fixed and spine development was analyzed. Ubc12-C111S-expressing hippocampal CA1 pyramidal neurons showed a reduced number of spines on basal and apical dendrites compared to control-electroporated neurons. Scale bars represent 200 µm. SO, stratum oriens, SP, stratum pyramidale, SR, stratum radiatum, SL, stratum lacunosum moleculare.

In summary, neuronal cell culture and *in vivo* experiments clearly demonstrated that neddylation critically controls spine and synapse formation during neuronal development independently of E3 ubiquitin ligases.

4.6. Neddylation regulates spine and synapse stability in mature neurons of the adult brain

In the previous chapters we described specific roles of the Nedd8 pathway during brain development. Taking into consideration the high and persistent expression of members of the neddylation system in the adult mammalian brain (see 4.1), we then explored the subcellular distribution and putative function of Nedd8 conjugation in the mature synapse.

4.6.1. Neddylation in the synapse of the adult brain

To characterize neddylation in pre- and post-synaptic compartments, we separated specific membrane fractions of adult mouse hippocampi by differential ultracentrifugation. Immunoblotting of the isolated samples revealed that Ubc12 and Ubc12-Nedd8 conjugates are present in all different brain and synaptosomal fractions as well as in the pre-synaptic vesicle and, since it is a small enzyme, in the Triton-soluble fraction of the post-synaptic density (PSD) (Figure 55). Neddylated proteins, detected by α-Nedd8 Western blot, were found in all isolated brain fractions, including pure synaptosomes, pre-synaptic vesicles and the PSD purifications. Furthermore, we observed changing patterns of neddylated targets between the different synaptic samples, suggesting that in each synaptic compartment specific proteins are differentially neddylated (Figure 55). The purity of the isolated fractions was confirmed by Western blotting against Synaptophysin, a protein specifically present in the pre-synapse and PSD-95, a protein exclusively present in the post-synapse (Figure 55).

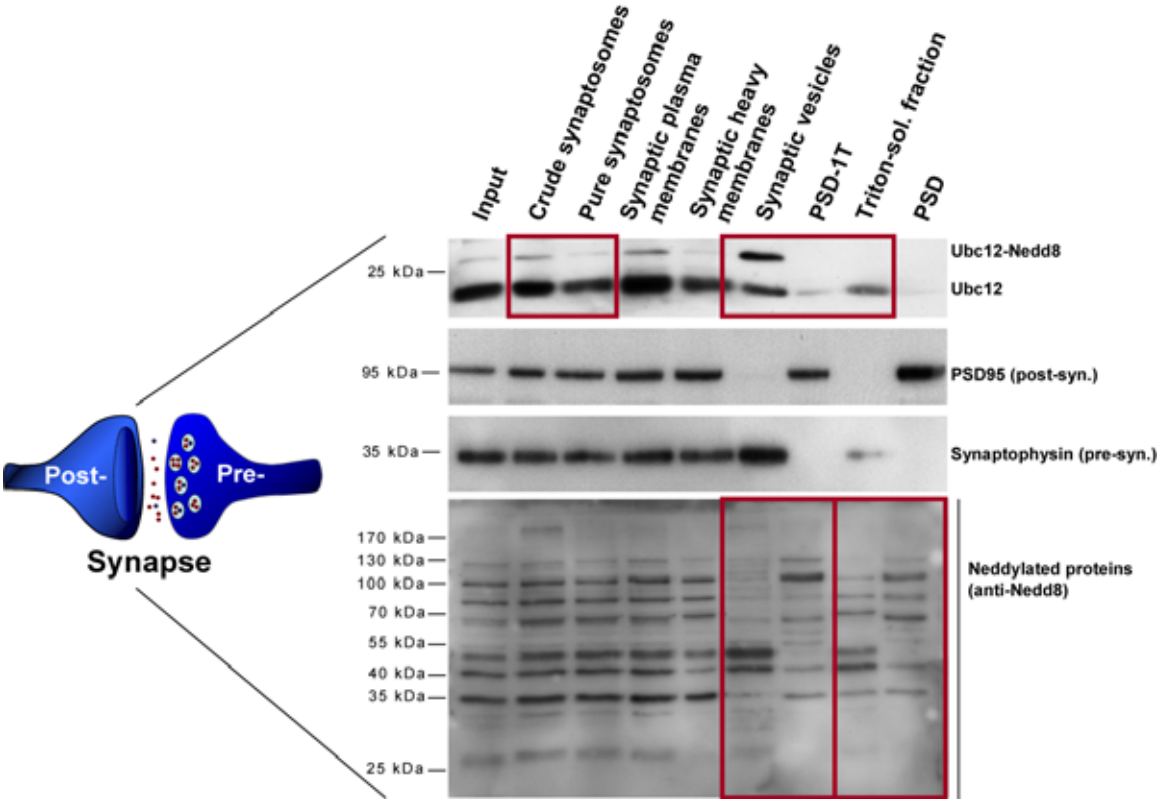


Figure 55: Ubc12 and neddylated proteins are present in pre- and post-synaptic compartments in the adult mouse brain.

Hippocampi of adult mice were homogenized, fractionated by differential centrifugation and analyzed by immunoblotting using α-Ubc12, α-PSD-95, α-Synaptophysin and α-Nedd8 antibodies. Red boxes show Ubc12 expression in the different synaptic fractions and differentially neddylated target proteins, present in the different synaptic fractions.

Once established during development, synapses were shown to be maintained for long periods of time (Grutzendler et al., 2002; Zuo et al., 2005; Holtmaat et al., 2005; Yang et al., 2009a). To uncover possible functions of Nedd8 in the regulation of synapse stability and maintenance we performed *in vitro* and *in vivo* experiments using inducible genetic tools or pharmacological inhibition combined with live-cell imaging microscopy.

4.6.2. Regulation of spine turn-over by Nedd8 in primary neurons

To study the function of Nedd8 in spine stability and maintenance, neddylation has to be blocked in neurons with already formed mature spines. Nevertheless, transfection efficiency declines dramatically in mature neuronal cultures beyond DIV 20. To overcome this technical difficulty, we generated a floxed-STOP tamoxifen-inducible Ubc12-C111S expression vector, that when co-expressed with a Cre-ERT2 plasmid (Matsuda and Cepko, 2007) allows the inducible expression of Ubc12-C111S after Tamoxifen treatment. Application of 1 µM Tamoxifen for 48 h was sufficient to induce Cre-mediated excision of the floxed STOP cassette in the inducible vectors and to achieve high and robust expression of Ubc12-C111S as well as reporter genes, such as eGFP (Figure 56).

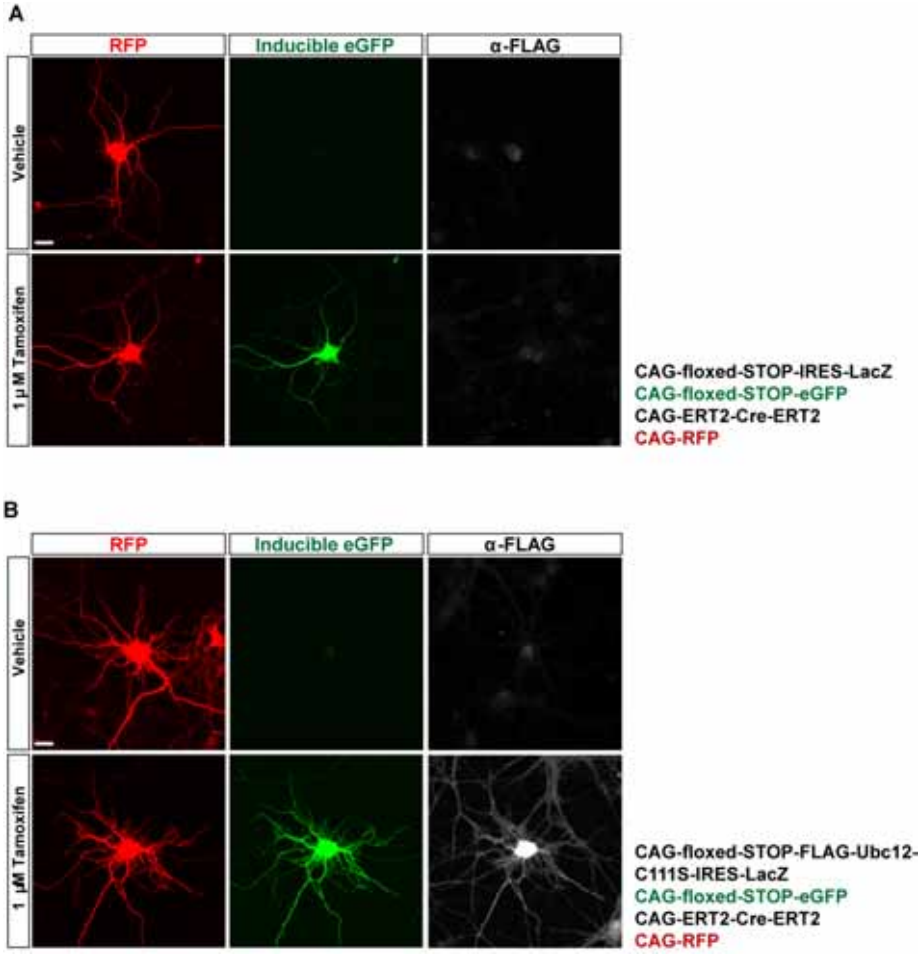


Figure 56: Validation of the tamoxifen-inducible plasmid system.

Primary hippocampal neurons were transfected at DIV 10 with the tamoxifen-inducible plasmids, ERT2-Cre-ERT2 and RFP constructs. **A)** Control transfection with CAG-floxed-STOP-IRES-LacZ and CAG-floxed-STOP-eGFP and **B)** inducible Ubc12-C111S transfection with CAG-floxed-STOP-FLAG-Ubc12-C111S-IRES-LacZ and CAG-floxed-STOP-eGFP. Application of 1 µM Tamoxifen for 48 h was sufficient to induce Cre-mediated excision of the floxed-STOP cassettes in the inducible vectors and to achieve expression of Ubc12-C111S, shown by α-FLAG immunostaining, and the eGFP reporter gene. In the absence of Tamoxifen, no leaky expression of Ubc12-C111S or eGFP was observed. Scale bars represent 20 µm.

After transfection with calcium-phosphate protocols, repeated live cell imaging of GFP- and RFP-based reporters in mature (> DIV 20) neurons with a confocal microscope systematically triggered neuronal death within 24 h, most probably elicited by phototoxicity. Neither very low laser settings, nor very fast imaging, nor short exposures to the laser, nor extensive testing of different fluorescent proteins, including eGFP, farnesylated-eGFP, eYFP, Venus, myr-Venus, RFP, dsRed and cherry could prevent neuronal death. Therefore, we used an epifluorescence microscope setting (Olympus) to be able to image repeatedly, for up to 6 days, living mature neurons. In order to do so, we acquired images with an ultrasensitive digital camera specially set-up for this purpose. Induction of Ubc12-C111S expression by Tamoxifen application at DIV 22 and concomitant blockade of Nedd8-conjugation, resulted in a reduction of the number of mature spines 48 h after Tamoxifen application, compared to control neurons, in which expression of the β-Galactosidase reporter was induced (Figure 57).

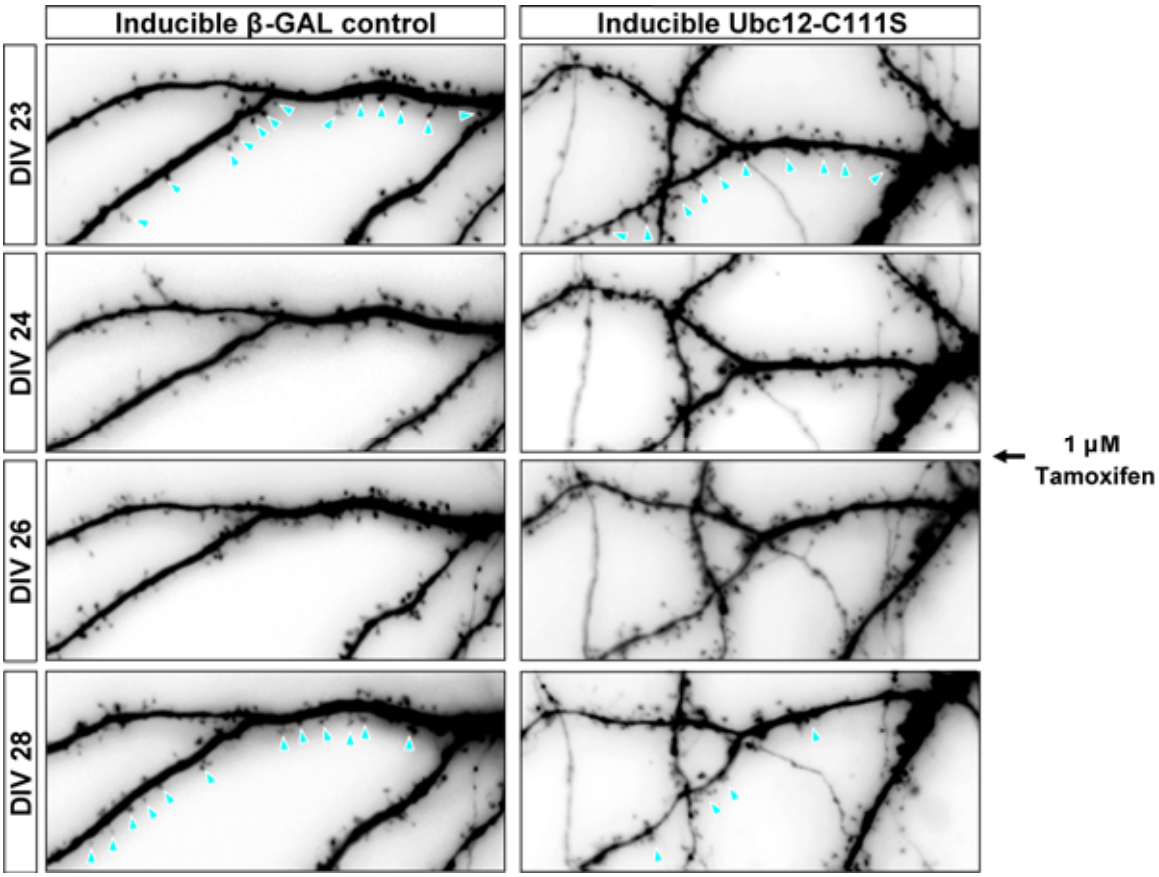


Figure 57: Neddylation blockade reduces spine number in mature neurons.

Primary hippocampal neurons were transfected at DIV 16 with the tamoxifen-inducible plasmids, ERT2-Cre-ERT2 and RFP constructs. **A)** Control neurons, transfected with CAG-floxed-STOP-IRES-LacZ and **B)** neurons transfected with the inducible Ubc12-C111S construct CAG-floxed-STOP-FLAG-Ubc12-C111S-IRES-LacZ were imaged under basal conditions at DIV 23 and 24. Induction of Ubc12-C111S expression by application of 1 µM Tamoxifen for 48 and 96 h resulted in a decreased density of mature spines, whereas β-Galactosidase induction in control neurons did not alter spine numbers.

However, careful analysis of the long-term live cell imaging revealed a high spine turn-over, characterized by elimination of old and formation of new spines, in mature hippocampal neurons in culture within 24 to 48 h. Therefore, a new question had to be formulated at this point: Does Nedd8 conjugation blockade favor the removal of existing spines or instead impede the generation of new spines? To address this question and to resolve more accurately the effects of Ned8 on spine maintenance and stability within narrower time frames, we used the pharmacological Nedd8-inhibitor MLN4924.

4.6.3. Spine stability is affected by MLN4924 treatment

In contrast to the inducible genetic tools presented above, in which the slow onset of Nedd8 inhibition depends on the gradually increasing levels of Ubc12-C111S after tamoxifen-induced transcription of the transgene, pharmacological inhibition of Nedd8 by MLN4924 treatment is able to block neddylation within 30 to 60 min and therefore allows the short-term treatment of neurons to evaluate spine stability and maintenance on the level of single spines with the high resolution of confocal microscopy. Treatment of mature hippocampal neurons in culture at DIV 20 with 1 µM MLN4924 resulted in severe shrinkage of spine head size and loss of spines within 150 min. Interestingly, this process was reversible as shown by the recovery of spine size and growth within 120 min after washout of the Nedd8 inhibitor (Figure 58).

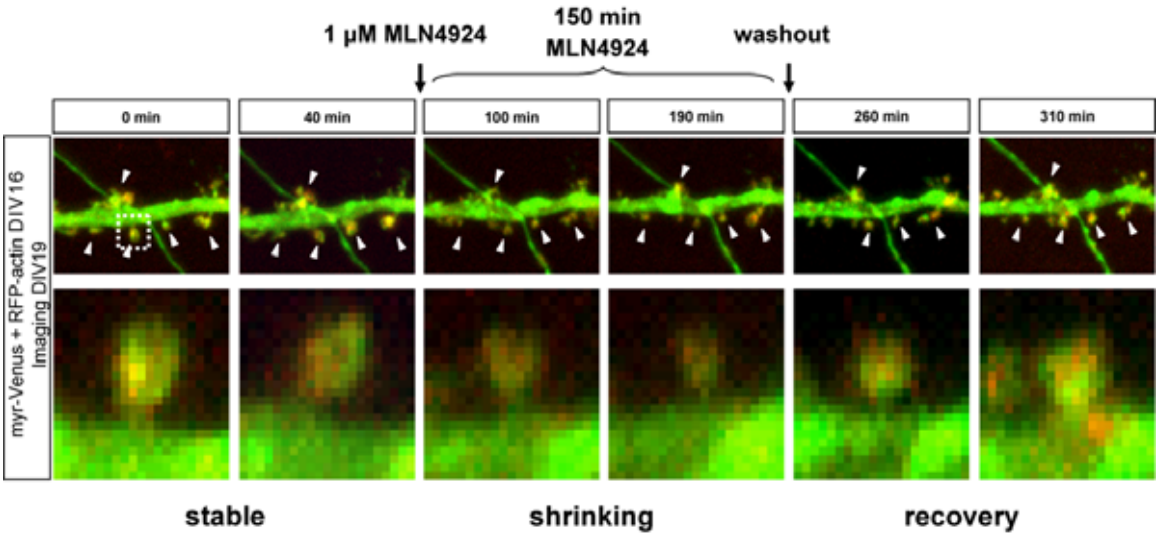


Figure 58: Inhibition of neddylation by MLN4924 treatment results in shrinkage and loss of mature spines.

Primary hippocampal neurons were transfected with myr-Venus and RFP-actin reporter constructs at DIV 16. At DIV 19, repeated live cell imaging of mature mushroom-shaped spines was performed. First, two basal images were acquired with a time interval of 40 min. Blockade of neddylation by treatment with 1 µM MLN4924 resulted in shrinkage of spine heads within 150 min. Washout of the NAE-inhibitor rescued spine head size within 120 min.

In summary, these experiments provided the first evidence that neddylation is required to maintain the structure of spines in primary neurons *in vitro*.

4.6.4. Neddylation controls spine maintenance *in vivo*

To further explore and confirm the function of Nedd8 in spine stability *in vivo* we modified the *in utero* injection and electroporation protocol to permit the tamoxifen-inducible expression of Ubc12-C111S in principal forebrain neurons in CamKIIα-Cre-ERT2 mice. Plasmid DNA, including inducible Ubc12-C111S-IRES-β-Galactosidase or inducible β-Galactosidase control vectors, inducible eGFP and constitutively expressed RFP plasmids, was *in utero* microinjected into the ventricle of E15.5 mouse embryos. After electroporation directed to target neurons in the hippocampus, embryos were allowed to develop. The offspring was genotyped for Cre-ERT2-recombinase, and at P35 (5 weeks) mice were injected twice per day with 1 mg Tamoxifen for 5 consecutive days to induce expression of Ubc12-C111S-IRES-β-Galactosidase or β-Galactosidase and eGFP in principal forebrain neurons of Cre-ERT2-positive mice. At P50, 15 days after the last Tamoxifen injection, adult mice were transcardially perfused, brains were subsequently cut and spines were analyzed.

The inducible plasmids, e.g. inducible eGFP were only expressed after Tamoxifen application in mice expressing Cre-ERT2 (iCre/+) and not in their wild-type littermates (+/+) (Figure 59.A, Figure 60.A), confirming the specificity and the thight regulation of the Cre-ERT2/loxP-based expression system *in vivo*. Tamoxifen-induced expression of Ubc12-C111S in Cre-positive animals resulted in a severe reduction of spines on basal and apical dendrites of hippocampal CA1 pyramidal neurons as well as in granule neurons of the dentate gyrus compared to tamoxifen-injected and inducible Ubc12-C111S-electroporated wild-type littermates (Figure 59.B-D). In Cre-positive control electroporated animals tamoxifen-induced expression of β-Galactosidase did not alter spine number or density in hippocampal pyramidal or granule neurons compared to tamoxifen-injected wild-type littermates (Figure 60.B-D).

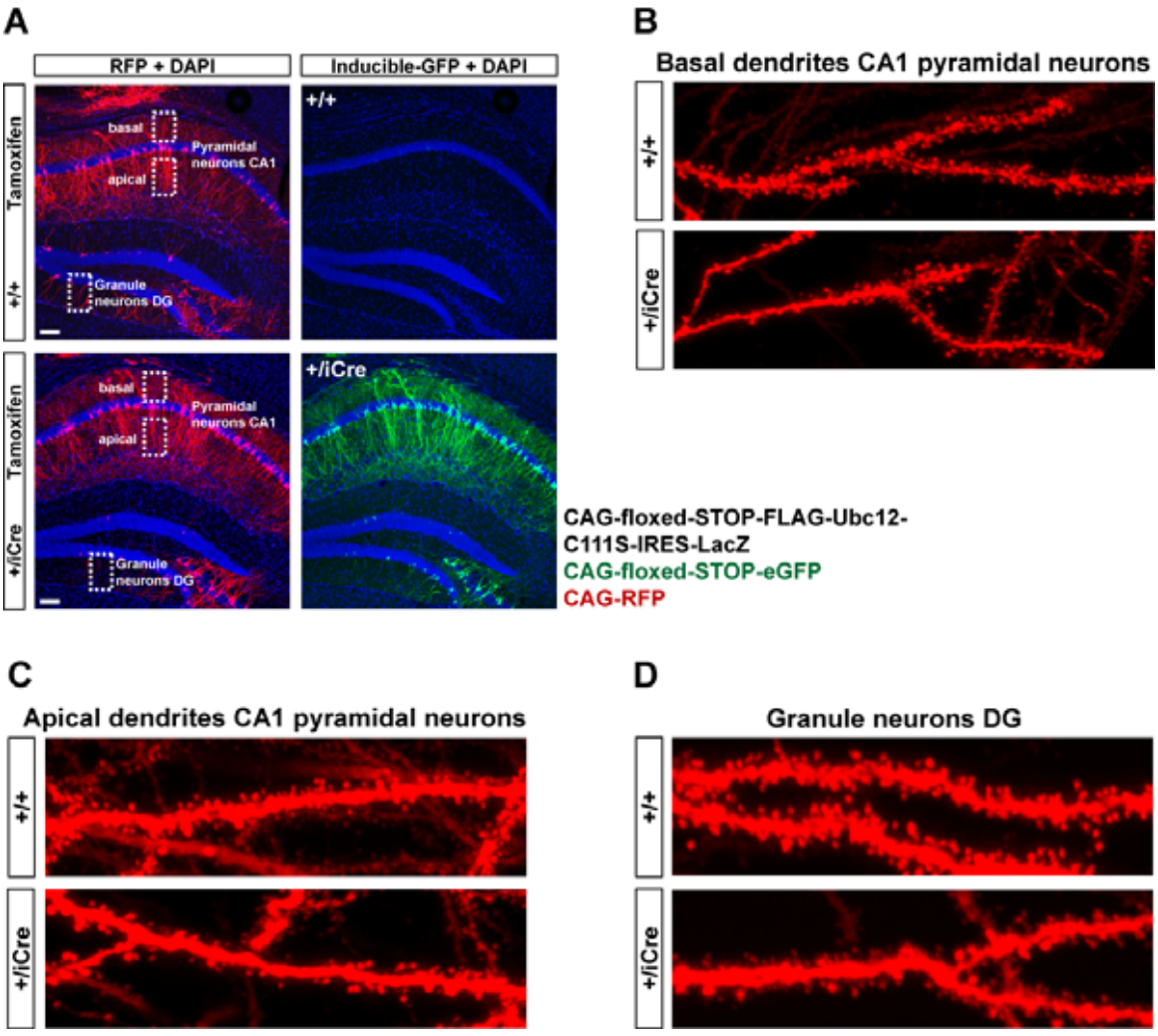


Figure 59: Neddylation is required for spine maintenance *in vivo*.

A) At E15.5 mouse embryos from CamKIIα-Cre-ERT2 x CD1 breedings were microinjected with inducible Ubc12-C111S, inducible eGFP and constitutive RFP plasmids and electroporated to target the developing hippocampus. After birth, electroporated embryos were genotyped by PCR and Cre-ERT2-positive animals (iCre/+) and wild-type (+/+) littermates were allowed to develop. At P35 Cre-ERT2-positive and wild-type littermates were injected with 1 mg Tamoxifen, twice per day for 5 consecutive days. At P50, 15 days after the last Tamoxifen injection all animals were sacrifized and electroporated brains were analyzed. Expression of inducible e-GFP, and therefore inducible Ubc12-C111S, was only detected in iCre/+ animals. Scale bars represent 100 µm. Inhibition of neddylation in mature neurons of the hippocampus resulted in reduced spine number and density in (**B**) basal and (**C**) apical dendrites of CA1 pyramidal neurons as well as in (**D**) granule neurons of the dentate gyrus, indicating that an intact neddylation pathway is necessary to maintain spines *in vivo*.

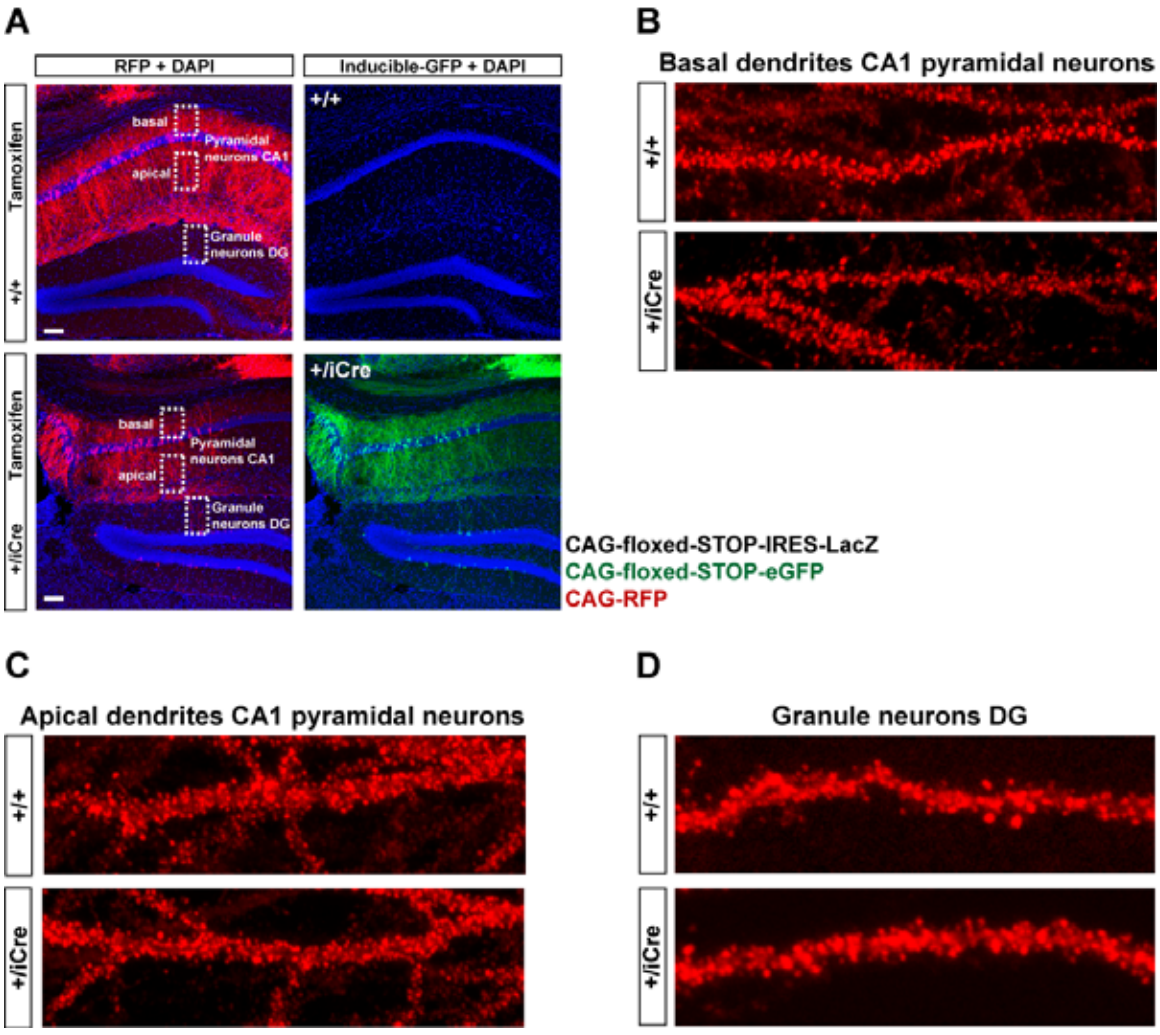


Figure 60: Inducible expression of β-Galactosidase does not exert influences on spine stability *in vivo*.

A) At E15.5 mouse embryos from CamKIIα-Cre-ERT2 x CD1 breedings were microinjected with inducible LacZ control, inducible eGFP and constitutive RFP plasmids and electroporated to target the developing hippocampus. After birth, electroporated embryos were genotyped by PCR and Cre-ERT2-positive animals (iCre/+) and wild-type (+/+) litteremates were allowed to develop. At P35 Cre-ERT2-positive and wild-type littermates were injected with 1 mg Tamoxifen, twice per day for 5 consecutive days. At P50, 15 days after the last Tamoxifen injection all animals were sacrifized and electroporated brains were analyzed. Expression of inducible e-GFP, and therefore inducible β-Galactosidase, was only detected in iCre/+ animals. Scale bars represent 100 µm. Expression of β-Galactosidase in mature neurons of the hippocampus didn't result in alterations of spine number or density in (**B**) basal or (**C**) apical dendrites of CA1 pyramidal neurons or in (**D**) granule neurons of the dentate gyrus.

Considering the *in vitro* and *in vivo* experiments described above, we concluded that neddylation critically controls processes involved in maintaining spine stability. To characterize the molecular mechanisms underlying neddylation-mediated spine stabilization, our next aim was to identify putative targets of Nedd8 that regulate synapse stability and spine maintenance.

Apart from focusing on the function of Nedd8 in stabilization and maintenance of synapses, in future experiments we will explore the possible activity-dependent regulation of the neddylation system in neurons and the role of neddylation during activity-induced synapse remodeling. In the following section (4.6.5, see also 4.7.7) prelimary results are presented which suggest a link between the neddylation pathway and neuronal activity.

4.6.5. Activity-dependent accumulation of Ubc12 in spines

Long term changes in synaptic strength, occuring in response to activity-dependent remodelling of synapses, commonly refered to as neuroplasticity, are thought to be synaptic correlates for learning and memory (Bliss and Collingridge, 1993; Malenka and Bear, 2004; Citri and Malenka, 2008). It is clearly established, that different types of learning and memory processes as well as some of their underlying plasticity mechanisms are dependent on *de novo* protein synthesis. Following this evidence it became obvious that the control of protein degradation (the counterpart of protein synthesis) should also be an important mechanism controlling neuroplasticity. In fact, protaeasome function and ubiquitylation in the synapse are required in different types of neuronal plasticity, including LTP and LTD (Frey et al., 1993; Colledge et al., 2003; Fonseca et al., 2006; Dong et al., 2008; Citri et al., 2009). Moreover, it was shown that neuronal activity regulates the UPS system, e.g. the proteasome shuttles into spines in response to KCl stimulation (Bingol and Schuman, 2006).
To gain insight into activity-dependent regulation of the neddylation pathway, we studied synaptic localization of Venus-tagged Ubc12 in response to glutamate treatment in primary hippocampal neurons. Mature hippocampal neurons, transfected with Venus-tagged Ubc12 and RFP were stimulated with 10 µM glutamate for 10 min and imaged

first under basal conditions and 1 h after stimulation. It was shown that activity-induced synaptic strengthening is accompanied by and correlates with a persistent increase in spine size (Holtmaat et al., 2006; Holtmaat and Svoboda, 2009). Analysis of Venus/RFP signal intensities revealed that Venus-Ubc12 accumulated in spines, which responded to glutamate treatment by increasing their size, whereas no change in Venus-Ubc12 signal intensities were observed in spines that didn't grow after glutamate stimulation (Figure 61). Glutamate stimulation did not result in changes in Venus-Ubc12/RFP intensities in dendrite segments underneath the spines, confirming the accumulation of Ubc12 specifically in spines in response to neuronal activity.

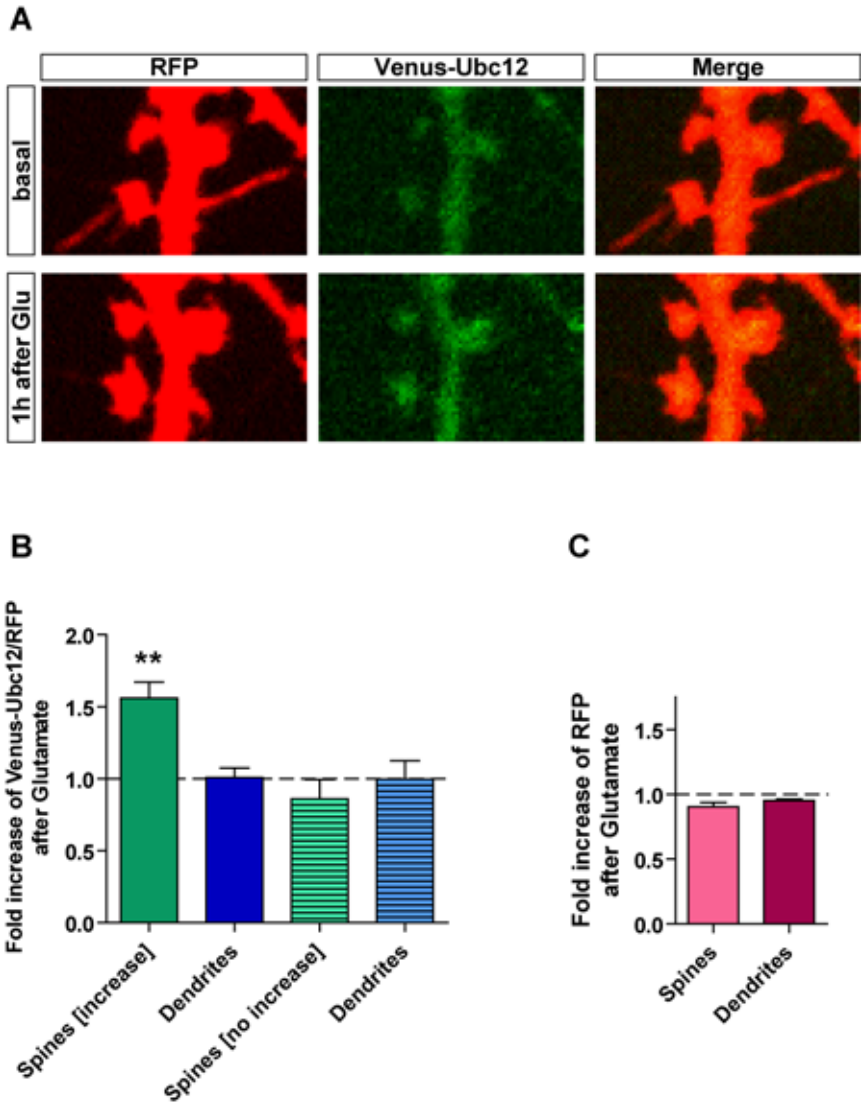


Figure 61: Glutamate stimulation induces Ubc12 accumulation in spines.

A) Mature primary hippocampal neurons, expressing Venus-Ubc12 and RFP, were treated at DIV 19 with 10 µM glutamate for 10 min. Neurons were imaged before (basal) and 1 h after glutamate stimulation. **B)** and **C)** Venus and RFP signal intensities were measured in spines and dendrite segments underneath spines before and after glutamate treatment in single slices of the Z-stacks, in which the signals appeared brightest. Spines were classified into two groups; spines that increased in their size and spines that didn't show an increase. Venus-Ubc12 accumulated specifically in spines that responded to glutamate stimulation (baseline (before glutamate treatment) at 1 fold, dotted line). No changes in Venus/RFP signal intensities were observed in dendrite segments, nor in spines that didn't increase in size, nor in their respective dendritic segments; and glutamate didn't induce any change in RFP spine or dendrite signals. [Statistical analysis: t-test, **p<0.01.]

This experiment provides first evidence that neddylation might be regulated by neuronal activity. Further experiments are being performed in our group in order to unravel the requirement and function of neddylation during activity-induced synapse remodeling in different plasticity processes, including LTP and LTD.

4.7. Identification and validation of PSD-95 as a synaptic target of Nedd8 in the spine

In order to elucidate the molecular mechanism by which neddylation controls spine formation and stability we searched for novel substrates of Nedd8 in the synapse beyond the classically described cullin E3 ubiquitin ligases.

4.7.1. Seeking for new neuronal targets of Nedd8 beyond the cullin E3 ubiqutin ligases – approaches to identify neddylated proteins in the brain

Our group followed and continues to apply different experimental strategies in order to identify novel targets of Nedd8 in the brain.

Unbiased screening approaches, such as Yeast-2-Hybrid screens and immunoprecipiation experiments combined with mass spectrometry analysis are carried out to identify putative neddylated neuronal substrates. To accomplish this goal we generated a Neuro-2a cell line stably expressing a 3x-FLAG-Nedd8 construct at low endogenously physiological conditions. These challenging and time consuming unbiased screening approaches are complemented by hypothesis-driven analysis of putative candidates, derived from the phenotypes obtained in neuronal cell culture experiments and *in vivo* studies.

Hypothesis-driven and literature-based analysis of putative synaptic substrates of Nedd8 suggested that scaffold proteins of the PSD are likely candidates for the effects observed in spine development and stability. This is based on the fact that scaffold proteins were shown to regulate spine development and to mediate adaptations in the mature spine (Rao et al., 1998; Naisbitt et al., 1999; El-Husseini et al., 2000b; Sala et al., 2001; Stein et al., 2003; Usui et al., 2003; Romorini et al., 2004; Sala et al., 2005; Gerrow et al., 2006; Hung et al., 2008; Hung et al., 2010; Verpelli et al., 2011).

4.7.2. Searching for possible targets of Nedd8: screening of scaffold proteins of the PSD via spine rescue experiments

Based on the hypothesis, that scaffold proteins could mediate the effects of neddylation on spine development, we first screened the most important scaffold molecules present in the PSD, including PSD-95, Homer-1c, Shank3, and GKAP, for their abilility to rescue the MLN4924-induced spine loss in mature neurons, when overexpressed in neurons. We found that PSD-95 overexpression was sufficient to stabilize spines in mature neurons during MLN4924 treatment, whereas Homer 1c, Shank 3 or GKAP expression did not influence the spine shrinkage and spine loss induced by the blockade of neddylation (Figure 62).

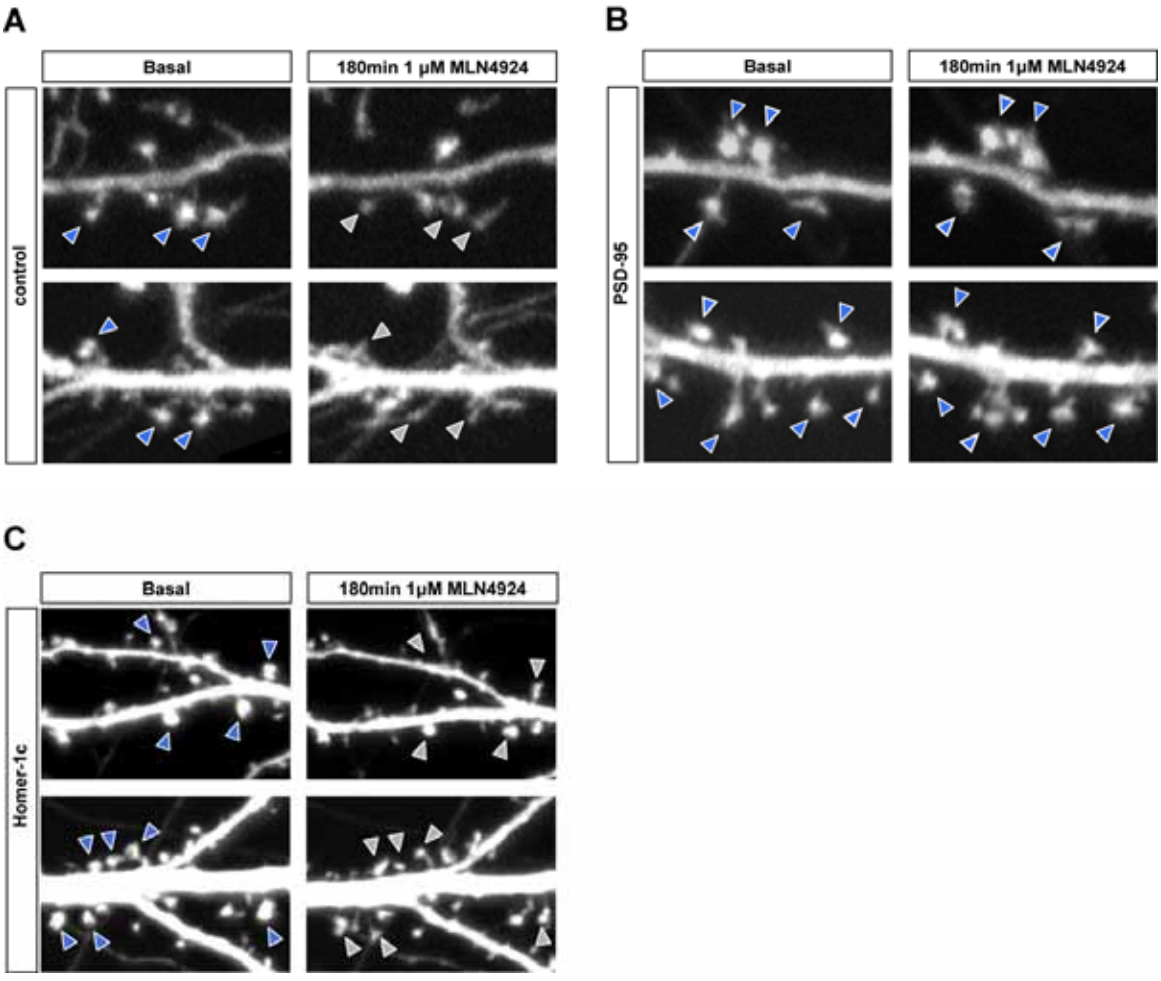


Figure 62: Effects of different scaffold proteins on MLN4924-induced spine loss in mature neurons.

Mature primary hippocampal neurons, expressing (**A**) control, (**B**) PSD-95 or (**C**) Homer-1c, were treated with 1 µM MLN4924 at DIV 19. Neurons were imaged before and 180 min of MLN4924 treatment. **A)** and **C)** In neurons expressing control or Homer-1c, blockade of neddylation induced spine shrinkage and loss of spines (marked by blue (basal) and grey (180 min MLN4924) arrowheads). **B)** In contrast, PSD-95 overexpression prevented the MLN4924-induced spine shrinkage and loss (marked by blue (basal and 180 min MLN4924) arrowheads).

To further validate PSD-95 as a possible synaptic target of Nedd8, we analyzed its effect in additional *in vitro* and *in vivo* experiments. *In vitro*, PSD-95 overexpression prevented the Ubc12-C111S- and MLN4924-induced filopodia phenotype during spine development (Figure 63.A, B). Moreover, when overexpressed in the hippocampus i*n vivo,* using *in utero* electroporation experiments, PSD-95 was also sufficient to rescue the Ubc12-C111S-induced reduced spine formation on apical and basal dendrites of CA1 pyramidal neurons (Figure 63.C-E). Interestingly, PSD-95 overexpression during development also restored Ubc12-C111S-induced impairments in dendritic growth and arborization *in vitro* and *in vivo*. As it will be discussed later, the restoration of dendritic growth might be a direct effect of PSD-95 at the dendritic level or might be indirect and secondary to synaptic and spine stabilization.

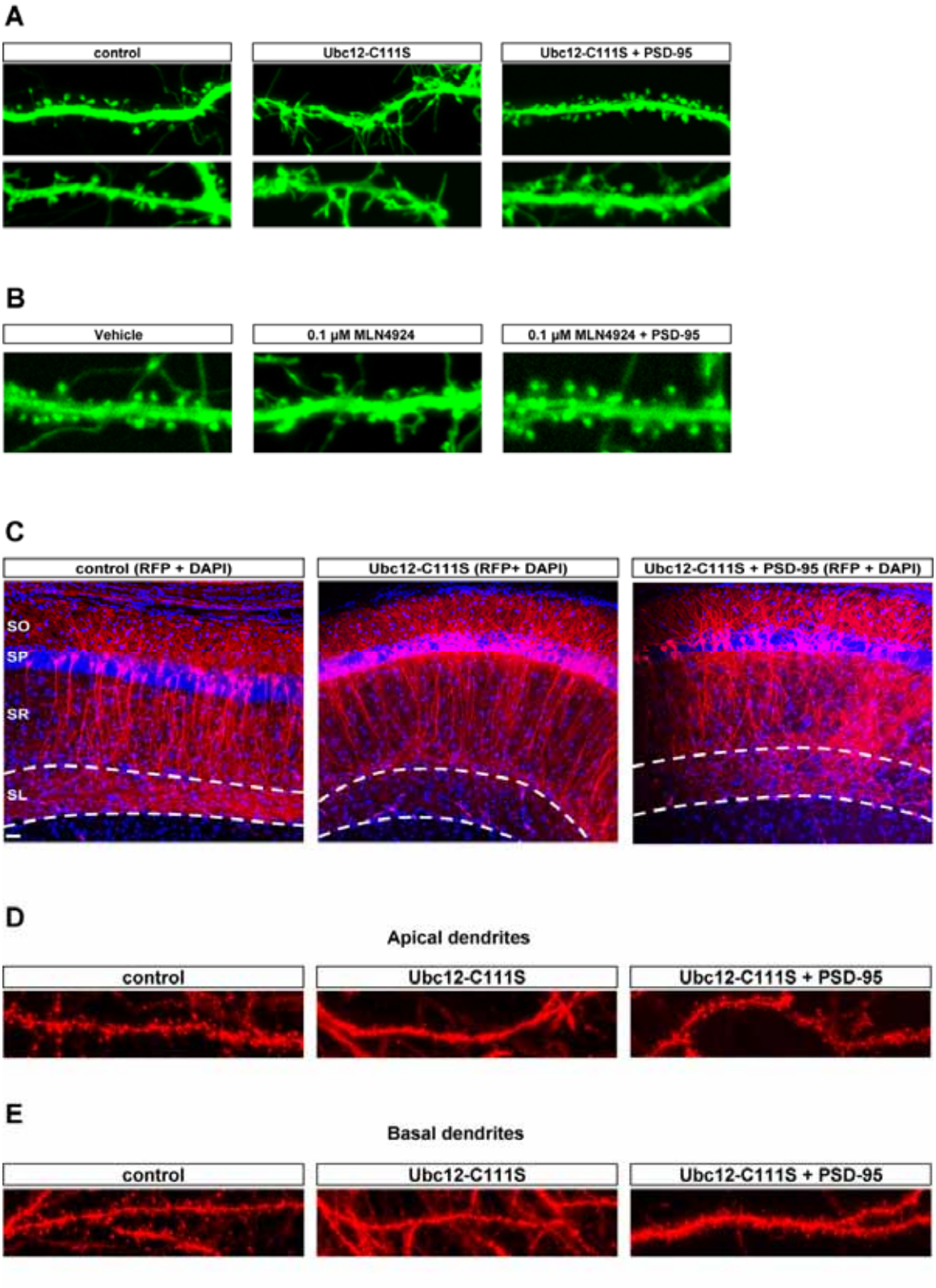

A
control
Ubc12-C111S
Ubc12-C111S + PSD-95
B
Vehicle
0.1 µM MLN4924
0.1 µM MLN4924 + PSD-95
C
control (RFP + DAPI)
Ubc12-C111S (RFP+ DAPI)
Ubc12-C111S + PSD-95 (RFP + DAPI)
SO
SP
SR
SL
D
Apical dendrites
control
Ubc12-C111S
Ubc12-C111S + PSD-95
E
Basal dendrites
control
Ubc12-C111S
Ubc12-C111S + PSD-95

Figure 63: PSD-95 rescue experiments *in vitro* and *in vivo* during spine development.

A) Primary hippocampal neurons were transfected with control, Ubc12-C111S or Ubc12-C111S plus PSD-95 constructs at DIV 13 and spine formation was analyzed at DIV 19. PSD-95 overexpression rescued the Ubc12-C111S-induced filopodia phenotype. **B)** Primary hippocampal neurons were transfected with control or PSD-95 constructs at DIV 14 and treated at DIV 16 with 0.1 µM MLN4924 for 48 h. PSD-95 expression was sufficient to rescue the MLN4924-induced filopodia phenotype. **C)** to **E)** Mouse embryos were *in utero* electroporated on E14.5 with control, Ubc12-C111S or Ubc12-C111S plus PSD-95 constructs to target the hippocampus and brains were analyzed at P28. PSD-95 overexpression rescued the Ubc12-C111S-induced (**C**) dendritic defects and restored spine formation on (**D**) apical and (**E**) basal dendrites of CA1 pyramdial neurons. Scale bar represents 50 µm. SO, stratum oriens, SP, stratum pyramidale, SR, stratum radiatum, SL, stratum lacunosum moleculare.

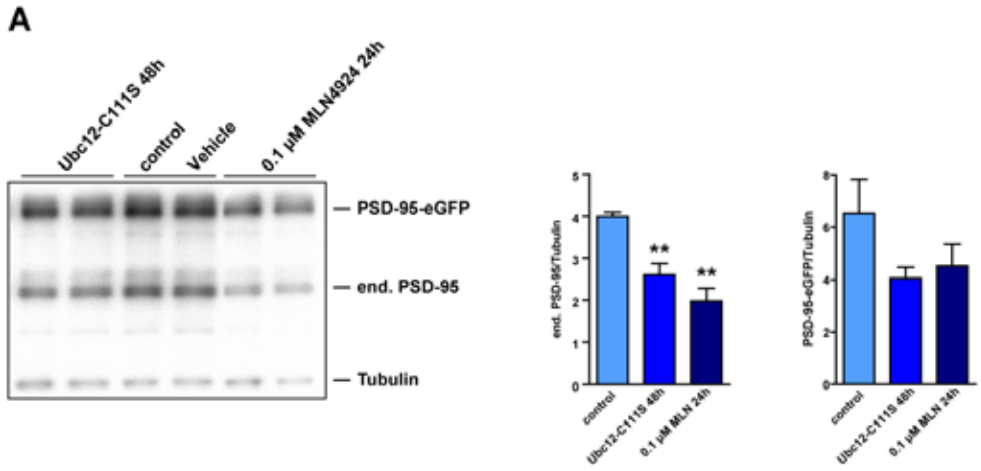
4.7.3. PSD-95 is neddylated on specific lysine residues

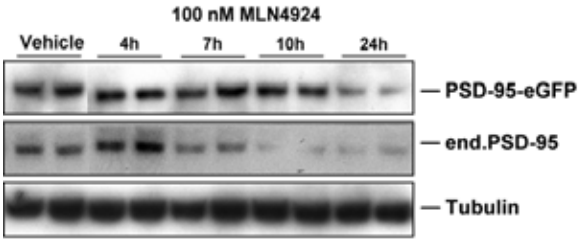
To address whether neddylation exerts its effect on the spine via direct neddylation of PSD-95 or indirectly via modulation of PSD-95 function, immunoprecipitation and Western blot experiments were performed in our group by Marisa Brockmann, that revealed that PSD-95 is a direct target of Nedd8 (unpublished data). PSD-95 was found to be neddylated when it was expressed in heterologous cells. PSD-95 neddylation was shown to be specific since its neddylation was dependent on Ubc12 and was reversed by MLN4924 treatment. Furthermore, PSD-95-Nedd8 conjugates were found endogenously under physiological conditions in primary neurons (data not shown). To identify specific lysine-residues in PSD-95 that are modified via Nedd8 conjugation, immunoprecipitation experiments were combined with subsequent mass spectrometry analysis. Using this approach we could identify particular lysine residues that can be specifically neddylated or ubiquitylated or modified by both ubiquitin and Nedd8 (data not shown).

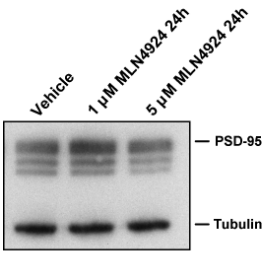
Using site-directed mutagenesis we generated several PSD-95 mutants that carry lysine to arginine (K→R) amino-acid substitutions, which cannot be modified by Nedd8 or ubiquitin conjugation anylonger. For instance, PSD-95-K193R and PSD-95-K202R mutants show decreased PSD-95 neddylation in immunoprecipitation assays, whereas PSD-95-K157R and PSD-95-K211R are mutants in which the lysine residue was found to be specifically ubiquitylated (M. Brockmann, unpublished data). Given that PSD-95 is neddylated, the next step was to analyze how neddylation affects PSD-95 expression or function.

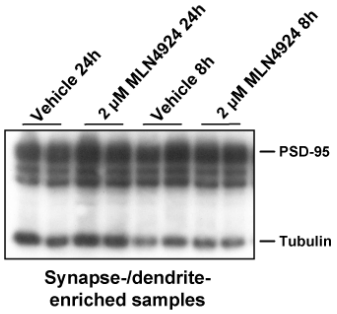
4.7.4. Neddylation does not regulate PSD-95 protein stability in primary neurons

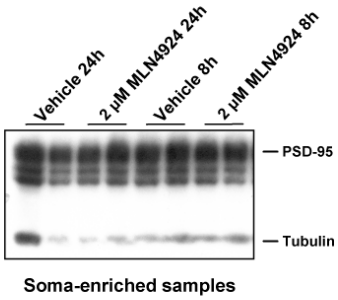
Our first hypothesis was that neddylation might regulate protein stability, either by synergizing with ubiquitin and inducing proteasome-mediated degradation or counteracting ubiquitination and stabilizing PSD-95. Interestingly, neddylation blockade produced differential effects on PSD-95 protein stability depending on the cellular systems used. We found contradictory results when we compared neuronal cell lines and primary neurons in culture. Protein levels of endogenous as well as transfected PSD-95-eGFP decreased in Neuro-2a cells in response to neddylation blockade via MLN4924 treatment or transfection of Ubc12-C111S (Figure 64.A, B), suggesting that neddylation stabilizes PSD-95. However, in primary neurons inhibiting neddylation via MLN4924 treatment for 24 h did not alter total PSD-95 protein levels measured by Western blot (Figure 64.C). Furthermore to assess the effect of neddylation on the synaptic PSD-95 pool only, neurons were grown on special filter plates, that allow isolation of synapse-/dendrite-enriched and soma-/nucleus-enriched protein samples/fractions. Inhibition of neddylation for 8 and 24 h did not induce changes in PSD-95 content neither in the synapse/dendrite-enriched nor in the soma-enriched samples (Figure 64.D). However prolonged treatment of primary neurons for 72 h with 1 and 5 μM MLN4924 decreased PSD-95 levels by 15% and 46%, respectively (Figure 64.E).


A
Ubc12-C111S 48h
control
Vehicle
0.1 µM MLN4924 24h
PSD-95-eGFP
end. PSD-95
Tubulin
end. PSD-95/Tubulin
PSD-95-eGFP/Tubulin
**
**
control
Ubc12-C111S 48h
0.1 µM MLN 24h


B
100 nM MLN4924
Vehicle
4h
7h
10h
24h
PSD-95-eGFP
end.PSD-95
Tubulin


C
Vehicle
1 µM MLN4924 24h
5 µM MLN4924 24h
PSD-95
Tubulin


D
Vehicle 24h
2 µM MLN4924 24h
Vehicle 8h
2 µM MLN4924 8h
PSD-95
Tubulin
Synapse-/dendrite-enriched samples


Vehicle 24h
2 µM MLN4924 24h
Vehicle 8h
2 µM MLN4924 8h
PSD-95
Tubulin
Soma-enriched samples

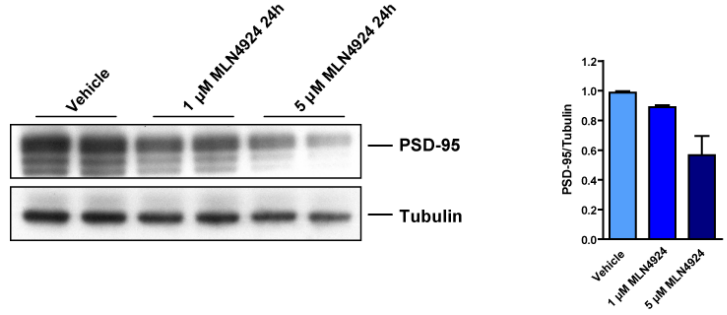

E
Vehicle
1 µM MLN4924 24h
5 µM MLN4924 24h
PSD-95
Tubulin
PSD-95/Tubulin
Vehicle
1 µM MLN4924
5 µM MLN4924

Figure 64: Effect of neddylation on protein stability of PSD-95 in Neuro-2a cells and primary neurons.

A) Neuro-2a cells were differentiated by serum withdrawal and transfected with control, Ubc12-C111S and PSD-95-eGFP constructs for 48 h and treated with vehicle (DMSO) or 0.1 µM MLN4924 during the last 24 h as indicated. Protein extracts were subjected to immunoblotting and PSD-95-eGFP and endogenous PSD-95 levels were analyzed. Blockade of neddylation via expression of Ubc12-C111S or MLN4924 treatment decreased PSD-95-eGFP and endogenous PSD-95 levels compared to control cells. **B)** Neuro-2a cells were differentiated by serum withdrawal and transfected with PSD-95-eGFP. 18 h after transfection cells were treated with vehicle (DMSO) or 0.1 µM MLN4924 for 4, 7, 10 and 24 h. Protein extracts were subjected to immunoblotting and PSD-95-eGFP and endogenous PSD-95 levels were analyzed. Inhibition of neddylation resulted in a time-dependent decrease of both, PSD-95-eGFP and endogenous PSD-95 levels. Here, the 24 h vehicle-treatment is shown as a representative control time point in the Western blot. **C)** Primary cortical neurons were treated at DIV 17 with 1 and 5 µM MLN4924 for 24 h and protein extracts were subjected to immunoblotting. Inhibition of neddylation did not alter PSD-95 levels in primary neurons. **D)** Primary hippocampal neurons were grown on filter-plates that allow the separation of synapse/dendrite-enriched and soma-enriched protein samples. At DIV 17 neurons were treated with vehicle (DMSO) or 2 µM MLN4924 for 8 and 24 h and protein extracts were subjected to immunoblotting. Blocking neddylation did not change PSD-95 levels neither in the synapse/dendrite-enriched nor in the soma-enriched samples. **E)** Long-term treatment of primary cortical neurons at DIV 15 with vehicle (DMSO) or 1 and 5 µM MLN4924 for 72 h slightly decreased PSD-95 levels, analyzed by Western blot. Tubulin served as house-keeping protein. [Statistical analysis: t-test, **p<0.01.]

In summary, we can conclude that in neurons, neddylation is not primarly involved in regulating PSD-95 protein stability, but rather controls other aspects of PSD-95 function in the spine.

4.7.5. Neddylation of PSD-95 is necessary for synaptic localization and oligomerization of PSD-95

To confine the analysis of the effect of neddylation on PSD-95 to post-synaptic spines, we blocked neddylation in mature primary neurons by MLN4924 application and immunofluorescently stained pre- and post-synaptic sites by α-Synaptophysin and α-PSD-95 antibodies, respectively.

Mature primary hippocampal neurons, that were treated with 1 µM MLN4924 for 1 4, 8 and 10 h, showed a reduction in the number and intensity of synaptic PSD-95 clusters compared to vehicle-treated control neurons, while pre-synaptic Synaptophysin immunofluorescence staining was not affected during short-term MLN4924 applications (Figure 65.A-D).

The intensity of PSD-95 puncta was decreased as early as 1 h after neddylation blockade (Figure 65.C, D), whereas the number of PSD-95 puncta was significantly reduced in a time-dependent manner after 4, 8 and 10 h MLN4924 treatment compared to vehicle-treated neurons (Figure 65.A, B).

To unambiguously visualize mature mushroom-like spines that form synapses, neurons were transfected with a GFP-expressing plasmid, treated with MLN4924 or vehicle and stained with α-Synaptophysin and α-PSD-95 antibodies. As previously, blocking neddylation for 8 h resulted in a decrease in spine size. In addition, we observed a weaker intensity of PSD-95 puncta within the spine head, whereas pre-synaptic Synaptophysin immunofluorescence staining was not affected during this short-term MLN4924 application (Figure 65.E, F). Taken together these experiments suggested a specific role of neddylation on PSD-95 function in the spine compartment.

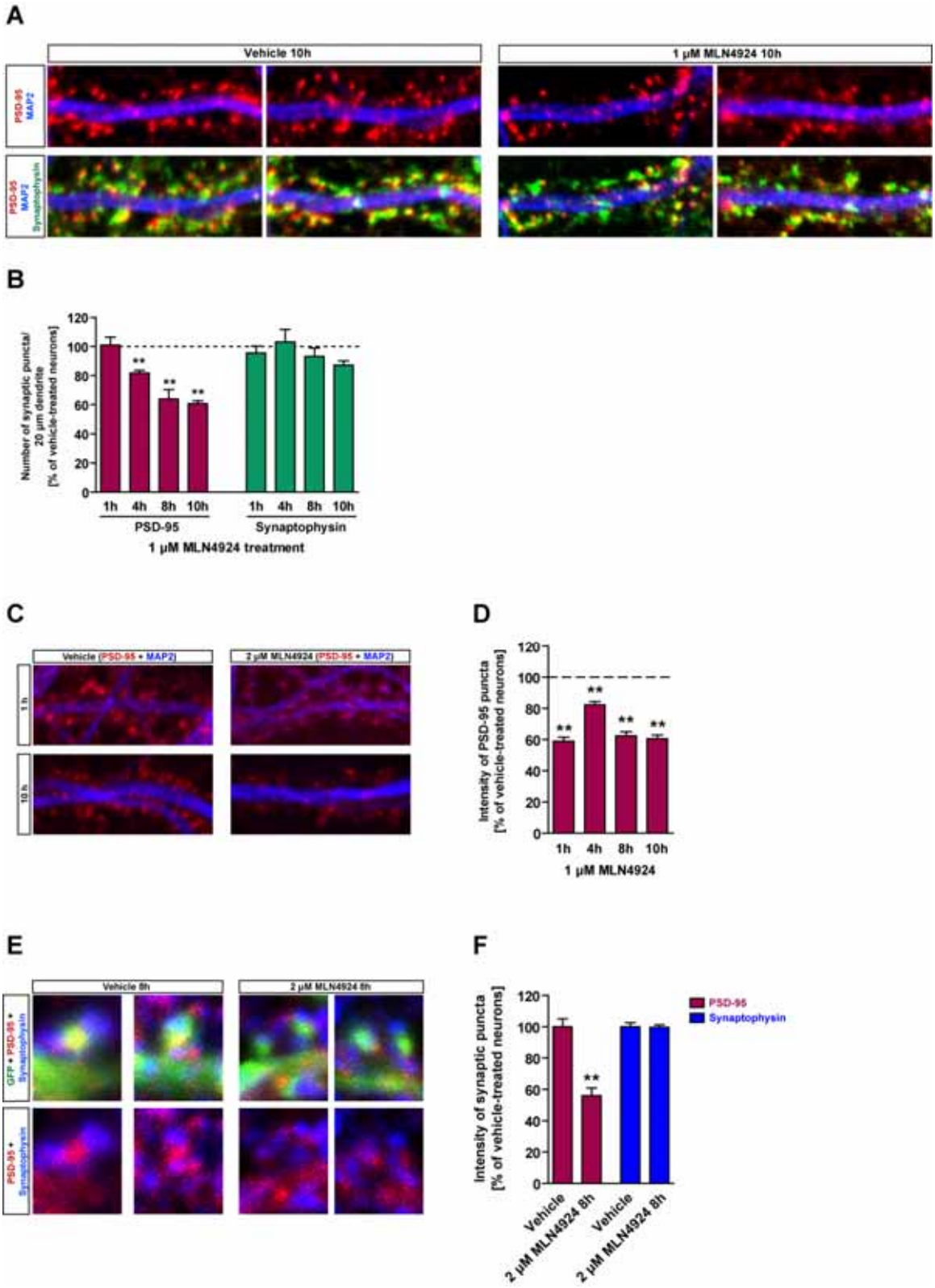

A
Vehicle 10h
1 µM MLN4924 10h
PSD-95
MAP2
Synaptophysin
B
Number of synaptic puncta/
20 µm dendrite
[% of vehicle-treated neurons]
120
100
80
60
40
20
0
**
1h 4h 8h 10h
PSD-95
1h 4h 8h 10h
Synaptophysin
1 µM MLN4924 treatment
C
Vehicle (PSD-95 + MAP2)
2 µM MLN4924 (PSD-95 + MAP2)
1 h
10 h
D
Intensity of PSD-95 puncta
[% of vehicle-treated neurons]
1h 4h 8h 10h
1 µM MLN4924
E
Vehicle 8h
2 µM MLN4924 8h
GFP + PSD-95 + Synaptophysin
PSD-95 + Synaptophysin
F
Intensity of synaptic puncta
[% of vehicle-treated neurons]
PSD-95
Synaptophysin
Vehicle
2 µM MLN4924 8h
Vehicle
2 µM MLN4924 8h

Figure 65: Effect of neddylation on post-synaptic PSD-95 and pre-synaptic Synaptophysin puncta in primary neurons

A) Mature hippocampal neurons at DIV 18 were treated with DMSO (vehicle) or 1 µM MLN4924 for 1, 4, 8 and 10 h. Immunofluorescence staining was performed to label synapses and dendrites. Post-synaptic PSD-95 clusters are labeled in red, pre-synaptic Synaptophysin clustes in green and dendrites are stained by MAP2 in blue. Pictures shown depict the 10 h treatment time. **B)** The number of post-synaptic PSD-95 and pre-synaptic Synaptophysin clusters was counted on 20 µm long dendritic segments. The diagram shows the number of synaptic puncta after MLN4924 treatment in % of the vehicle treatment (vehicle treatment in 100%, dotted line). Blocking neddylation resulted in a specific time-dependent reduction of PSD-95 clusters. [Statistical analysis: 2 Way ANOVA, treatment x time interaction, $F_{(3,62)}$=12.6, p<0.001; Bonferroni post-hoc test, **p<0.01.] **C)** and **D)** Intensity of PSD-95 puncta was measured at 1, 4, 8 and 10 h after treatment in single slices of the Z-stacks, in which the PSD-95 signals appeared brightest. Inhibition of the Nedd8 pathway led to a decrease in the intensity of PSD-95 puncta. The diagram depicts the intensity of PSD-95 puncta after MLN4924 treatment in % of the vehicle treatment (vehicle treatment in 100%, dotted line). [Statistical analysis: 2 Way ANOVA, treatment x time interaction, $F_{(3,355)}$=6.8, p<0.001; Bonferroni post-hoc test, **p<0.01.] **E)** At DIV 16, primary hippocampal neurons were transfected with GFP constructs, and on DIV 20, when neurons already developed mature synapses, they were treated with vehicle or 2 µM MLN4924 for 8 h. Post-synaptic PSD-95 puncta were labeled in red, pre-synaptic Synaptophysin clustes are labeled in blue and GFP expression is shown in green. Inhibition of neddylation resulted in a specific decrease in intensity of PSD-95 clusters, while the intensity of Synaptophysin puncta was not changed. **F)** The diagram shows the quantification of the intensities of PSD-95 and Synaptophysin clusters on MLN4924-treated neurons in % of vehicle-treated control neurons. [Statistical analysis: t-test, **p<0.01.]

PSD-95 function in the spine relies on the interaction and oligomerization of PSD-95 with itself and other synaptic proteins via its PDZ domains (Christopherson et al., 2003). Using co-immunoprecipitation assays and immunoblotting we established a role of neddylation in PSD-95 oligomerization. Transfection experiments in HEK cells, that do not express PSD-95 endogenously, showed that in response to MLN4924 treatment the oligomerization of PSD-95 measured via the interaction of PSD-95 and GFP-tagged PSD-95 is strongly decreased (Marisa Brockmann, data not shown). Moreover, PSD-95 lysine mutants (PSD-95-K193R, PSD-95-K202R), in which the lysine residue that was found to be neddylated was mutated to arginine, were unable to interact with wild-type PSD-95-eGFP, when co-expressed together in primary neurons. In contrast, PSD-95 lysine mutants that were found to be ubiquitylated (PSD-95-K157R, PSD-95-K211R) did not produce the same effect (unpublished data).

4.7.6. Neddylation-defective mutants of PSD-95 impair spine development *in vitro*

Primary neurons transfected with the neddylation-defective PSD-95 lysine mutants, PSD-95-K193R and PSD-95-K202R, showed impaired development of mature spines and increased number of immature filopodia structures, resembling the phenotype induced by neddylation blockade via MLN4924 treatment, transfection of Ubc12-C111S or transfection of shRNAs against Nedd8 and Ubc12 (Figure 66). In contrast, expression of PSD-95 constructs, in which lysine targets for ubiquitylation were mutated, PSD-95-K157R and PSD-95-K211R, did not affect spine development in primary neurons (Figure 66).

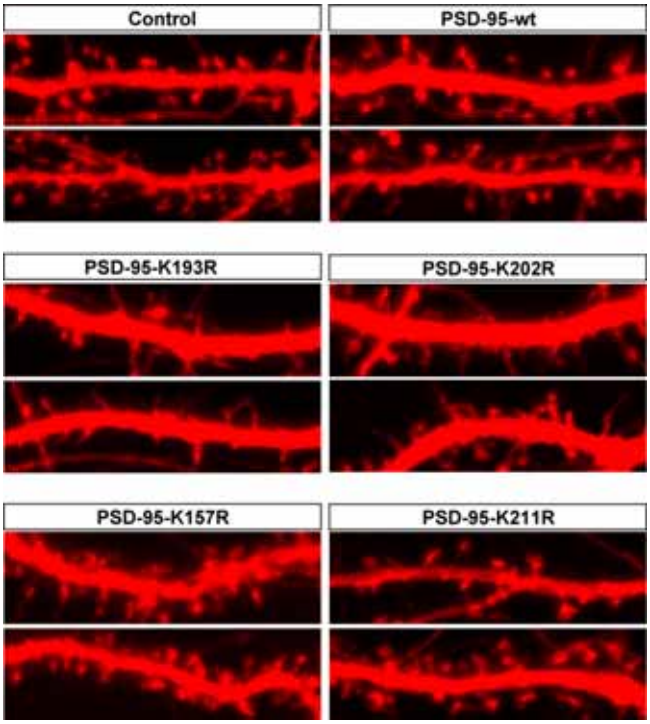


Figure 66: Effect of PSD-95 lysine mutants in spine development *in vitro*.

Primary hippocampal neurons were transfected at DIV 14 with RFP and control, PSD-95-wt, PSD-95-K193R, PSD-95-K202R, PSD-95-K157R or PSD-95-K211R constructs and spine development was analyzed at DIV 19. Neurons expressing control, PSD-95-wt as well as ubiquitin PSD-95 mutants K157R and K211R developed normal spines. In contrast spine formation was severly impaired in neurons expressing the neddylation-deficient PSD-95 mutants K193R and K202R.

In summary these experiments implicate direct neddylation of PSD-95 at specific lysine residues (K202 and K193) as an important regulatory mechanism in spine development in primary neurons.

Additional experiments are being performed in order to further dissect the role of neddylation of PSD-95 at specific lysine residues in different aspects of PSD-95 function during spine development, stability as well as during activity-induced neuronal plasticity in primary neurons and *in vivo*.

4.7.7. Neddylation controls activity-dependent glutamate-induced synaptic increase in PSD-95

Many studies showed that PSD-95 is involved in mediating plasticity-induced and activity-dependent spine growth, stabilization and synapse strengthening, and that LTP and neuronal activity leads to an increase in synaptic PSD-95 content (Stein et al., 2003; Ehlers, 2003; Malenka and Bear, 2004; Citri and Malenka, 2008; Steiner et al., 2008; Xu, 2011).

To functionally test if neddylation controls activity-dependent synaptic accumulation of PSD-95, we stimulated primary neurons, grown on special filter plates in order to isolate spine-/dendrite-enriched vs. soma-enriched protein fractions, with 10 µM glutamate. Protein samples were isolated before stimulation (basal), 15, 30, 60 and 120 min after glutamate application and subjected to immunoblotting with α-PSD-95 and α-Tubulin antibodies. In the spine-/dendrite-enriched samples, we observed a transient increase in PSD-95 levels, whereas in the soma-enriched protein samples, PSD-95 levels transiently decreased, suggesting that protein trafficking between different cellular compartments as well as regulation of PSD-95 stability might underlie the transient increase in synaptic and dendritic PSD-95 (Figure 67). Pre-treatment of neurons with 1 µM MLN4924 for 2 h before glutamate stimulation, strongly prevented the synaptic and dendritic accumulation of PSD-95, implicating that neddylation might regulate activity-dependent functions of PSD-95 (Figure 67).

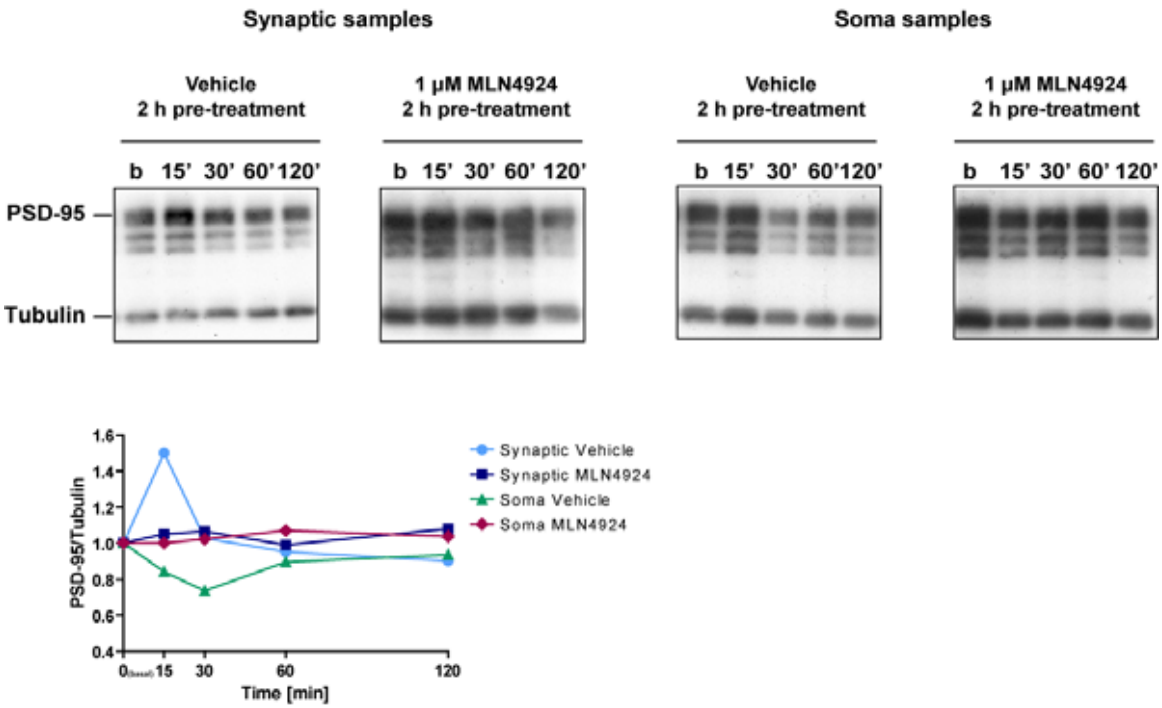


Figure 67: Glutamate-induced increase in synaptic PSD-95 is prevented by MLN4924 pre-treatment.

Primary hippocampal neurons were grown on filter-plates to allow the separation of synapse/dendrite-enriched and soma-enriched protein samples. At DIV 16 neurons were pre-treated with vehicle (DMSO) or 1 µM MLN4924 for 2 h. The neurons were then treated with 10 µM glutamate for 5 min. Protein extracts were isolated before and 15, 30, 60 and 120 min after glutamate stimulation. Spine-/dendrite-enriched and soma-enriched protein samples were subjected to immunoblotting with α-PSD-95 and α-Tubulin antibodies. Glutamate stimulation led to a transient increase in synaptic and to a transient decrease in soma PSD-95 levels in vehicle pre-treated neurons. Blocking neddylation via MLN4924 treatment abolished the fast and transient increase in synaptic PSD-95 in response to the brief glutamate stimulation. b, basal; Tubulin served as a house-keeping protein. One representative Western blot experiment with its respective quantification is shown, from a total of three independent experiments with comparable results.

Further experiments are planned to dissect the function of PSD-95 neddylation at specific lysine residues in different aspects of activity-dependent synaptic processes, including LTD.

In summary we described PSD-95 as a synaptic target of Nedd8, we identified specific lysine residues in PSD-95 that can be modified by Nedd8-conjugation and we showed that neddylation of PSD-95 accomplishes specific functions during spine development.

4.8. Generation of mouse mutants to analyze the role of the neddylation pathway in vivo

To further dissect the functions of neddylation *in vivo* during brain development and in the postnatal brain we developed conditional transgenic mouse models based on the Cre/loxP system. Breeding to specific Cre mouse lines, expressing Cre-recombinase in defined and restricted cell populations in the brain and in specific neurotransmitter circuits will allow us to inhibit the neddylation pathway in a spatio- and temporal-controlled manner.

During the first year of this project, and once we confirmed the relevance of our findings in cell culture and *ex vivo* experiments, we initiated the development of the first conditional neddylation-defective mutant mouse line. As a first approach, we had chosen, a Ubc12 gene trap clone, in which the *Ubc12* gene is conditionally disrupted by the random insertion of the gene trap cassette. This selection was essentially based on the availability of this ES cell clone, which had been already generated by the German Gene Trap Consortium (GGTC). This ES cell clone gave many chimeric mice, which successfully gave germline transmission of the modified *Ubc12* allele (see also 4.8.1). Unfortunately, due to a cryptic splicing site in the gene trap cassette the trapped allele behaved as a null mutant (and not like a conditional) allele. Therefore, in the second year of the project, we started the generation of an alternative Nedd8 mouse mutant, following a targeted trapping strategy of the *Ubc12* gene locus. Although we injected a number of positive ES cell clones repeatedly into blastocysts, we just obtained chimeras that didn't transmit the mutant locus to their offspring. Of note, the European Consortium for Mouse Mutants (EUCOMM) also failed in conditionally targeting Ubc12, indicating that the *Ubc12* gene might be a "difficult locus" for gene targeting by homologous recombination (http://www.knockoutmouse.org/about/eucomm). Thus, during the third year, we developed again two new complementary strategies. One the one hand, we generated a Nae1 conditional KO mouse and on the oher hand, we developed a mouse line conditionally overexpressing Ubc12-C111S in the *ROSA26* locus. These animals will be described in detail in the next sections.

4.8.1. Ubc12 conditional KO mouse (German Gene Trap Consortium – GGTC) – Ubc12-LacZ reporter mouse

We obtained a *Ubc12* gene trap clone from the German Gene Trap Consortium (GGTC) in collaboration with the IDG at the Helmholtz-Zentrum München. A gene trap cassette, containing the β-Galactosidase gene and a Neomycin-resistance gene – rsFlipROSAßgeo(Cre)∗ – was randomly inserted into intron 1 of the mouse *Ubc12* gene (ENSMUSG00000005575), in mouse E14 ES cells (Figure 68.A) (http://tikus.gsf.de/). The integration site was confirmed by 5' and 3' Splinklerette-PCRs and mutant ES cells were used to generate chimeric mice by BALB/c blastocyst injections, in collaboration with Dr. Ralf Kühn at the Helmholtz-Zentrum München. Germline transmission of the modified *Ubc12* allele was confirmed in offspring from high percentage male chimeras bred to wild-type C57BL/6J mice. These positive mice of the offspring of the chimera breedings are heterozygous (het.) conventional KO mice for Ubc12 and at the same time Ubc12-LacZ reporter mice because the insertion of the gene trap vector disrupts the Ubc12 allele and results in splicing of Exon 1 of Ubc12 to the β-Galactosidase reporter gene. This KO-allele was confirmed by LacZ staining as well as by *in situ* hybridization for *Ubc12* in brain sections of adult mice (12 weeks old) (Figure 68.B, C). The *in situ* hybridization signal was reduced as expected by ~50% in the heterozygous Ubc12-conventional KO mice compared to wild-type littermates. Furthermore this Ubc12-LacZ reporter mouse was used to characterize the cell type specific expression pattern of Ubc12 in the mouse brain via LacZ staining (see 4.1.2.) From breedings of het-Ubc12 KO (-/+) x het-Ubc12 KO (-/+) we never obtained a homozygous (hom) Ubc12 KO mouse, indicating that the conventional homozygous KO of Ubc12 is lethal. From a total of 676 pubs from het-Ubc12 KO x het-Ubc12 KO breedings, 286 pubs were wild-type (+/+), 390 were het. KO (-/+) and 0 were hom. KO (-/-). Further characterization of the timepoint of lethality using genotyping and LacZ staining of embryos of timed pregnant breedings revealed that the conventional homozygous KO of Ubc12 is embryonic lethal before E9.5. For analysis of neuronal morphology, het-Ubc12 KO were crossed to Thy1-eGFP (M) mice, that express GFP in a subset of neurons, including CA1 pyramidal neurons in the hippocampus, granule neurons in the DG as well as pyramidal neurons in the cortex (Feng et al., 2000) However we didn't observe any major morphological changes, neither in dendritic arborization nor in spine morphology, in pyramidal

hippocampal or cortical neurons of het-Ubc12 KO-GFP mice compared to control-GFP mice, implying that the remaining functional allele of *Ubc12* is sufficient for the proper functioning of the Nedd8 pathway (data not shown).

In order to obtain a conditional KO of Ubc12, the next step was to breed the heterozygous conventional Ubc12 KO mouse to Flp-deleter (hACTB::Flp) mice, resulting in turning around and inactivation of the gene trap vector (Dymecki, 1996; Rodriguez et al., 2000). Unfortunately in the case of Ubc12, FLP-mediated inactivation of the gene trap vector did not result in restoring the wild-type expression of the *Ubc12* allele, as shown by *in situ* hybridization on brain sections, but led to splicing of exon 1 of *Ubc12* to a cryptic splice site in the spacer between the 5' LTR sequence and the frt/lox5157/loxP sites of the gene trap vector confirmed by sequencing of RT-PCR products (Figure 68.D-F). Due to this cryptic splicing, a conditional Ubc12 KO line couldn't be established from this gene trap ES cell clone.

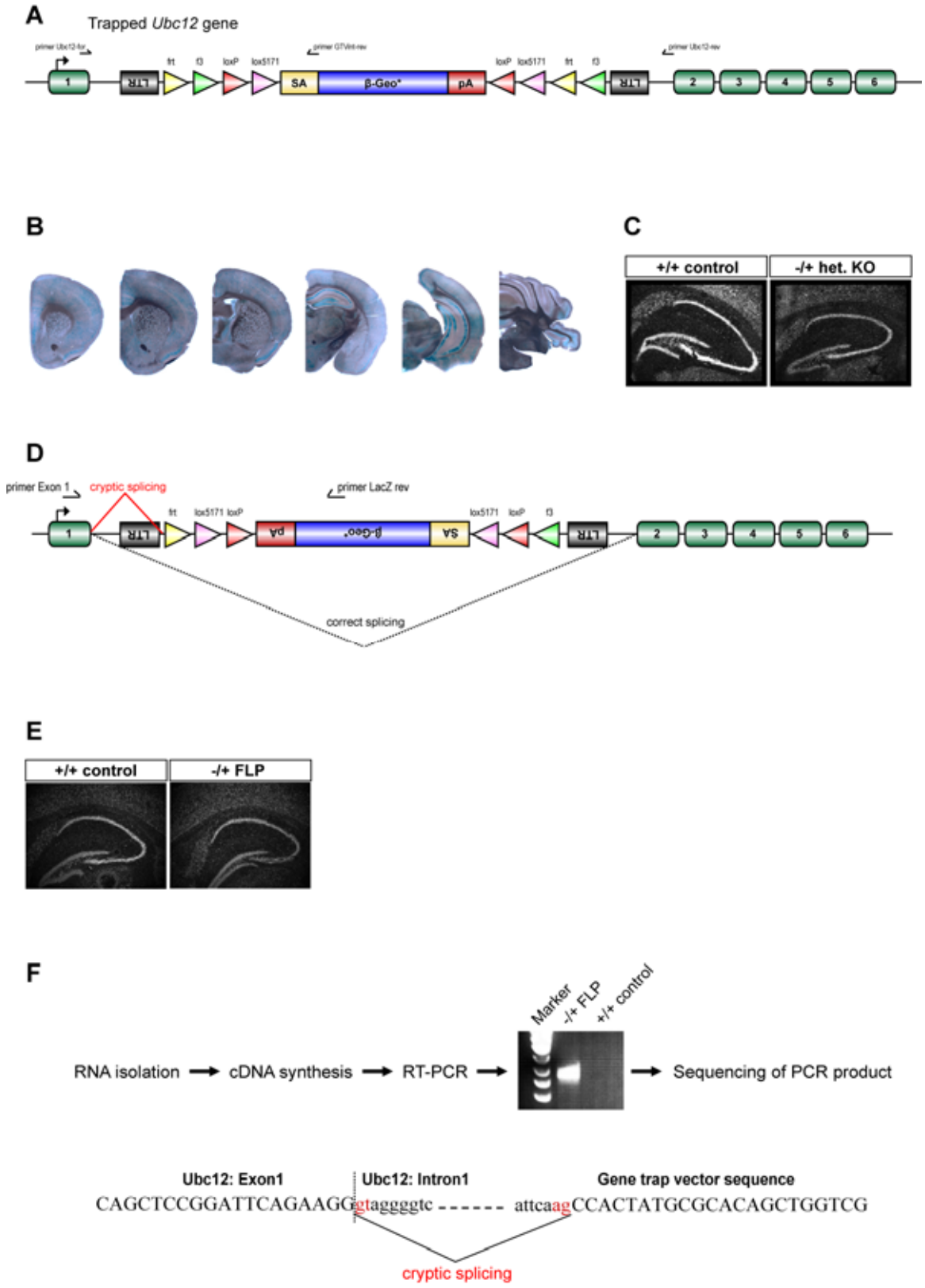

A
Trapped Ubc12 gene
primer Ubc12-for
primer GTVint-rev
primer Ubc12-rev
frt
f3
loxP
lox5171
1
LTR
SA
β-Geo*
pA
2
3
4
5
6
B
C
+/+ control
-/+ het. KO
D
primer Exon 1
cryptic splicing
primer LacZ rev
correct splicing
E
+/+ control
-/+ FLP
F
RNA isolation
cDNA synthesis
RT-PCR
Marker
-/+ FLP
+/+ control
Sequencing of PCR product
Ubc12: Exon1
Ubc12: Intron1
Gene trap vector sequence
CAGCTCCGGATTCAGAAGGgtaggggtc ------ attcaagCCACTATGCGCACAGCTGGTCG
cryptic splicing

Figure 68: Generation of Ubc12-LacZ reporter mice.

A) Trapped *Ubc12* allele. A gene trap cassette, containing the β-Galactosidase gene and a Neomycin-resistance gene – rsFlipROSAßgeo(Cre)∗ – was randomly inserted into intron 1 of the mouse *Ubc12* gene (ENSMUSG00000005575), in mouse E14 ES cells. **B)** LacZ staining and **C)** *in situ* hybridizations for *Ubc12* on coronal brain sections of adult (12 weeks old) wt and het. KO mice confirmed the KO allele of *Ubc12*. **D)** FLP-mediated inactivation of the gene trap vector resulted in cryptic splicing of exon 1 of Ubc12 to a cryptic splice acceptor located the spacer between the 5' LTR sequence and the frt/lox5157/loxP sites of the gene trap cassette. **E)** *In situ* hybridizations confirmed a reduced expression of *Ubc12* in -/+ FLP mice compared to wild-type littermates. **F)** The cryptic splice site was identified by sequencing of qRT-PCR products with primers located in exon 1 of Ubc12 and in the β-Galactosidase sequence of the gene trap vector. (The blastocyst injections to obtain chimeras were done in collaboration with Dr. Ralf Kühn at the Helmholtz-Zentrum München.)

4.8.2. Nae1 conditional KO mouse

We obtained targeted ES cell clones for a conditional KO mouse of the Nedd8-activating enzyme subunit 1 (Nae1), Appbp1, from Eucomm (http://www.knockoutmouse.org/about/eucomm). In these three ES cell clones (EPD0441_1_B07, _F07, and _C08) a gene trap cassette, consisting of an engrailed 2 splice acceptor (En2 SA)-IRES-LacZ sequence followed by a pGK promoter-neomycin-resistance gene construct, was inserted by homologous recombination in JM8A3.N1 ES cells into intron 3 of mouse *Nae1* gene, leading to disruption of the Nae1 gene and to splicing of exon 3 of *Appbp1* to the SA-IRES-LacZ sequence. The whole gene trap cassette is flanked by frt sites for FLP-mediated excision, resulting in the floxed *Appbp1* allele, in which exon 4 of *Nae1* is flanked by loxP sites (Figure 69.A). Correct targeting of the *Nae1* locus was confirmed by long range PCRs in the quality control step of the Eucomm pipeline and by us. In collaboration with Dr. Ralf Kühn from the IDG at the Helmholtz-Zentrum München we injected the Nae1 ES cell clones into BALB/c blastocysts from donor females. Male chimeric mice were bred to C57BL/6J as well as CD1 females. Although we obtained in total 15 high percentage (70-100%) male chimeras from all three ES cell clones, we only found germline transmission of the mutant *Appbp1* allele in two out of 600 pubs confirmed by PCRs for the 5' and 3' regions of the genetrap cassette as well as for LacZ. These germline mice were first bred to C57BL/6J females, and offspring carrying the mutant *Nae1* allele were further bred among each other to establish the conventional Nae1 KO mouse line. LacZ-reporter

staining and *in situ* hybridization for *Appbp1* on brain sections of heterozygous conventional Nae1 KO mice and wild type littermates showed high expression of Appbp1 in the adult mouse brain in a similar pattern like it was found for Ubc12 (Figure 69.B, C; see also 4.1.1 and 4.1.2), further underlining the neuronal enrichment of components of the Nedd8 pathway in the brain. Furthermore, *in situ* hybridization for *Appbp1* showed a ~50% reduction of the signal on sections of heterozygous Nae1 KO mice compared to wild type littermates, confirming the correct targeting of the *Nae1* locus (Figure 69.B). Western blot analysis of hippocampus extracts revealed a decrease in neddylated proteins and a slight increase in free Nedd8 in het-Nae1 KO mice as well as in het-Ubc12 KO mice compared to wild-type control mice (Figure 69.D). This result was the first proof that knockout of the Appbp1 subunit of Nae diminishes Nedd8 conjugation in the murine brain. In breedings of het-Nae1 KO x het-Nae1 KO, no homozygous Nae1 KO pubs could be obtained, indicating that the conventional homozygous KO of Appbp1 is embryonic lethal similar to the conventional homozygous KO of Ubc12 (comapare to 4.8.1). To obtain a conditional KO of Nae1, conventional heterozygous KO mice were first bred to hACTB::Flp-deleter mice resulting in the floxed wild-type allele of *Nae1* ($Nae1^{flox}$) after excision of the gene trap cassette (Dymecki, 1996; Rodriguez et al., 2000) (Figure 69.E). To dissect the role of the Nedd8 pathway in the CNS, $Nae1^{flox/flox}$ mice will be bred to various Cre-recombinase mouse lines to achieve conditional inactivation of the *Appbp1* gene in specific brain regions, cell populations or neurotransmitter circuits.

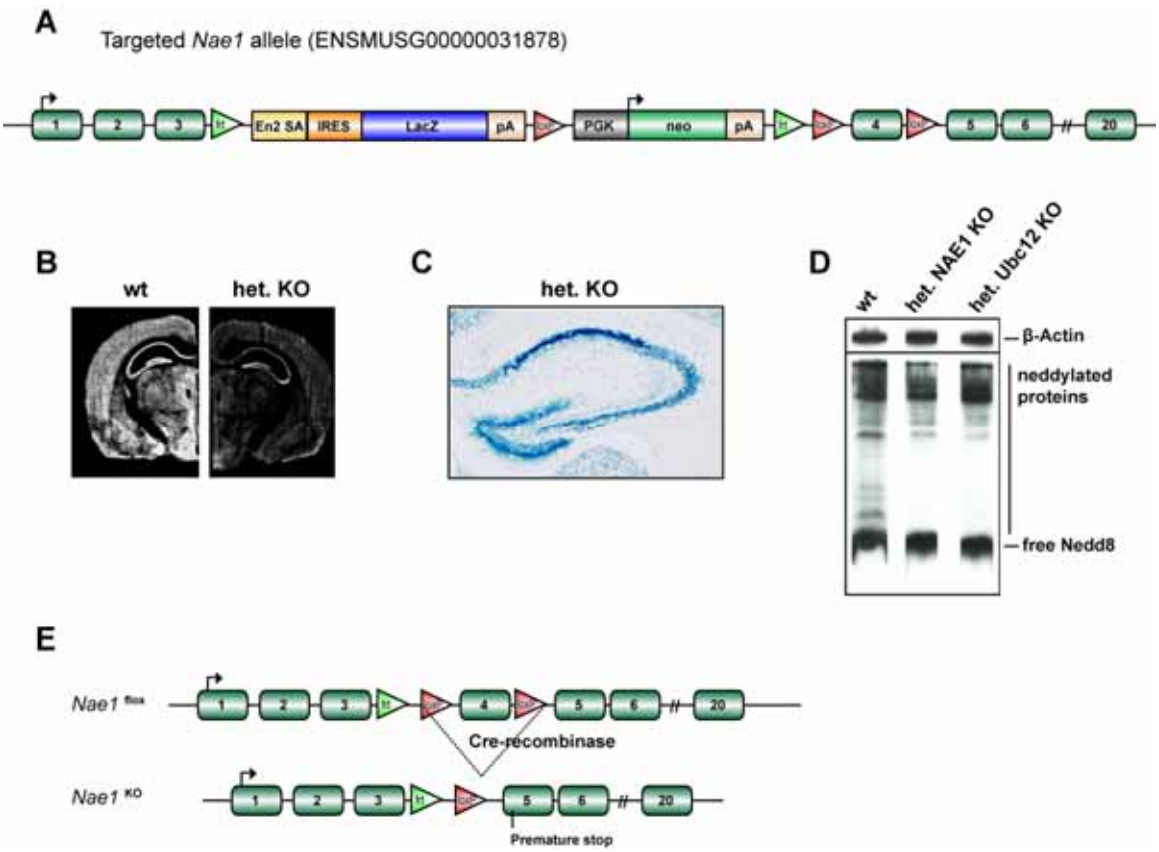


Figure 69: Generation of conditional Nae1 KO mice

A) Targeted *Nae1* allele. A gene trap cassette, consisting of an engrailed 2 splice acceptor (En2 SA)-IRES-LacZ sequence followed by a pGK promoter-neomycin-resistance gene construct, was inserted by homologous recombination into intron 3 of the mouse *Nae1* gene, leading to disruption of the *Appbp1* gene and to splicing of exon 3 of *Appbp1* to the SA-IRES-LacZ sequence. The whole gene trap cassette is flanked by frt sites for FLP-mediated excision, resulting in the floxed *Appbp1* allele, in which exon 4 of *Nae1* is flanked by loxP sites. **B)** *In situ* hybridization for *Appbp1* on wt and het. KO mice confirmed the correct targeting of *Nae1*. **C)** LacZ staining on coronal brain sections of adult (12 weeks old) het. KO (Nae1-LacZ reporter mouse) confirmed the ubiquitous and high expression of Nae1, especially in neurons, as shown for the hippocampus. **D)** Western blot analysis showed a reduction of Nedd8 conjugation in het. KOs of Nae1 and Ubc12. Hippocampus protein extracts from wt, het Nae1 KO and het Ubc12 KO mice, were subjected to immunoblotting with α-Nedd8 antibodies (Epitomics). β-Actin served as loading control. **E)** The conditional KO of *Nae1* will be achieved by Cre-mediated excision of the floxed exon 4 of *Appbp1*, resulting in a premature stop codon. (The blastocyst injections to obtain chimeras were done in collaboration with Dr. Ralf Kühn at the Helmholtz-Zentrum München.)

4.8.3. Conditional Ubc12-C111S overexpression in the *ROSA26* locus

In a second approach we developed a Ubc12-C111S conditional overexpressing mouse line using homologous recombination in ES cells in the *ROSA26* locus as described previously in Lu *et al.* (Lu et al., 2008) with some modifications. Figure 70 depicts the construct and targeting strategy in the *ROSA26* locus. The FLAG-tagged human Ubc12-C111S cDNA sequence was cloned together with the IRES-LacZ sequence into the targeting vector containing 5' and 3' homology arms to target the *ROSA26* locus. To achieve conditional overexpression, upstream of the Ubc12-C111S-IRES-LacZ construct a STOP cassette flanked by loxP sites was inserted, which contains the neomycin-resistance gene expressed under the pGK promoter for G418 selection of ES cells. Cre-mediated excision of the floxed STOP cassette would result in expression of Ubc12-C111S and LacZ under the control of the ROSA26 promoter. In addition to achieve a high overexpression of Ubc12-C111S especially in neurons, the CAG (cytomegalovirus immediate early enhancer/chicken β-actin) promoter flanked by frt sites for Flp-mediated excision was inserted upstream of the floxed STOP cassette and downstream of exon 1 of the *ROSA26* gene. Furthermore the targeting vector contains a diphtheria toxin a fragment (DTA) as a negative selection marker during ES cell culture.

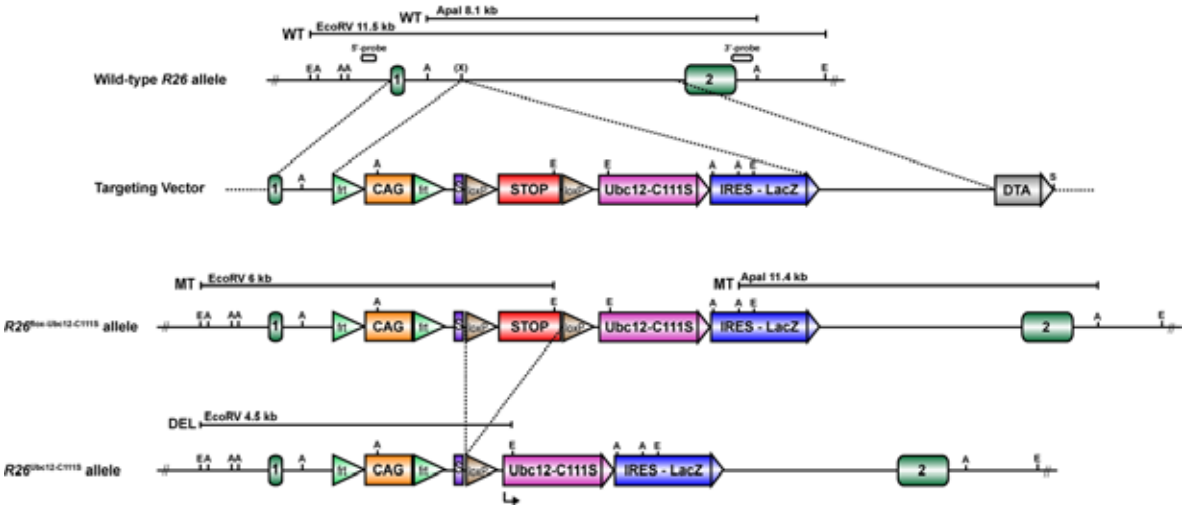


Figure 70: Targeting strategy for conditional overexpression of Ubc12-C111S in the *ROSA26* locus.

Partail restriction maps of wild-type *ROSA26* (*R26*) locus, targeting vector, recombined $R26^{flox\text{-}Ubc12\text{-}C111S}$ allele and activated $R26^{Ubc12\text{-}C111S}$ allele are shown. Ubc12-C111S-IRES-LacZ cassette was cloned into the targeting vector downstream of the floxed (flanked by loxP sites) STOP cassette, which contains the neomycin-resistance gene for selection in ES cells. The targeting vector carries 5' and 3' homology regions to facilitate homologous recombination in the *R26* locus and the minimal CAG promoter sequence flanked by frt sites was introduced upstream of the STOP cassette to achieve high expression of Ubc12-C111S in neurons. Cre-mediated excision of the STOP cassette activates $R26^{Ubc12\text{-}C111S}$ and results in expression of Ubc12-C111S and LacZ. 5'-probe and 3'-probe indicate Southern blot probes. WT, wild-type fragment, MT, mutant fragment following homologous recombination, and DEL, deletion fragment resulting from Cre-mediated excision of the STOP cassette, detected by Southern blot analysis. A, ApaI. E, EcoRV, DTA, diphtheria toxin fragment a, used as negative selection marker, S, SwaI, S, splice acceptor.

IDG3.2 ES cells, kindly provided by Ralf Kühn from the IDG at the Helmholtz-Zentrum München, were electroporated with the linearized targeting vector and selected with G418. Neomycin-resistant ES cell colonies were screened via Southern blot for homologous recombination in the *ROSA26* locus using specific 5'- and 3'- probes (depicted in Figure 70). Genomic DNA was extracted from frozen ES cell clones and digested with EcoRV and ApaI for hybridization with the 5'-ROSA Southern blot probe and 3'-ROSA Southern blot probe, respectively, as previously described (Lu et al., 2008). Several positive ES cell clones were identified by a 5' mutant band of 6 kb and a 5' wt band of 11.5 kb as well as a 3' mutant band of 11.4 kb and a 3' wt band of 8.1 kb (Figure 71.A, B). Positive ES cell clones were expanded and injected via laser-assisted microinjection into blastocysts of superovulated donor females from BDF-1 intercosses (C57BL/6J x DBA). (The blastocyst injections were done in collaboration with Dr. Ralf Kühn at the Helmholtz-Zentrum München.) For germline transmission high percentage male chimeric mice, identified by coat colour and PCR genotyping, were bred to female C57BL/6J mice. The offspring was genotyped by PCR, using primers either to amplify a 3' fragment of the β-Galactosidase/LacZ sequence (LacZ-for and -rev) or a 5' band with ROSA-for and CAG-rev primers (depicted in Figure 71.C-D). Positive mice will be further bred to mouse lines expressing Cre-recombinase to restrict Ubc12-C111S expression to specific brain areas and neurotransmitter circuits. In this line, specific inhibiton of neddylation in the brain of adult mice will be achieved either by breeding to tamoxifen-inducible Cre-ERT2 mouse lines or by stereotactic injection of Cre-expressing AAVs (adeno-associated viruses).

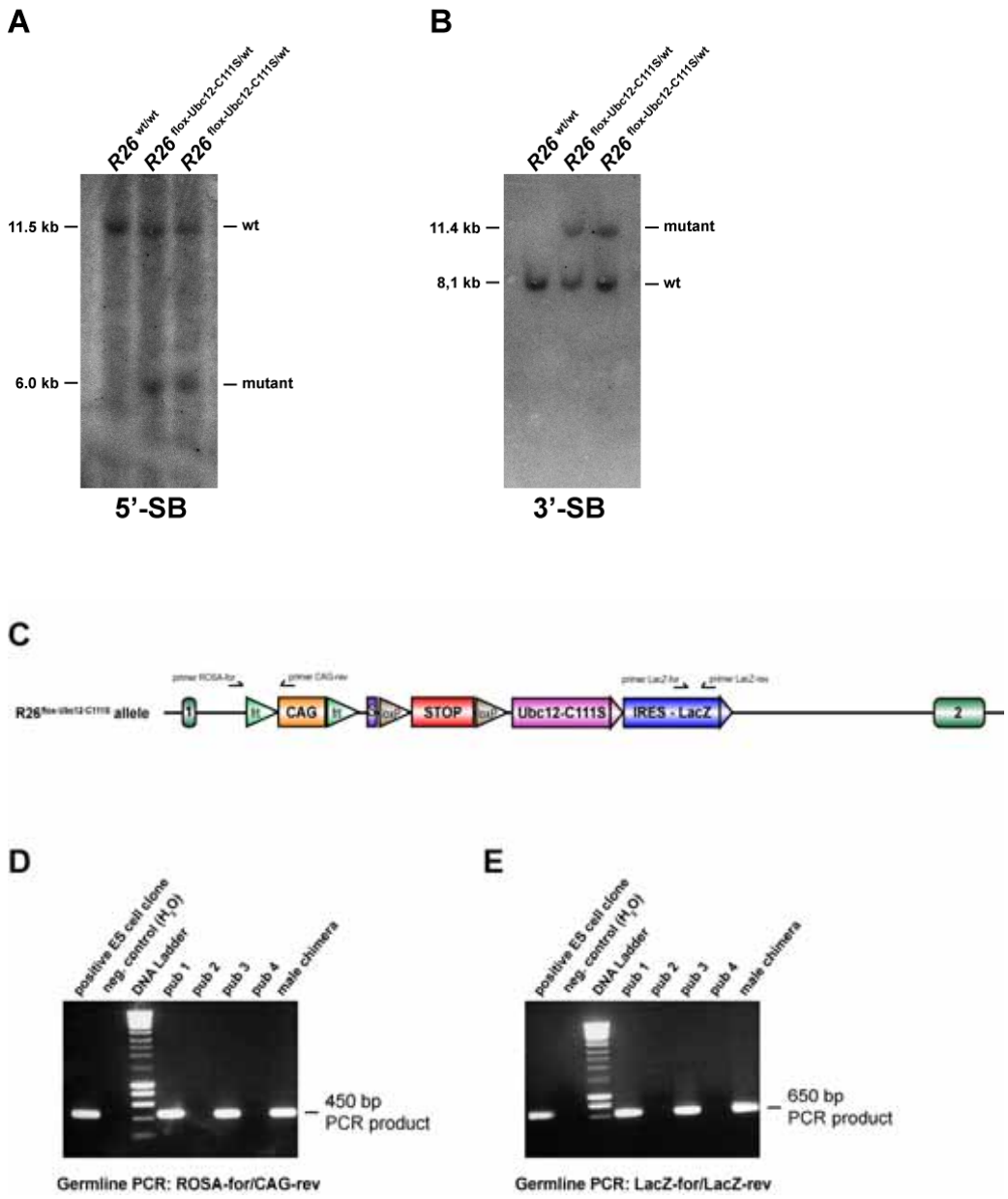


Figure 71: Generation of mice conditionally overexpressing Ubc12-C111S in the *ROSA26* locus.

A) Southern blot analysis of wild-type (wt) and targeted embryonic stem (ES) cell clones. The *R26* 5'-probe was hybridized to EcoRV-digested genomic ES cell DNA. The targeted allele (*ROSA26*$^{flox\text{-}Ubc12\text{-}C111S}$) was indicated by the presence of an additional mutant 6.0 kb fragment. **B)** The *R26* 3'-probe was hybridized to ApaI-digested genomic DNA from the same ES cell clones confirming homologous recombination by detection of an additional mutant fragment at11.4 kb. **C)** Location of primers in the *R26*$^{flox\text{-}Ubc12\text{-}C111S}$ allele, used to screen the offspring of chimera x wt breedings for germline transmission of the targeted allele. **D)** and **E)** Germline transmission of the targeted *R26* allele was detected via PCR in the offspring of chimeric mice. ROSA for/CAG rev primers amplify a mutant PCR product of 450 bp, LacZ for/LacZ rev primers amplify a mutant

band of 650 bp. (The blastocyst injections to obtain chimeras were done in collaboration with Dr. Ralf Kühn at the Helmholtz-Zentrum München.)

In summary, in addition to the Ubc12-LacZ reporter mouse line, we generated two conditional mouse models of the Nedd8 pathway, Nae1 conditional KO mice, in which the Appbp1 subunit of the Nedd8-activating enzyme is deleted, and mice conditionally overexpressing the dominant-negative version of the Nedd8-conjugating enzyme (Ubc12-C111S), that will allow us to dissect the function of the neddylation pathway in the central nervous system in detail, on the molecular, morphological as well as on the behavioral level.

5. DISCUSSION

Ubiquitylation and subsequent proteasome-mediated protein degradation have been found as critical regulators of multiple aspects of neuronal development and function, including neurogenesis, axon and dendrite growth, synaptogenesis, synapse elimination, pre-synaptic function and post-synaptic remodeling during activity-dependent plasticity (Bingol and Schuman, 2005; reviewed in Yi and Ehlers, 2007; Haas et al., 2007; Haas and Broadie, 2008; Segref and Hoppe, 2009; Mabb and Ehlers, 2010; Kawabe and Brose, 2011). Moreover, the dysregulation of the ubiquitin-proteasome system has been implicated in various diseases of the central nervous system (CNS) in humans (reviewed in Ciechanover and Brundin, 2003; Tai and Schuman, 2008; Ding and Shen, 2008; Schwartz and Ciechanover, 2009).

During the last years, several new ubiquitin-like proteins (UBLs) were discovered, including NEDD8, SUMO, ISG15 and many others, whose functions are just to be elucidated (reviewed in Schwartz and Hochstrasser, 2003; Kerscher et al., 2006). So far, Nedd8 has been implicated mainly in the regulation of cell cycle progression and cellular proliferation, stimulating cullin RING ligase-dependent and proteasome-mediated degradation of diverse molecules with key functions in these processes (reviewed in Hori et al., 1999; Pan et al., 2004; Cardozo and Pagano, 2004; Herrmann et al., 2007; Hotton and Callis, 2008). Accordingly, dysregulation of the Nedd8 system is closely related to the aetiology of cancers in human (reviewed in Watson et al., 2011). However, little is known apart from its role in cell cycle control and cellular growth, and the putative actions of Nedd8 in post-mitotic neurons remained almost completely unexplored.

In this work we studied the functions of the neddylation pathway in neuronal development and in the mature neuron in the mouse brain.

The aims of the presented work were I) to characterize the expression pattern of the neddylation pathway in the mammalian CNS; II) to analyze the function of neddylation during neuronal development; III) as well as in the adult neuron; IV) to identify cellular targets of Nedd8 and the molecular mechanism underlying Nedd8 function during neuronal development and in mature neurons and V) to generate conditional mouse models of the Nedd8 pathway in order to dissect the functions of neddylation *in vivo*.

5.1. Expression analysis of the members of the Nedd8 pathway in the mouse brain

The identification of the expression pattern of a specific gene is the first and mandatory step when searching for the unknown function of a particular candidate. Thus, the first aim was to characterize the expression profiles of the members of the Nedd8-conjugation pathway in the mammalian CNS during development and adulthood, both at mRNA and protein level, in order to deduce their cell type-specific expression and to reveal their intracellular localization.

By means of *in situ* hybridizations and qRT-PCRs, we observed that the key members of the neddylation pathway, including Nedd8, Ubc12 (Nedd8-conjugating enzyme) and Nae1 (Nedd8-activating enzyme), are highly, ubiquitously and persistently expressed in the mouse brain at embryonic, postnatal and adult stages. These results were further validated by β-Galactosdidase stainings in brain sections of Ubc12- and Nae1-LacZ reporter mouse lines, which were generated during this work. Moreover, these members of the neddylation pathway were found to be enriched in neurons by means of co-labeling procedures and analysis of expression profiles in primary neuronal cell cultures by qRT-PCR and immunoblotting (see 4.1).

Nedd8 was originally identified as one of different neural precursor cell-expressed, developmentally downregulated (Nedd) genes in a cDNA library screen by Kumar *et al.* (1992).

In our study, we performed a detailed expression analysis of every component of the neddylation system by qRT-PCR in neuronal primary cell cultures and we could not find any significant change in any of the analyzed mRNAs, including Nedd8, Nae1, Uba3, Ubc12, Senp8, Rbx1 and the complete set of cullins. Similar results were observed when the mRNA expression of Nedd8, Ubc12 and Nae1 was assessed by *in situ* hybridization in the CNS. Again, we observed high and ubiquitous expression of these key components of the neddylation system in the CNS throughout development as well as in adulthood. Finally, potential changes during development in the protein expression levels of Ubc12 and free Nedd8 were studied by Western blot analysis but the levels of these proteins remained unchanged during the course of development, supporting the results obtained at mRNA level.

Although in the original description, Kumar and coworkers claimed that Nedd8 is gradually downregulated during brain development, a careful analysis of the original Northern blot pictures shows hardly any embryonic or postnatal decrease in Nedd8 expression (Kumar et al., 1992; Kumar et al., 1993). In fact, a decrease in the Nedd8 mRNA signal can be observed only when adult brain tissue is compared against any other developmental stage. This decrease in the adult brain was not observed in our anatomical mapping by *in situ* hybridization, where neuronal vs. non-neuronal tissues can be clearly discriminated anatomically. This spatial resolution allowed us to detect the neuronal enrichment of Nedd8 and Ubc12 expression. This difference in expression observed between both methods is most probably secondary to the fact that *in situ* hybridization can visually discriminate between different cell types and brain regions, whereas in Northern blot approaches the whole tissue is used as a source of RNA. This is particularly relevant in this case, since brain development in rodents is characterized by dramatic changes in the cellular composition of the brain (Bandeira et al., 2009). Accordingly, neurons are mainly generated during embryonic development, whereas gliogenesis increases dramatically after birth. These changes in the cellular composition of the brain, in particular the addition and growth of new cells and the loss of cells by cell death, are underlying brain growth after birth. In this line, the rat brain contains ~75 x 10^6 neurons and ~4 x 10^6 glial cells (ratio of neuron/glia=18.7) at birth (P0), but 60 days later (P60) the net number of neurons moderately increased to ~200 x 10^6 (due to the postnatal generation of the cerebellum, olfactory bulb and part of the hippocampus), whereas the net number of non-neuronal elements rises to ~130 x 10^6 cells (ratio of neuron/glia=1.5) (Bandeira et al., 2009). This net incease in glial and non-neuronal cells changes the composition of the postnatal and adult brain compared to the embryonic brain. Our results demonstrated that the expression of Nedd8, Ubc12 and Nae1 is enriched in neurons, compared to the lower expression levels detected in glial cells. Therefore, it is obvious, that the large increase in the number of non-neuronal components in the postnatal brain would lead to the apparent decrease in the total amount of Nedd8, Ubc12 or Nae1, detected by Northern blot analysis. With a similar bias, Kamitani *et al.* claimed that Nedd8 is downregulated during embryogenesis after embryonic day (E) 11 in the mouse using Northern blot analysis (Kamitani et al., 1997). However, in this experiment whole mouse embryos were analyzed what precludes any

conclusion about the regulation of Nedd8 in CNS tissue, mainly considering the very high expression of Nedd8 in the heart and skeletal muscle in the adult.

Regarding the expression of Nae1 (also named Appbp1), it has been been reported by Joo and coworkers that Nae1 mRNA expression decreases in the brain of rats by postnatal day 12. However, in the study of Joo *et al.* Nae1 expression was detected in the ventricular zone, where neural precursor cells proliferate, at later time points (P16), evaluated by *in situ* hybridization experiments (Joo et al., 2010).
Our *in situ* hybridizations of Nae1 mRNA in the mouse brain do not suggest any downregulation of Nae1 expression in adult stages, thus supporting similar findings obtained in primary neuronal cell culture systems.
However, a confounding factor in *in situ* hybridization experiments and antibody-based immunodetection systems might be the sensitivity and specificity of the *in situ* hybridization probes and antibodies used. Taking this into consideration and in order to further support and validate our previous anatomical findings, we took advantage of Ubc12- and Nae1-LacZ reporter mouse lines that offer the unique opportunity to detect Ubc12 and Nae1 expression without any (error-prone) PCR-, hybridization- or immunohistochemical-based method (see 4.8). In these reporter mice, the β-Galactosidase sequence was inserted into the endogeneous Ubc12 or Nae1 gene locus and is expressed under the control of the endogenous Ubc12 or Nae1 promoter, thereby unambiguously reporting the endogenous expression of the respective gene. Highly sensitive LacZ stainings, reporting the activity of β-Galactosidase, showed high and ubiquitous expression of Ubc12 and Nedd8 during embryogenesis, postnatal development and in adulthood in the CNS, especially enriched in neurons, as well as in additional tissues and organs.

The identification of the subcellular localization is of particular interest when searching for the unknown function of a specific candidate protein. To the best of our knowledge, the intracellular localization of Nedd8 and Ubc12 was never addressed systematically so far. To approach this question, we first tested whether a set of different commercially available antibodies raised against Nedd8 and Ubc12 might be suitable for immunohistochemical and immunocytochemical studies. Using brain sections and neurons, either overexpressing Ubc12 and Nedd8 or carrying shRNA constructs targeted against Ubc12 and Nedd8 as positive and negative controls, respectively, we

tested the sensitivity and specificity of selected antibodies under different immunohisto- and immunocytochemical conditions (different fixation methods, epitope unmasking protocols, permeabilization reagents and antibody incubation conditions). Unfortunately, none of the tested antibodies proved to be good enough in terms of both, sensitivity and specificity in immunohisto- or immunocytochemistry protocols. Thus, due to the lack of antibodies suitable to specifically detect Ubc12 and Nedd8 with immunofluorescence on a subcellular level in neurons, we generated eGFP- and FLAG-tagged Ubc12 and Nedd8 constructs. In primary neurons, eGFP-Ubc12 and eGFP-Nedd8 fusion proteins showed somatodendritic and axonal localization, and in mature neurons eGFP-Ubc12 and eGFP-Nedd8 were also present in dendritic spines. To rule out that the 238 amino acids long eGFP tag mislocalizes Ubc12 and Nedd8, FLAG-tagged version of Ubc12 and Nedd8 were expressed and a highly similar intracellular localization was detected following α-FLAG immunofluorescence staining.

Two studies reported that Nedd8 detected by immunocytochemical means is predominantly located in the nucleus of tumor cell lines (Kamitani, 1997, Stenoien, 1999). However, our detailed screening of antibodies against Nedd8, including appropriate negative and positive controls, indicated that the Nedd8 antibodies are unspecific and of low sensitivity in immunocytochemical staining protocols. Furthermore, Kamitani *et al.* used a HA-tagged Nedd8 construct transfected into COS-M6 cells and analyzed Nedd8 expression by α-HA (α-HA mAb (16B12)) avidin-biotin-HRP-enhanced immunocytochemistry. In our lab, experiments with different tagged Nedd8 constructs, including FLAG-, HA- and eGFP-tags, transfected into various cell lines, such as HEK, HeLa, HT22 and Neuro-2a, systematically indicated cytoplasmic and nuclear localization of Nedd8. Therefore, though these discrepancies are difficult to reconciliate, it is plausible that the result obtained by Kamitani *et al.* represents an artifact of the HA-tagged Nedd8 construct in combination with the α-HA antibody and the cell line used.

We also observed Nedd8 in the nucleus of primary neurons, but apart from the nucleus, Nedd8 and Ubc12 were found in the cytoplasm, dendrites and axons of neurons. Furthermore, brain fractionation by ultracentrifugation demonstrated that Ubc12 and Nedd8 are present in the synaptic compartments of mature neurons, indicating that neddylation might be involved in more diverse processes in neurons than previously thought.

We also analyzed the activity and targets of neddylation in the developing and adult mouse brain and during development of primary neurons in culture by immunoblotting. Nedd8 and Ubc12 were found at protein level throughout embryonic and postnatal brain development and in the adult mouse brain, as well as in developing and mature primary neuronal cell cultures. Interestingly, we detected many neddylated target proteins in total brain, cortex as well as primary neuron samples, ranging from 30 to 200 kDa. These findings indicate that there are many additional Nedd8-targets in neurons apart from the cullin E3 ubiquitin ligases, the classical described Nedd8 targets, whose molecular weights only range from 98 to 110 kDa. During neuronal maturation *in vitro* as well as during brain development *in vivo*, we observed a peak in neddylation coinciding with the time window of dendritogenesis and synaptogenesis, followed by a change in the neddylation pattern.
Altogether, these experiments clearly indicate that the Nedd8 conjugation activity, but not the expression of any key component of the pathway, is regulated during brain development.

Finally, brain fractionation by ultracentrifugation and subsequent Western blotting with α-Nedd8 antibodies revealed differential patterns of neddylated target proteins in the synaptosomal, pre-synaptic vesicle, and the two different post-synaptic fractions of mature neurons. Until now, almost all effects of Nedd8 are thought to be mediated by the cullin RING ligases (Ou et al., 2002; Kurz et al., 2002; Pintard et al., 2003; Pan et al., 2004; Saha and Deshaies, 2008; Merlet et al., 2009; Boh et al., 2011); and only very few additional cullin-independent targets of Nedd8 have been described so far, mainly tumor suppressor genes, oncogenes and ribosomal proteins (Xirodimas et al., 2004; Stickle et al., 2004; Oved et al., 2006; Watson et al., 2006; Gao et al., 2006; Abida et al., 2007; Xirodimas et al., 2008; Russell and Ohh, 2008; Lee et al., 2008; Ryu et al., 2011) (reviewed in Rabut and Peter, 2008; Xirodimas, 2008). Our expression data clearly indicates that neddylation might accomplish different physiological functions during brain development and in post-mitotic mature neurons, independent of cullins and obviously beyond the regulation of the cell cycle.

5.2. Different loss-of-function approaches to inhibit the neddylation pathway at different steps, to a different degree

Different tools are currently available to block the Nedd8 conjugation pathway at specific steps, including a dominant-negative version of Ubc12 (Ubc12-C111S), siRNA against key members of the neddylation cascade and the NAE-inhibitor MLN4924. First we determined the efficiency of the genetic and pharmacological loss-of-function approaches in primary neurons and in the hippocampal Neuro-2a cell line (described in 4.2).

The dominant-negative Ubc12-C111S abolishes neddylation of target proteins by forming a stable heterodimeric complex with Nedd8 (Wada et al., 2000; Oved et al., 2006). In primary neurons as well as in cell lines, expression of Ubc12-C111S from strong and constitutive promoters, such as the CMV (cytomegalovirus immediate early promoter) and CAG (chicken β-Actin promoter coupled with CMV early enhancer), efficiently blocked neddylation at single cell level. Moreover, we successfully used CAG-Ubc12-C111S constructs to inhibit neddylation in developing neurons of the mouse brain *in vivo*. Additionally, we cloned a construct for tamoxifen-inducible expression of Ubc12-C111S. This plasmid contains a floxed-STOP cassette upstream of the Ubc12-C111S coding sequence, to activate neddylation blockade in adult neurons in *in vitro* and *in vivo* experiments by Cre-mediated excision of the floxed-STOP sequence. This construct was further employed to generate the knockin mouse line in the *ROSA26* locus, in which the neddylation pathway can be blocked in a spatial- and temporal-controlled manner by conditional overexpression of Ubc12-C111S achieved by Cre- and Cre-ERT2- mediated excision of the floxed-STOP cassette. In contrast to the ubiquitin pathway, which depends on ~30 different E2-conjugating enzymes (in humans), Ubc12 is the main E2-conjugation enzyme of the Nedd8 pathway, together with the recently identified Ube2f that specifically neddylates cullin 5, (Gong and Yeh, 1999; Huang et al., 2009; Ye and Rape, 2009; van Wijk and Timmers, 2010; Wenzel et al., 2011). This allows the effective blockade of the neddylation pathway by inhibiting Ubc12 function.

Another well established tool to knock-down the expression of particular genes *in vitro* and *in vivo* is RNA interference (RNAi) (Krichevsky and Kosik, 2002; Hommel et al., 2003; Thakker et al., 2005; Wang et al., 2005; Janas et al., 2006; Guissouma et al., 2006; Salahpour et al., 2007; Peters et al., 2009; Fallini et al., 2010). To stably knock-down the expression of specific genes of the neddylation pathway in primary neurons by calcium-phosphate transfection, we used short hairpin RNA (shRNA) constructs expressed from U6 snRNA gene or H1 RNA Pol III promoters. The efficiency of each shRNA construct was assessed in neuronal cell lines by immunoblotting and a control shRNA sequence containing 5 basepair mismatches to any known human or mouse gene served as control. To minimize confounding off-target effects, experiments were performed at least with two different shRNA sequences efficiently targeting the same gene. Furthermore, in this work, we used shRNA experiments to validate previous results, obtained by transefction of dominant-neagtive constructs or pharmacological inhibition, by an independent method, thereby strengthening our findings.

The specific NAE-inhibitor MLN4924, recently developed by Millenium Pharmaceuticals (Soucy et al., 2009), offers the unique opportunity to block neddylation in a dose-dependent manner in whole neuronal cell populations and networks. MLN4924 was purchased either at Active Biochem or synthesized and kindly provided by Lak Shin Jeong from the Department of Bioinspired Science and Laboratory of Medicinal Chemistry, College of Pharmacy at the Ewha Womans University in Seoul, South Korea (Lee et al., 2011). MLN4924 from both sources inhibited the Nedd8 pathway in a dose-dependent manner, in neuronal cell lines and primary neurons. Efficient neddylation blockade was achieved by treatment of cells with 0.1 to 1 μM MLN4924 within 2 to 24 h, as shown by Western blotting with α-Nedd8 and α-Cullin 1 antibodies.

All three approaches, Ubc12-C111S, shRNAs targeting Ubc12 or Nedd8 and pharmacological inhibition by MLN4924 treatment, gave highly consistent results and thereby, underscored the relevance and importance of the neddylation pathway in neuronal development. Furthermore, experiments in primary neurons with MNL4924 validated the specificity of the α-Nedd8 antibodies used in this work, further emphasizing that many different proteins are neddylated in neurons apart from cullins.

To evaluate, if the effects we observed upon neddylation blockade in neurons are dependent or independent of cullin RING ligases, the known targets of Nedd8, we used three different approaches to interfere with the function of cullin RING ligases. First, we transfected dominant-negative cullin 1, 2, 3, 4A, 4B and 5 constructs, which lack the C-terminal region containing the conserved Nedd8-conjugation site, developed by the lab of Wade Harper (Shirogane et al., 2005). These mutant cullins sequester adaptors and substrate receptors but have no ubiquitin ligase activity, thus acting as dominant negatives. Second, another target to inhibit cullin 1 through 4 at the same time is the ring protein Rbx1, which is necessary for the activity of every cullin. Thus, we used dominant-negative Rbx1 constructs (Rbx1-C42S-C45S and Rbx1-C75S), that lack enzymatic RING ligase activity (Kamura et al., 1999a; Kamura et al., 1999b). And third, we knocked down the expression of Rbx1 via shRNAs.
As a functional read-out of the different loss-of-function approaches, we evaluated the neddylation of cullin 1 by immunoblotting. All different methods applied, resulted in a substantial decrease of neddylated cullin 1, thereby proofing the efficiency of the different genetic and pharmacological tools to inhibit the Nedd8 pathway. In most of the validation experiments performed, the unneddylated cullin 1 was reduced as well, indicating that Nedd8 contributes not only to the activity but also to the stability of cullin 1.

Interestingly, compared to shRNA- and MLN4924-mediated blockade of neddylation, expression of Ubc12-C111S produced slightly stronger and more severe morphological phenotypes in neurons, probably due to the stable sequestration of Nedd8 and total depletion of Nedd8 from the cell.

By combining primary neuronal cell culture and *in vivo* experiments with the tools to inhibit neddylation discussed above, we provide evidence that neddylation critically controls migration, axon formation, dendritic growth and synaptogenesis during brain development and regulates spine stability in the adult neuron (largely) independent of cullin RING ligases.

5.3. The Nedd8 pathway controls axonal growth: role of the cytoskeleton

We found that neddylation controls neuronal polarization and axonal growth during early development, in the first 72 h after plating of primary neurons in culture. Inhibition of neddylation, by means of Ubc12-C111S transfection, expression of shRNAs targeting Nedd8 and Ubc12 and MLN4924 treatment, resulted in impaired neuronal polarization and axon formation in hippocampal neurons as well as strongly reduced axonal growth in cortical neurons. Axon formation and axonal growth were analyzed by classification of neurons into stage 1, 2 and 3 and measurement of axonal length and branching of neuron tracings, respectively, as previously decribed (Dotti et al., 1988; Delima et al., 1997; Bradke and Dotti, 2000b; Meijering et al., 2004; Barnes and Polleux, 2009; Polleux and Snider, 2010) (see 4.3). The differences in the extend of inhibition of axon formation observed between complete inhibition of neuronal polarization in hippocampal neurons and reduction of axonal growth in cortical neurons might reflect the different degree of polarization that primary hippocampal and cortical neurons already possess at the time of preparation (Barnes and Polleux, 2009; Polleux and Snider, 2010). Accordingly to well established protocols, we usually prepared primary neuronal cultures from embryonic day (E) 17.5-18.5 CD1 mice or E18.5–19.5 Sprague Dawley rats (Dotti et al., 1988; Goslin and Banker, 1991; de Hoop et al., 1997; Kaech and Banker, 2006). At this developmental time point, cortical neurons possess already a higher degree of polarization compared to hippocampal neurons that develop a bit delayed in time (Nakahira and Yuasa, 2005; Ko et al., 2005).

Interstingly, long-term live cell imaging studies revealed that hippocampal neurons expressing Ubc12-C111S, which completely fail to polarize and to develop neurites, do not undergo apoptosis, but they are able to survive for the whole length of the experiment (more than 5 DIVs). This suggests, that at early developmental stages, neddylation is rather controlling specific cell intrinsic mechanisms or programs that eventually lead to neurite formation and axonal growth, than interfering with general neuronal health or survival pathways. Furthermore, we could demonstrate that the effect on axon development is specific for the Nedd8-conjugation cascade, since inhibition of the sumoylation pathway did not alter axonal growth, indicating that among the UBLs, Nedd8 plays a unique role in neuronal development.

We then examined the contribution of the cullin RING ligases, the best known targets of Nedd8, to the axon phenotype. However, inhibition of cullin RING ligase function by transfection of the dominant-negative cullins (Shirogane et al., 2005) and shRNAs targeting Rbx1, didn't recapitulate the strong impairments in axonal development observed under neddylation blockade. Hence, we concluded that neddylation controls axon growth independently of cullin RING ligases in primary hippocampal and cortical neurons. Studies in *Drosophila* implicated cullin 3 in axonal arborization of mushroom body neurons (Zhu et al., 2005). Loss-of-function of cullin 3 in these neurons resulted in reduced axonal arborization and in defects of terminal morphogenesis of neurites. Similar phenotypes were observed in Nedd8 mutant neurons, suggesting that cullin 3-based ubiquitin ligase activity is required for neurite arborization, and that proper functioning of cullin 3 might depend on neddylation (Zhu et al., 2005). In our work, we didn't find any major contribution of the cullin E3 ligases to the impairments of axonal growth, induced by Nedd8 blockade in primay hippocampal and cortical neurons. Interestingly, we observed, that loss-of-function of cullin 3 and 4A resulted in slightly shorter axons, whereas inhibition of cullin 1 led to slightly longer and more branched axons. However, since inhibition of individual cullin RING liagses had opposing effects and knock-down of Rbx1, and thereby simultaneous inhibition of cullin 1 to 4, didn't produce strong defects in axonal growth, we inferred that neddylation controls neuronal polarization and axonal growth largely independent of cullin E3 ligases in hippocampal and cortical neurons.

In the past, elegant studies demonstrated, that dynamic rearrangements of the neuronal cytoskeleton are ultimately responsible for axon formation (Bradke and Dotti, 1999; Witte and Bradke, 2008; Witte et al., 2008; Tahirovic and Bradke, 2009; Hoogenraad and Bradke, 2009; Stiess and Bradke, 2011). In order to study the mechanisms possibly underlying the defects in neuronal polarization and axonal growth, we examined the consequences of neddylation blockade on the microtubular and actin cytoskeleton. We observed that blockade of neddylation, via expression of Ubc12-C111S or by MLN4924 treatment, resulted in strong distortions of the microtubular and actin cytoskeleton of primary neurons, evident I) in a decrease of stable microtubules (α-Acetylated-Tubulin), and therefore, in a higher ratio of dynamic/stable MTs in the axon; II) in a distorted distribution of dynamic microtubules (α-Tyrosinated-Tubulin); and III) in a higher amount of F-actin surrounding the neuronal periphery as well as in more dynamic behavior of

actin-based filopodia. Accordingly to these morphological changes, treatment with 1 µM Cytochalasin D, which disrupts actin filaments, and low concentration (10 nM) of Taxol, which promotes microtubule stabilization (Witte et al., 2008), proved to be effective to rescue polarization and axonal growth in Ubc12-C111S-expressing and MLN4924-treated neurons. Furthermore, Cytochalasin D and Taxol treatment decreased the amount of actin filaments and increased the amount of stable microtubules resulting in a higher ratio of stable/dynamic MTs in the axons of MLN-treated neurons comparable to vehicle-treated neurons and in the induction of formation of multiple axons in vehicle-treated as well as in MLN4924-treated neurons. These results are in line with previous findings, that local actin instability (Bradke and Dotti, 1999), which can be induced by Cytochalasin D treatment, and concomitant stabilization of microtubules (Witte et al., 2008), which can be induced by treatment with low concentrations of Taxol, in one of the neurites, eventually specifiy axon formation (reviewed in Witte and Bradke, 2008; Tahirovic and Bradke, 2009; Hoogenraad and Bradke, 2009; Stiess and Bradke, 2011; Neukirchen and Bradke, 2011b).

Interestingly, experiments with the NAE-inhibitor MLN4924 revealed a dose-dependent effect of Nedd8 in neuronal polarization and axonal growth. High concentrations (1 µM) of MLN4924 led to a complete failure of neuronal polarization, whereas low (0.1 µM) to moderate (0.5 µM) concentrations of MLN4924 only slightly affected neuronal polarization but severly reduced axonal growth. These findings might suggest that different mechanisms and different target proteins could be involved in mediating Nedd8 effects during early neuronal development.

Thus, our next goal was the identification of cellular regulators of actin filaments and microtubules, which are modified by direct Nedd8 conjugation, in order to at least partially uncover the molecular mechanisms how neddylation exerts its effects on the neuronal cytoskeleton. Many actin regulatory proteins, e.g. Ena/VASP proteins, Cdc42 and Cofilin (Garvalov et al., 2007; Kwiatkowski et al., 2007; Dent et al., 2007), and microtubule-associated proteins, e.g. cytoplasmic linker protein (CLIP)-115 and CLIP-170 (Neukirchen and Bradke, 2011a) and plus end tracking proteins (+TIPs), such as EB3 (Geraldo et al., 2008), were described to regulate neurite formation and axonal growth in primary neurons. In this line, we found that the microtubule end binding protein EB3, which promotes persistent microtubule growth and stability (Tirnauer and Bierer, 2000; Geraldo et al., 2008; Komarova et al., 2009), might be a direct target of

neddylation. Immunoprecipitation experiments in hippocampal cell lines showed that EB3-GFP is covalently modified with 3x-FLAG-Nedd8. Furthermore, we could show that neddylation regulates EB3 protein levels in neuronal cell lines as well as in primary neurons and accordingly, overexpression of EB3 partially rescued the impaired axonal growth induced by Ubc12-C111S expression in hippocampal and cortical neurons. Ongoing studies in our laboratory aim to identify putative Nedd8 E3 ligases, which neddylate EB3, as well as to identify by mass spectrometry analysis specific lysine residues in EB3 that are modified by Nedd8.

So far, our biochemical and functional experiments indicate that EB3 might be a promising target of Nedd8, possibly involved in mediating the effects of neddylation on axonal growth. Recently, it has been shown that inhibition of EB3-function, and especially the loss of the interaction between the F-actin binding protein drebrin and EB3, results in a severe impairment of neuritogenesis in cortical neurons (Geraldo et al., 2008), further establishing a crucial role for the coordinated and orchestrated interactions of microtubules and actin filaments in neurite growth. Further experiments are planned with the aim to characterize the influence of neddylation of EB3 on its protein function, especially in regards to the interaction with other microtubule-binding proteins, e.g. CLIPs and +TIPs as well as the interaction with drebrin.

Previous studies in *C. elegans* implicated the Nedd8/cullin system in the regulation of microtubules during development. Neddylation and deneddylation of cullin 3 by the COP9 signalosome was shown to be required for proper degradation of the microtubule severing protein MEI-1/Katanin at the meiosis to mitosis transition (Kurz et al., 2002; Pintard et al., 2003). In contrast, our results demonstrate that in developing mammalian neurons the Nedd8 pathway regulates microtubules and actin filaments in a direct and cullin-independent manner.

As discussed above, due to the remarkable differences in axon formation between *in vivo* and *in vitr*o models, we then attempted to validate our previous findings in *in vivo* studies. Therefore, we applied *in utero* gene delivery in mouse embryos to express Ubc12-C111S in neurons of the retrosplenial cortex and in pyramidal neurons of CA1 of the hippocampus. As a read-out of axon growth, we analyzed the axon projections to and terminals in the contralateral brain hemispheres via co-expression of fluorescent proteins. Inhibition of neddylation *in vivo* resulted in strong defects of axonal growth and strongly reduced terminal targeting, confirming a role of neddylation in axon

development. Interestingly, as observed in primary neurons, also *in vivo*, hippocampal neurons seemed to be affected more severly by the blockade of neddylation compared to cortical neurons. Although EB3 overexpression was sufficient to restore axon growth during Nedd8-blockade in primary neurons, in *in utero* electroporation experiments EB3 overexpression could not rescue the defects in axon growth and terminals induced by Ubc12-C111S expression, neither in the cortex nor in the hippocampus (data not shown). This result suggests that additional yet unidentified Nedd8 targets contribute to the regulation of axon formation and growth during neuronal development in the brain.

Since gene expression is altered only in a subpopulation of individual neurons and general environmental guidance cues are not affected, the *in utero* electroporation technique allows us to study cell-autonomous processes during axon growth as well as the intracellular response to extracellular cues. Using *in utero* electroporation, Hand and Polleux showed in a recent study, that Neurogenin2 regulates the initial axon guidance of cortical pyramidal neurons, that project medially to the corpus callosum (Hand and Polleux, 2011). *In vivo* axons are guided by extracellular repellent and attractant cues to their final destination, where they eventually establish synaptic contacts. In this sense, we are convinced that in the target identification studies, our research group is performing, molecular candidates related to guidance and navigation will be found.

Although, *in utero* electroporation is commonly used to study axon development *in vivo* (Chen et al., 2005b; Wang et al., 2007; Mizuno et al., 2007; Sehara et al., 2010; Mizuno et al., 2010; Kolk et al., 2011; Hand and Polleux, 2011), it is noteworthy, that there are clear limitations of this technique for studying axonal projections *in vivo*. The variability in the targeting procedure is one of the main drawbacks of this approach. Since we evaluate axon projections to the contralateral brain side it is important and necessary to compare similar populations of electroporated neurons and that these cells express the fluorescent reporter protein at equal levels. In order to avoid wrong interpretations of the presence or absence of axon projections, we choose for analysis only highly similar electroporated brains, both in terms of region and expression levels. This circumstance clearly limits the number of brains available and diminishes the efficiency of this technique. Hence, the generation of conditional Nae1 knockout and conditional Ubc12-C111S overexpressing mice crossed to reporter mice, expressing fluorescent proteins in cortical and hippocampal brain regions, will allow a more detailed analysis of axonal growth, branching, guidance as well as synaptic targeting and pruning during brain

development *in vivo*. Furthermore, by means of breeding to different Cre-transgenic mouse lines, the function of neddylation in axon development can be addressed in different neuronal populations in the brain as well as in neurons in the spinal cord and the peripheral nervous system.

5.4. Neddylation controls dendrite development

The proper formation of dendrites and the establishment of their particular branching patterns are fundamental to neuronal circuit functioning in the brain and provide the basis for neurons to receive, process and propagate information (Hausser et al., 2000; Vetter et al., 2001; London and Hausser, 2005). Dysregulation of dendrite development and abnormal dendrite remodeling is associated with several severe neurodevelopmental as well as neurological brain diseases (Emoto, 2011).

The increase in neddylation during a temporal window of the brain development that coincides with the terminal remodeling of dendritic arborizations and synaptic contacts, is suggestive for a role for the Nedd8 conjugation pathway in dendritic development. Indeed, we found that inhibition of the Nedd8 conjugation resulted in impaired dendrite formation in primary neurons in culture as well as in pyramidal and granule neurons *in vivo*, evidenced by a decrease in total dendritic length and a reduced dendritic branching pattern measured by Sholl analysis (Sholl, 1953; Meijering et al., 2004).
Remarkably, the lack of changes observed after transfection with dominant-negative cullin and Rbx1 constructs indicated that neddylation regulates dendrite formation seemingly through cullin RING ligase-independent mechanisms.
Previous studies in *Drosophila* reported different and even opposite effects of cullin RING ligases in the development of dendrites in different types of neurons. The COP9 signalosome possesses a dual role, both stimulating and repressing dendritic growth in larval PNS (peripheral nervous system) neurons by controlling cullin 1 and cullin 3 levels, respectively (Djagaeva and Doronkin, 2009a; Djagaeva and Doronkin, 2009b). In this line, cullin 1 was shown to act with the substrate-specific F-box protein Slimb to target the Cubitus interruptus protein for degradation by the proteasome thereby stimulating dendritic growth, whereas cullin 3 was shown to reduce dendritic branching via proteasome-mediated degradation of the actin-crosslinking BTB-domain protein Kelch (Djagaeva and Doronkin, 2009a; Djagaeva and Doronkin, 2009b). Additionally, an earlier study showed the involvement of cullin 3 in axonal arborization and dendritic elaboration in mushroom-body (mb) neurons of *Drosophila*. In contrast to the findings in PNS neurons, loss-of-function of cullin 3 in mushroom-body neurons of *Drosophila* resulted in decreased dendritic elaboration (Zhu et al., 2005). Similar phenotypes were

observed in Nedd8 mutant neurons, suggesting that the Cul 3-based ubiquitin ligase is required for neurite arborization, and its proper function might require neddylation (Zhu et al., 2005). In contrast, our results indicate that neddylation controls dendritic growth and branching in the mammalian brain, mainly by targeting alternative, so far unknown substrates. This might reflect different influences of cullins on evolutionary different brains.

Two recent studies identified targets of E3 ubiquitin ligases that regulate dendrite development in primary neurons and in the mammalian brain. The ubiquitin ligase Nedd4-1 was shown to promote dendrite extension by ubiquitinating Rap2, a ras-related protein, that together with TNIK modulates the cytoskeleton and cell morphology (Kawabe et al., 2010). On the other hand, the ubiquitin ligase $Cul7^{Fbxw8}$ was found to induce the ubiquitination and degradation of Grasp65, by this means it organizes the structure of the Golgi complex and drives dendrite elaboration (Litterman et al., 2011).
Our *in utero* electroporation experiments highlight the Nedd8 pathway as a critical regulator of dendritic growth and arborization in pyramidal neurons of the cortex and hippocampus in the mouse brain. Inhibition of neddylation led to reduced dendritic growth and strongly reduced dendritic complexity, fully recapitulating *in vivo* the effects observed in primary neuronal cultures.
Interestingly, inhibition of Nedd8 function during cortical and hippocampal development *in vivo* also produced defects in radial migration of cortical pyramidal neurons as well as slight disturbances in migration of hippocampal pyramidal neurons. These migratory defects were evident in ‘overmigration phenotypes’, characterized by an excessive migration of neurons that did not stop in the correct place, but instead migrated too far. This suggests that signalling pathways that normally regulate neuronal migration are distorted. In this sense, the reelin signaling system, that controls neuronal guidance and cortical layering (Caviness and Sidman, 1973; Caviness, 1975; Goffinet, 1979; Caviness, 1982) appears as a reasonable target candidate for neddylation. Reelin is secreted by Cajal–Retzius cells in the marginal zone, and activates Vldlr and ApoER2 as wells as the downstream effector molecule Dab1 in cortical neurons that migrate radially towards the pial surface. Binding of reelin to Vldlr or ApoER2 results in tyrosine phosphorylation of Dab1, which is necessary for the activation of downstream signaling cascades that ultimately mediate the cellular effects of reelin and stop migrating neurons

(Howell et al., 1997; Rice et al., 1998; Howell et al., 1999; Hiesberger et al., 1999; Howell et al., 2000; Kerjan and Gleeson, 2007).

Two recent studies, analyzed the function of cullin 5 in reelin signaling and migration of cortical neurons by means of *in utero* electroporation of mouse embryos. Cullin 5 was identified as the ubiquitin ligase mediating ubiquitination and subsequent degradation of phosphorylated Dab1 in response to reelin, thereby regulating neuron layering in the developing cortex, controlling migration speed and stopping point of developing neurons. Knock-down of cullin 5 by RNAi, caused accumulation of active, phosphorylated Dab1 protein and a defect in cortical layering, characterized by excess migration of cortical neurons (Feng et al., 2007; Simo et al., 2010). Unfortunately, the role of neddylation of cullin 5 in these processes was not addressed. However, these experiments suggest, that cullin 5-based E3 ligase function might be involved in the migration phenotype induced by Nedd8 inhibition in the developing cortex. Nevertheless, the effects we observed *in vivo* by inhibiting neddylation in the developing cortex were more severe than those reported for cullin 5 knock-down, suggesting that the Nedd8 system regulates additional targets and signaling pathways beyond cullin 5 in order to control neuronal migration. In this line, inhibition of neddylation in migrating upper layer cortical neurons caused additional migratory phenotypes. For instance at P7, some neurons expressing Ubc12-C111S were consistently found below layers II and III in the cortex. Furthermore, the migratory defects in pyramidal neurons in the developing hippocampus and in newborn granule neurons in the dentate gyrus of adult animals induced by inhibition of neddylation rely on alternative targets and signaling mechanisms, since reelin signaling is unique in the developing cortex.

As mentioned, previous work in fruitflies suggested that neddylation might exert differential influences on different set of neurons. To gain more insight into the diverse roles of neddylation during neuronal development and to further study the function of the Nedd8 pathway in another type of neuron, we analyzed the generation of new granule neurons in the dentate gyrus (DG) during adult neurogenesis. Therefore, we employed retroviral injection strategies to label and manipulate gene expression in adult newborn neurons. Adult neurogenesis refers to the production of new neurons in an adult brain. Adult neurogenesis is a prominent example of adult neuroplasticity that occurs in most vertebrate species including humans (Eriksson et al., 1998; Gross, 2000; Curtis et al., 2003; Sanai et al., 2004; reviewed in Ming and Song, 2005). The two neurogenic niches

in the rodent brain are the subgranular zone of the DG and the subventricular zone of the lateral ventricle, from where newborn neurons migrate to the olfactory bulb (OB) via the rostral migratory stream (RMS) (Kempermann and Gage, 2000; varez-Buylla and Garcia-Verdugo, 2002; van Praag et al., 2002; Carleton et al., 2003). For us, the process of adult neurogenesis represented an interesting model to study the role of neddylation in basic aspects of neuronal development and neuroplasticity *in vivo* because it is controlled by well-described developmental programs which are different from those controlling embryonic neuronal development (Cameron and Mc Kay, 2001; Ming and Song, 2005; Balu and Lucki, 2009). By means of labeling of newborn neurons by retroviral injections, we found that neddylation controls dendrite formation in newborn granule neurons of the DG as well as migration of these newborn DG neurons. Dendritic length and complexity of dendritic arborization was severly decreased in newborn granule neurons expressing Ubc12-C111S compared to control neurons. Interestingly, inhibition of neddylation resulted in aberrant bipolar and multipolar dendritic morphologies and misprojecting axons of new granule neurons of the DG. Furthermore, the survival of Ubc12-C111S-expressing newborn neurons was reduced compared to control neurons. The decreased survival rates upon neddylation blockade are most probably secondary to the strong morphological distortions, a phenomenon known to affect the functional integration into the local hippocampal circuit, what eventually results in elimination of these neurons (van Praag et al., 2002). These experiments further established the Nedd8 pathway as a necessary and facilitatory regulator of dendrite patterning in the central nervous system. Due to the intrinsic methodological difficulty that implies the necessity of making a retrovirus batch for every plasmid wished to be transfected, we could not infect animals with retroviral vectors coding for dominant negative forms of every cullin. Nevertheless, we speculated that the Nedd8 effects in adult neurogenesis might be independent of cullin RING ligases, because progenitor cell proliferation seemed not to be affected in our experiments.

A major challenge for the near future is to identify novel Nedd8 substrates that mediate effects on dendritic elaboration and arborization as well as on migration of developing neurons. In order to achieve this, we perform unbiased screening approaches (see later 5.5.3).

Dysregulation of dendritic growth and patterning in development causes severe neurodevelopmental disorders and dendritic shrinkage is related to neurodegenerative diseases in humans (Duyckaerts et al., 2008; Luebke et al., 2010; Emoto, 2011). Accordingly, the spatio- and temporal-controlled ablation of Nedd8 function in conditional Nae1 knockout and Ubc12-C111S knockin mice in the context of neurodevelopmental and neurodegenerative disease mouse models, will provide insight into the involvement of the neddylation system in developmental and remodelling processes affecting dendritic morphology and function causing CNS diseases.

5.5. The Nedd8 pathway controls spine development and stability via PSD-95

One of the main interests of our laboratory is to uncover the functions of the neddylation pathway in spine development and synaptic function in the mammalian brain. During the work presented in this thesis, we gained insight into the different functions of Nedd8 in dendritic spine formation during brain development and synapse stability in mature neurons by means of primary neuronal cell culture models and the *in utero* electroporation technique. We demonstrated that neddylation is required for dendritic spine development as well as for spine stability in the adult brain. These effects were cullin-independent, and our lab identified and characterized PSD-95 as a novel neuron- and synapse-specific Nedd8 target. Furthermore, we could establish a crucial role of neddylated PSD-95 in spine formation. These results will be discussed in the following section.

5.5.1. Neddylation regulates spine development in primary neurons in culture and *in vivo*

During later stages of brain developmental, neurons establish synaptic contacts among each other. Accordingly to the prevalent hypothesis, the process of synapse formation in neo- and allocortical brain structures occurs via the transition of filopodia structures to mature spines (Dailey and Smith, 1996; Ziv and Smith, 1996; Fiala et al., 1998; Maletic-Savatic et al., 1999; Marrs et al., 2001; Trachtenberg et al., 2002; Zuo et al., 2005; de Roo et al., 2008).

Inhibition of neddylation in primary hippocampal and cortical neurons between DIV14 and 20 resulted in severe defects in spine development. Pharmacological inhibition and genetic inactivation of the neddylation pathway produced numerous and aberrantly long filopodia instead of the classical mature mushroom-shaped dendritic spines on excitatory pyramidal neurons. Neddylation-deficient neurons displayed a reduction in synapse numbers at the morphological level, which was functionally reflected in a reduced spiking frequency of mEPSPs (miniature Excitatory Post-Synaptic Potentials) determined in

electrophysiological recordings. mEPSPs originate from spontaneous single synaptic vesicle fusion events, and the glutamate release, which activates receptors in the post-synaptic membrane, is detected. Of note, the fact that mEPSP amplitudes in Ubc12-C111S-expressing neurons were similar to control neurons indicates, that the number and density of post-synaptic glutamate receptors at the remaining synapses directly formed on the dendritic shaft are normal in neddylation-defective neurons. Interestingly, similar results were recently observed by Kawabe *et al.* in Nedd4-1 KO neurons (Kawabe et al., 2010).

The large number of filopodia-like structures might be secondary to disturbances in the dynamic behavior of filopodia that is normally necessary to explore the surrounding neuropil in search for pre-synaptic partners (Dailey and Smith, 1996; Portera-Cailliau et al., 2003). This hypothesis would indeed help to explain the decrease in synapse numbers and the lack of pre-synaptic contacts near filopodia tips in neddylation-deficient neurons. However, recordings of filopodia motility in neddylation-defective neurons by time-lapse microscopy clearly displayed an opposite picture, with highly dynamic and motile filopodia, further discarding this possibility. Thus, we hypothesize that neddylation-deficient, highly motile filopodia are impaired in their intrinsic capability to stabilize synaptic contacts. In turn, this defect results in the observed impairments in synapse formation.

Further analysis of remaining synapses in neddylation-deficient neurons by immunocytochemical stainings with antibodies detecting specific proteins in the post-synapse, e.g. gephyrin and GluR1, will also provide information about the type and quantity of synaptic contacts, inhibitory vs. excitatory synapses, established on dendrites of Ubc12-C111S-expressing or MLN4924-treated neurons compared to control neurons.

Inhibition of neddylation by different means showed consistent effects on synapse formation but, are these effects also taking place in the normal developing brain? Indeed, we confirmed the function of neddylation in spine development *in vivo* by means of *in utero* electroporation experiments. In pyramidal neurons of the mammalian forebrain two different modes of spine formation are observed depending on the developmental stage. During early postnatal development spine formation is based on the growth of dynamic filopodia and their subsequent transformation into spines. In this line, dendritic filopodia are observed in pyramidal neurons of the neocortex mainly

between P2 and 12 (Ziv and Smith, 1996; Portera-Cailliau et al., 2003). Accordingly, our analysis of cortical and hippocampal pyramidal neurons expressing Ubc12-C111S revealed that blocking of Nedd8 conjugation *in vivo* leads to an increased filopodia formation in young postnatal stages (P5 to 21), as it was observed in primary neurons in culture.

In older animals (>P21), the alterations in spine development were reflected as a reduced spine density. These findings indicate that the Ubc12-C111S-induced developmental alterations cannot be compensated by alternative mechanisms and indeed impact on the adult brain. Recent studies report that during later stages of postnatal brain development (third postnatal week) and in the adulthood, spines also seem to grow directly from dendritic shafts without filopodia precursors (Engert and Bonhoeffer, 1999; Trachtenberg et al., 2002; Jourdain et al., 2003; de Roo et al., 2008). We cannot rule-out that this second form of spine formation is also compromised by Nedd8 inhibition in our *in utero* electroporation experiments. Future, two-photon imaging studies of eGFP-labeled spines in cortical neurons in *in utero* electroporated and transgenic animals carrying a cranial window will help to understand this issue.

Prospectively, the conditional mouse mutants of the Nedd8 pathway, we generated, will provide the basis to globally understand the complete role of this pathway in synapse formation at different developmental stages and brain regions. In addition, these mouse models will allow us to explore the putative contribution of neddylation in synapse growth, not only under physiological but also in pathological CNS conditions, via differential breeding with mouse models of neurodevelopmental disorders.

As observed in axon and dendrite development, Nedd8 effects on spine development were primarily independent of the cullin E3 ligases, suggesting that yet unknown targets of Nedd8 mediate synaptic effects in mammalian neurons. Notably, in *Drosophila*, cullin 5 was implicated in cell fate specification and synapse formation at the neuromuscular junction (NMJ) of motor neurons during development (Ayyub et al., 2005). It is important to state, that in our experiments, the involvement of cullin RING ligases in mediating Nedd8 effects was tested solely on the morphological level. Therefore, we do not exclude contributions of cullin E3 ligases in Nedd8 function during the development of mammalian neurons. However, since the strong effects we observed upon neddylation blockade in different developmental stages cannot be addressed to any individual cullin E3 ubiquitin ligase, we searched for novel targets beyond the cullins, that are modified

by Nedd8, especially concentrating on excitatory synapses. This hypothesis is further supported by our previous Western blotting results, showing that many different proteins, apart from cullins, are differentially neddylated during brain development.

5.5.2. Spine stability and maintenance are susceptible to Nedd8-conjugation blockade

In adult animals, maintenance of spine stability as well as plastic remodeling of synapses is thought to underlie memory and learning processes. Chronic long-term live imaging studies in living animals revealed that individual dendritic spines can be maintained for the entire lifetime of organisms (Grutzendler et al., 2002; Zuo et al., 2005; Holtmaat et al., 2005; Yang et al., 2009a). These findings suggest that apart from spine formation and pruning during development, also spine maintainance of already formed spines impacts on the density and distribution of spines in the adult brain, thereby affecting the brain's information processing. In this sense a critical experiment of the present work was the evaluation of Nedd8 proteins in synaptosomal membrane preparations. These studies clearly showed that dozens (probably hundreds) of different proteins are differentially neddylated in pre- and post-synaptic compartments and displayed also differences in the patterns of neddylated proteins between the Triton-soluble and insoluble fractions of the post-synaptic density. These results represent the first description of neddylation as a relevant and active pathway within the synaptic compartment. Therefore, and taking into account the previous results showing effects of Nedd8 at morphological spine level, we decided to extend our exploration towards a putative role of neddylation on spine stability and maintenance in primary neurons and *in vivo* (see 4.6).

Since the transfection efficiency drops dramatically in older neurons after DIV 20, we either used an inducible set of plasmids, based on the Cre-ERT2/loxP system, or MLN4924 treatment of neurons that were previously transfected with fluorescent proteins, in order to achieve inhibition of neddylation in neurons with mature spines. For this purpose, Ubc12-C111S was cloned into an inducible construct downstream of a floxed-STOP cassette, which allows efficient inhibition of Nedd8 conjugation by

coexpression of Cre-ERT2 and Tamoxifen treatment. This inducible system was combined with repeated imaging of living neurons before and after the rather slow onset of Ubc12-C111S expression after Cre-mediated excision of the floxed-STOP cassette. However, repeated imaging of >DIV20 neurons, transfected with calcium phosphate protocols, with the confocal microscope systematically triggered neuronal death within 24 h. To overcome this limitation, we successfully established an epifluorescence-based live-imaging microscope set-up with fast shutters and a highly sensitive fluorescent camera, which combined with deconvolution-software for image post-processing, allowed repeated imaging of fluorescent primary neurons over the time period of several days. Blocking neddylation led to a net loss of spines at 48 to 96 h after tamoxifen administration compared to control neurons. After careful analysis of the long-term live cell imaging studies, which revealed a high spine turn-over in mature hippocampal neurons in culture within 24 to 48 h, the next step was to experimentally address if Nedd8 conjugation blockade favors the removal of existing spines or instead impedes the generation of new spines. In order to do so, we resolved more accurately the effects of Nedd8 on spine maintenance and stability at the single spine level within narrower time frames by treatment of neurons with the Nedd8-inhibitor MLN4924. Inhibition of neddylation in response to MLN4924 treatment resulted in reversible shrinkage of spine heads, indicating that neddylation primarily controls the stability of existing spines. In addition and in agreement with the previous findings, Nedd8 most probably also controls the generation of new spines in mature neurons.

These results obtained in primary neurons were confirmed *in vivo* employing the *in utero* electroporation technique in CamKIIα-Cre-ERT2 mice. In adult animals, the induction of Ubc12-C111S expression, after Tamoxifen injections at postnatal days 35, resulted in a decrease in mature spines in pyramidal hippocampal neurons as well as in granule neurons of the dentate gyrus. In the future, we will analyze the contribution of different signaling pathways, which are required for spine stability, e.g. BDNF-signaling and RhoA/Rac1-signaling (Xu et al., 2000; Nakayama et al., 2000; Gorski et al., 2003; Tashiro and Yuste, 2004; Carvalho et al., 2008), to the effects exerted by Nedd8.
In summary, we demonstrated that neddylation is not only involved in neuronal development, as its name suggests, but also regulates synaptic function during adulthood.

5.5.3. PSD-95 is a neuron-specific target of the Nedd8 pathway

In order to identify new targets of Nedd8 conjugation and to uncover the molecular mechanism underlying Nedd8 function during neuronal development and in mature neurons, our group followed and continues to apply different experimental strategies. We perform unbiased screening approaches, such as Yeast-2-Hybrid screens and immunoprecipiation experiments combined with mass spectrometry analysis, to identify neddylated neuronal substrates apart from the cullin proteins. These challenging and time consuming unbiased screening approaches were complemented by hypothesis-driven and literature-based analysis of putative candidates, derived from the phenotypes obtained in neuronal cell culture experiments and *in vivo* studies. Therefore, we tested the hypothesis that scaffold proteins of the PSD could likely be candidates for the effects observed in spine development and stability, since scaffold proteins were shown to regulate spine development and to mediate adaptations in the mature spine (Rao et al., 1998; Naisbitt et al., 1999; El-Husseini et al., 2000b; Sala et al., 2001; Stein et al., 2003; Usui et al., 2003; Romorini et al., 2004; Sala et al., 2005; Gerrow et al., 2006; Hung et al., 2008; Hung et al., 2010; Verpelli et al., 2011). Moreover, chronic alterations in synaptic activity lead to reversible changes in the protein composition of the post-synaptic density (PSD) (Sheng and Hoogenraad, 2007; Okabe, 2007; Piccoli et al., 2007). On the one hand, some PSD-proteins, including the scaffold molecules Shank and GKAP/ASAP undergo ubiquitin-proteasome system (UPS)-mediated degradation while others, e.g. CamKIIα, increase in the PSD in response to neuronal activity (Ehlers, 2003). On the other hand, PSD-95, one of the main scaffold proteins of the PSD, shows a differential response, in which NMDA-induced LTD results in UPS-mediated degradation of PSD-95 (Colledge et al., 2003), whereas LTP and chronic synaptic stimulation leads to synaptic increases in PSD-95 content (Shen and Meyer, 1999; Ehlers, 2003; Kim et al., 2007b; Steiner et al., 2008).

Based on the hypothesis that scaffolds might be instrumental targets of neddylation in the spine, we initially performed a screening experiment overexpressing the most important scaffold proteins of the PSD, including PSD-95, Shank, GKAP and Homer, and tested their ability to affect the MLN4924-induced spine loss. In these experiments, we identified and confirmed PSD-95 as a candidate, since PSD95 was the only scaffold that effectively reverted the neddylation blockade-induced spine loss (see 4.7). It is

important to mention, that this kind of screening approach was performed under the assumption that overexpression of a putative Nedd8 target protein will be sufficient to overcome the effect of Nedd8 inhibition. However, this screening represented for us only the starting point and many additional biochemical and functional experiments were performed to validate and establish PSD-95 as a direct neuron-specific target protein of Nedd8 conjugation (Marisa Brockmann, unpublished data).

Interestingly, PSD-95 overexpression did not only rescue the defects in spine development and stability induced by inhibition of neddylation, but also rescued the dendritic defects in hippocampal neurons *in vitro* and *in vivo*. On one side, this could be thought as a direct effect of PSD-95 at dendritic level. However this scenario is not very likely, because it has been shown that PSD-95 negatively regulates dendritic growth in an activity-independent manner (Charych et al., 2006). In addition and accordingly, knock-down of PSD-95 via RNAi leads to increased dendritic branching, while overexpression of PSD-95 restricted dendritic branching patterns (Charych et al., 2006).

It's more likely that the rescue of dendritic growth and arborization is secondary to the synaptic effects compensated by PSD-95 and stems from the stabilization of spines and synapses (Wu and Cline, 1998; Rajan et al., 1999; Niell et al., 2004; reviewed in Lin and Koleske, 2010).

In order to shed light on the functional consequences of neddylation of PSD-95, we first analyzed the possibility that the post-translational modification with the UBL protein Nedd8 alters protein stability of PSD-95, as it is the case for ubiquitination (Colledge et al., 2003). However, we obtained contradictory results depending on the cellular model used. In neuronal cell lines, e.g. in hippocampal Neuro-2a cells, inhibition of neddylation consistently resulted in strongly decreased PSD-95 levels, suggesting that normally Nedd8 conjugation stabilizes PSD-95. In contrast, in primary neurons, inhibition of neddylation did not result in significant alterations neither in total nor in synaptic PSD-95 contents, and only prolonged treatment with high concentrations of MLN4924 slightly reduced PSD-95 levels. These results indicated that regulation of PSD-95 protein stability is not the prevalent mechanism, by which neddylation affects PSD-95 function. These experiments also clearly demonstrated the importance to study neuronal processes and effects of neuron-specific molecules in the appropriate cellular model systems, e.g. in primary neurons or sclice cultures instead of neuroblastoma cell lines. This is particularly important in cases like PSD-95, a protein enriched in the post-

synapse, mainly considering that neuronal cell lines develop just rudimentary neurites with almost a complete lack of true spines and post-synaptic sites.
We then continued analyzing the effect of Nedd8 conjugation on other aspects of PSD-95 functioning in dendritic spines. We first studied the effect of neddylation on PSD-95 localization in primary neurons. Synaptic localization of PSD-95 relies on anchoring of the protein in the synaptic membrane and oligomerization of PSD-95 proteins mediated by its N-terminal region, including two palmitoylation sites (Cys3 and 5) and the PDZ domains (Hsueh et al., 1997; Craven et al., 1999; Firestein et al., 2000; Christopherson et al., 2003; Schluter et al., 2006; Fukata and Fukata, 2010). Immunofluorescence stainings of synapses showed that inhibition of neddylation via MLN4924 treatment resulted in a time-dependent selective loss of post-synaptic PSD-95 clusters, indicating a specific role of neddylation in synaptic stabilization of PSD-95.

Previously, it was shown that PSD-95 is ubiquitinated and degraded in response to NMDA-induced LTD (Colledge et al., 2003), and specific lysine residues were identified that are ubiquitinated in a Cdk5-dependent manner (Morabito et al., 2004; Bianchetta et al., 2011).
Using mass spectrometry, we identified several Nedd8- as well as new ubiquitin- specific lysine conjugation sites in PSD-95 (Marisa Brockmann, unpublished data). In order to analyze the specific function of neddylation of PSD-95, we generated neddylation-defective PSD-95 mutants by site-directed mutagenesis, and confirmed in immunoprecipitation assays that the neddylated lysine residues K193, K393 and K202 are required for PSD-95 oligomerization (unpublished data).
We found that the neddylation-defective PSD-95 mutants K193R and K202R impaired spine development and led to increased formation of filopodia in primary hippocampal neurons, resembling the effect of Nedd8 inhibition. On the other hand spine formation was preserved in neurons expressing the ubiquitin-defective PSD-95 variants, PSD-95-K157R and PSD-95-K211R. This experiment suggested that the neddylation-defective PSD-95 mutants act as dominant-negative PSD-95 versions. From this and the previous results, showing that MLN4924 treatment reduces synaptic PSD-95 clusters, we formulated a working hypothesis, which suggests that neddylation-defective PSD-95 mutants probably compete with wild-type PSD-95 for binding partners in the PSD, but due to their impaired ability to oligomerize and interact among each other, they cannot

function as scaffolds to assemble the PSD proteins and mediate spine formation and maturation.
To further test this hypothesis, in the future we will determine their ability to interact with other proteins in the PSD, e.g. GKAP, NMDA receptors and stargazing. Moreover, we will examine the function of neddylation-deficient PSD-95 mutants in spine development *in vivo* by means of *in utero* electroporation.

It would be also interesting to analyze spine stability and maintenance in the presence of the Nedd8 mutants of PSD-95. However, the imaging of changes in spine head size on the level of individual spines via confocal microscopy requires fast onset of inducible expression of PSD-95 constructs (see above 4.6 and 5.5.2). Therefore, in the future we plan to clone a tetracyclin-inducible PSD-95 constructs as well as plasmid constructs to induce the synaptic translocation of PSD-95 in response to rapamycin treatment (Belshaw et al., 1996; Bingol et al., 2010).

5.5.4. Activity-dependent regulation of the Nedd8 functions

The regulated degradation of proteins by the ubiquitin proteasome system has emerged as an important modulator of synaptic function and plasticity (Hegde et al., 1997; Campbell and Holt, 2001; Burbea et al., 2002; Colledge et al., 2003; Patrick et al., 2003; Pak and Sheng, 2003; Ehlers, 2003; Zhao et al., 2003; Steward and Schuman, 2003; Bingol and Schuman, 2004; Juo and Kaplan, 2004; van Roessel et al., 2004; Bingol and Schuman, 2005; Dreier et al., 2005; Kawabe et al., 2010) (reviewed in Yi and Ehlers, 2007; Haas et al., 2007; Haas and Broadie, 2008; Segref and Hoppe, 2009; Mabb and Ehlers, 2010; Kawabe and Brose, 2011). Both, protein synthesis and UPS-mediated protein degradation are required for the maintenance of LTP during synaptic potentiation (Frey et al., 1993; Fonseca et al., 2006; Dong et al., 2008) as well as for LTD (Colledge et al., 2003; Hou et al., 2006; Citri et al., 2009). Moreover, the proteasome itself translocates into dendritic spines in response to neuronal activity (Bingol and Schuman, 2006; Bingol et al., 2010). After having established the Nedd8 pathway as a critical regulator of spine development and synaptic function, we began to explore if the neddylation system itself is regulated by neuronal activity. We found that Ubc12

specifically accumulated into spines, which showed activity-dependent spine growth in response to brief glutamate treatment. It would have been interesting to study if Nedd8 itself or Nedd8-conjugated proteins are enriched in spines in an activity-induced manner. However, we couldn't achieve strong enough fluorescence signals of eGFP-Nedd8 and Venus-Nedd8 constructs for stable and repeated live-cell imaging conditions. Beside that, an additional caveat is that overexpression of Nedd8 might lead to conjugation of Nedd8 to unspecific targets via the ubiquitin enzyme cascade (Dimitris Xirodimas, personal communication) (Hjerpe et al., 2011), which would obscure the interpretation of our results.

Since Ubc12 was enriched in spines after neuronal activation, and considering that PSD-95 clusters increase in number and density after activation as well (Shen and Meyer, 1999; Ehlers, 2003; Kim et al., 2007b; Steiner et al., 2008), we wondered whether this two processes might be coupled and whether neddylation might affect activity-induced PSD-95 relocalization or stabilization in dendritic spines. During the course of the experiments we found that synaptic levels of PSD-95 transiently increased in response to a brief glutamate stimulation of primary hippocampal neurons, whereas PSD-95 levels transiently decreased in the soma-enriched protein fraction, suggesting that protein trafficking between different cellular compartments and/or differential regulation of PSD-95 stability might underlie the transient increase in synaptic PSD-95 content. Pre-treatment with the NAE-inhibitor MLN4924 prevented this differential regulation of PSD-95, suggesting a role for neddylation in activity-induced synaptic accumulation of PSD-95. In this context, the behavior of the neddylation-defective PSD-95 mutants (PSD-95-K193R and PSD-95-K202R) in response to neuronal stimulation will be analyzed in the near future.

These results are particularly important and underline that the role of neddylation within the synapse is not confined to phenomena that only take place under basal conditions, but that Nedd8 conjugation also affects processes downstream of neuronal activity, thereby regulating synaptic plasticity. In fact, we speculate that the observed effects of Nedd8 on PSD-95 are only "the tip of the iceberg" of a much broader constellation of effects of Nedd8 within the dendritic spine.

5.6. Generation of mouse models of the Nedd8 pathway

First evidence for a crucial role of the neddylation pathway in cell cycle control and regulation of proliferation stems from studies in invertebrate model organisms, *C. elegans* and *Drosophila* (Jones and Candido, 2000; Ou et al., 2002; Kurz et al., 2002; Zhu et al., 2005; Kim et al., 2007a). Neddylation controls the activity of the ubiquitin cullin RING ligases (Pan et al., 2004; Saha and Deshaies, 2008; Merlet et al., 2009; Boh et al., 2011), which in turn regulate the UPS-mediated degradation of important signal transduction effectors, such as Cubitus interruptus (Ci) and Armadillo (Arm), and cell cycle proteins, e.g. Cyclin E (CycE) (Ou et al., 2002). Accordingly, targeted disruption of the *Uba3* gene in mice, results in lethality *in utero*, due to accumulation of cyclin E and β-catenin causing early defects in mitotic division that give rise to endoreduplicative cell cycles that lead to an aberrant increase in DNA content (Tateishi et al., 2001). Unfortunately, within the big family of members controlling neddylation, so far only this Uba3 conventional KO mouse line was generated (Tateishi et al., 2001). These animals die very early during embryogenesis, precluding the analysis of putative functions of Nedd8 in embryonic and postnatal development of the central nervous system. In order to overcome this limitation and to dissect the functions of neddylation *in vivo* during brain development and in the postnatal brain, we developed conditional transgenic mouse models based on the Cre/loxP system. Differential breeding of these lines with specific Cre mouse lines, expressing the recombinase in defined cell populations and in specific neurotransmitter circuits, will allow us to inhibit the neddylation pathway in a spatial and temporal controlled manner. As discussed in 4.8, the generation of mouse mutants of the Nedd8 pathway was time consuming and technically challenging. Despite the failure of several approaches, we finally succeeded in generating three mouse lines, a Ubc12-LacZ reporter mouse line, a conditional KO mouse of Nae1 (Appbp1) and a transgenic mouse line conditionally overexpressing Ubc12-C111S. The concept and the process of the generation of these conditional mouse mutants, their validation as well as their inherent pros and cons are dicussed below.

Our first attempt was to generate a conditional KO mouse of *Ubc12* from a gene trap clone from the GGTC (German Gene Trap Consortium, http://tikus.gsf.de/). The homozygous conventional KO of *Ubc12* was embryonic lethal, as expected from *Uba3*

KO mice, whereas in the heterozygous conventional KO mice of Ubc12 we did not observe any obvious phenotype. Unfortunately, the conditional KO allele of *Ubc12* could not be obtained from this clone, because of unexpected splicing of exon 1 of Ubc12 to a cryptic splice acceptor site in the spacer sequence between the 5' LTR sequence and the frt/lox5157/loxP sites of the FLP-mediated inactivated gene trap vector. This cryptic splicing, confirmed by sequencing of RT-PCR products, resulted in disruption of the *Ubc12* allele and in reduced expression of Ubc12 leading to early embryonic lethality in homozygous animals. This aberrant splicing might be associated with the small size of intron 1 of Ubc12 (only 960 bp), possibly in combination with a weak splice acceptor in exon 2 of *Ubc12* (Thomas Floss, personal communication). Nevertheless, the heterozygous KO mouse, which in fact is a Ubc12-LacZ reporter mouse, was of significant value in determining the expression pattern of Ubc12 in the mouse brain during development and in adulthood (see 4.8.1 and 5.1).

Therefore, during the second year of this project, we started to develop our own Ubc12 conditional KO mouse line following a targeted trapping strategy of the *Ubc12* gene locus. However, the chimeric mice produced with these trapped ES cell clones didn't transmit the modified allele to their offspring. Interestingly, the European Consortium for Mouse Mutants (EUCOMM) also failed to conditionally target Ubc12, indicating that the *Ubc12* gene might be a "difficult locus" for gene targeting by homologous recombination (http://www.knockoutmouse.org/about/eucomm).

Afterwards, *Nae1* and *Uba3* were successfully targeted by Eucomm in JM8A3.N1 ES cells by means of a targeted gene trapping approach (http://www.knockoutmouse.org/about/eucomm and see 4.8.2). But due to a low quality of the ES cells, blastocyst injections of Uba3 clones did not produce any chimeric mice. In contrast, Nae1 ES cell clones gave high percentage male chimeras. However only two chimeras out of 15, obtained from the same Nae1 ES cell clone (EPD0441_1_B07), finally transmitted the modified *Nae1* allele to their offspring, after 6 to 8 negative litters. In fact, we obtained just two positive germline transmitted animals out of ~600 pubs born from the chimeric mice. This low efficiency of germline transmission and generation of chimeras might be due to the aberrant karyotypes found in many of the JM8A3 ES cell clones originally generated by EUCOMM and/or unwanted differentiation of the ES cells during the ES cell culture.

These NAE1-CKO animals are designed in order that the Cre-mediated excision of the floxed exon 4 results in a premature stop codon, which will conditionally inactivate the *Nae1* allele. PCR-based genotyping indicated that the floxed exon 4 is efficiently excised by the Cre recombinase. In agreement with that, we already observed a clear reduction of the smear of neddylated target proteins in hippocampal extracts of heterozygous conventional Nae1 KO mice compared to wild-type littermates. This result demonstrated, that the KO of Appbp1, an essential component of the E1 enzyme of the neddylation pathway, blocks neddylation in mice. This finding is in line with results obtained in null mutants of the Drosophila ortholog of Appbp1 (Kim et al., 2007a). Importantly, the reduction in neddylation, observed by immunoblotting of hippocampal lysates, confirmed the specificity of the smear of Nedd8 substrates in the brain. This finally proved the existence of many neddylated target proteins in the central nervous system apart from cullin proteins (see 4.1.5 and 5.1).
As in the case of the Ubc12 KO line, the conventional heterozygous Nae1 KO allele functions as a LacZ reporter of endogeneous Nae1 expression. By means of LacZ staining and *in situ* hybridization experiments, we demonstrated that Nae1 is also not downregulated during development (Joo et al., 2010) (see Figure 69.B, C and 5.1).

Additionally, following a knockin approach in the *ROSA26* locus, we created a mouse line conditionally overexpressing Ubc12-C111S using gene targeting by homologous recombination in mouse ES cells as described previously (Soriano, 1999; Lu et al., 2008). In order to achieve strong and constitutive expression of Ubc12-C111S after Cre-mediated excision of the floxed-STOP cassette, a minimal CAG promoter sequence was introduced upstream of the floxed-STOP cassette. In our construct, the CAG promoter is flanked by frt sites that allow FLP-mediated removal of the CAG promoter, resulting in lower expression of Ubc12-C111S from the endogeneous *ROSA26* promoter. In principle, this flexible approach allows us to express four gradual dosages of Ubc12-C111S, from high to low: CAG-hom., CAG-het., *ROSA26*-hom. and *ROSA26*-het.. This would result in inhibition of the neddylation pathway at variable degrees. Cre-mediated overexpression of FLAG-tagged human Ubc12-C111S and LacZ will be confirmed by *in situ* hybridization for Ubc12 (with a human specific probe) and the LacZ reporter, LacZ stainings and α-FLAG immunohistochemistry.

We first used JM8A3 cells to target the *ROSA26* locus by homologous recombination. Although the ROSA26 locus was successfully targeted in this ES cell line, we could not obtain high percentage chimeras from the blastocyst injections and the low percentage chimeras (10-20%) did not transmit the modified allele to their offspring. As discussed above, this low efficiency in generation of chimeras and the absent germline transmission might be due to aberrant karyotypes that were found afterwards in many of the JM8A3 ES cell clones, which were originally generated by EUCOMM. Although, at that time we did not karyotype our ES cell clones, since this was not recommended by the original producers and therefore, we can only speculate about this possibility. During the ES cell culture we observed many JM8A3 ES cell clones that showed increasing morphological signs of differentiation over the course of the culture. Of course, these ES cell clones were discarded and not injected into blastocysts since it is well known that signs of differentiation correlate with low efficiency rates of chimerism and germline transmission. This unwanted differentiation might be due to the culture conditions we used for the JM8A3 ES cells. Accordingly to established protocols for JM8A3 ES cells from EUCOMM, these cells were grown in the absence of feeder cells, directly on gelantine-coated plates. However, in traditional ES cell protocols, the ES cells are grown on a layer of embryonic mouse fibroblasts, called feeder cells, which prevent differentiation of ES cells and provide support and growth factors. Finally, we successfully targeted the *ROSA26* allele in the IDG3.2 ES cell line. These positive ES cell clones produced high percentage chimeras, that effectively gave germline transmission of the modified allele (see 3.19.3 and 4.8.3).

Conceptually, overexpression of Ubc12-C111S represents another approach to block the neddylation pathway compared to strategies that knockout key enzymes of the Nedd8 cascade. Ubc12-C111S covalently binds Nedd8, thereby depleting Nedd8 from the cell, whereas in KO animals of Nedd8 pathway enzymes, Nedd8 could still be conjugated by yet unidentified enzymes or even by the ubiquitin enzyme cascade under compensatory circumstances (Dimitris Xirodimas, personal communication) (Hjerpe et al., 2011).

With these animal models at hand, we will perform a detailed dissection of the functions of Nedd8 during developmental processes and in different neuronal populations in the postnatal and adult brain at the morphological, functional and behavioral level. Furthermore, combining Nae1 KO and Ubc12-C111S overexpressing mice with mouse models of neurodegenerative diseases, will allow us to study the involvement of the Nedd8 system in the pathology and aetiology of neurodegenerative disorders. Previous findings showed, that Appbp1 expression is upregulated in lipid rafts from hippocampi of Alzheimer disease brains (Chen et al., 2003b), as well as in brains of a Alzheimer disease mouse model (Tg2576 mice) (Yang et al., 2010). Furthermore, accumulations of Nedd8 were found in neuronal and glial inclusions of neurodegenerative disorders (Mori et al., 2005). In accordance with these findings, a putative link of the neddylation system and amyloid β (Aβ)-induced Alzheimer pathology has been suggested. In conclusion, our conditional mouse models offer the unique possibility to analyze Nedd8 functions in various tissues and organs during development and adulthood, as well as in the context of mouse models of important human diseases.

6. GENERAL CONCLUSION AND OUTLOOK

We demonstrated, in cell culture experiments and *in vivo,* in the mouse brain, that an intact neddylation system is necessary for neuronal development as well as for synapse stability in mature neurons. Our results describe in detail novel functions of neddylation in neurons of the mammalian brain, beyond the classical regulation of the cell cycle via cullin RING ligases.

During this work we found that,

- Nedd8, Ubc12 and other members of the Nedd8 conjugation systems are highly and ubiquitously expressed throughout the mammalian brain.
- Neither Nedd8 nor any of the other members of the neddylation pathway are developmentally regulated in the CNS at expression level; but neddylation, which is the active transference of Nedd8 to final targets, does change over time, both in neurons in culture and in the entire mammalian brain.
- In neurons, Ubc12 and Nedd8 are localized in axons and dendrites, and neddylation is specifically and differentially active in the different synaptic compartments and brain fractions.
- Neddylation is necessary for normal development of primary neurites and axons and later it controls dendrite formation and arborization during neuronal development.
- A normal functioning of the neddylation machinery is required for the filopodia to mature spine transition and synaptogenesis during neuronal development. Without neddylation, mature dendritic spines can not be formed.
- Neddylation affects multiple targets within the neuron beyond cullins. The neddylation of EB3 affects the stability of this microtubular binding protein and this explains part of the effects of neddylation on axon and dendrite formation.
- PSD-95 is the most important scaffold protein of the postsynaptic density and not much is known about the post-translational control of PSD-95 function. Here we describe that via direct Nedd8 conjugation, the neddylation pathway controls the oligomerization of PSD-95. Thereby neddylation affects one of the critical functions of the PSD-95 scaffold molecule. By this means, Nedd8 regulates the generation and size of the dendritic spine. These findings represent the first description of a post-translational modification controlling the oligomerization of PSD-95.

We believe that a good project is one that answers important questions but also opens new and relevant questions in the field of research. In this regard, we consider that our work significantly contributes to the field of UBLs and neurobiology and essentially opened up three new main questions. 1. What are the functions of neddylation in neurons under activation conditions? 2. Which are the targets, the 'NEDDYLOME', in the brain? Which proteins are neddylated under basal and stimulated conditions? 3. How can we better model and study the role of neddylation in the entire brain of living organisms?

To answer the first question, our lab is currently addressing the functions of neddylation in activity-dependent plasticity and spine remodeling processes, e.g. LTP and LTD. Electrophysiological experiments will shed light on the role of PSD-95 neddylation on specific lysine residues in plasticity processes in the hippocampus, e.g. during LTD. Furthermore, we plan to study the function of PSD-95 neddylation in spine growth induced by glutamate uncaging.

To characterize the brain's 'NEDDYLOME', we will conduct unbiased screening approaches, including automated yeast-2-hybrid screenings and detection of neddylated target proteins from brain tissue or neurons via mass spectrometry. This will allow us to identify novel brain- and neuron-specific Nedd8 modified substrates, which might be involved in the synaptic, axonal or dendritic phenotypes observed.

And to explore the role of neddylation *in vivo* in more detail, we generated mouse models of the Nedd8 pathway, including a conditional knockout mouse of Nae1 (Appbp1) and a *ROSA2*6 knockin mouse, conditionally overexpressing the dominant-negative version of Ubc12 (Ubc12-C111S). These mutant mice will allow the detailed dissection of Nedd8 functions *in vivo*, in the developing and adult brain as well as in many additional body systems and organs. Moreover, our neddylation-deficient mouse models will provide insight into the contribution and regulation of the Nedd8 system in different neurodevelopmental and neurological diseases.

We hope that our work will contribute to the field of molecular neurobiology and biochemistry of UBLs not only from the merely conceptual point of view, but also with new and innovative biological tools.

We have generated a large battery of tools, including mutant cDNAs, RNAi-based constructs, viral plasmids and inducible expression vectors, which might be very useful

for the scientific field. In addition, we are convinced that the conditional Nae1 KO and the conditional Ubc12-C111S overexpressing mouse line, which were generated during this project, will be heavily requested by the “neddylation community”. These fundamental tools will help to understand the role of neddylation, not only in the brain but also in many additional tissues and organs, both under physiological and pathological conditions.

Our results might also have clinical implications. MLN4924, originally developed by Millenium Pharmaceuticals, has been recently approved by the FDA for the treatment of multiple myeloma. New clinical trials are currently being conducted to extend its use to solid tumors. In this thesis work, we found that Nedd8 inhibition has profound consequences on different aspects of neuronal function and health. We have been told, that due to its intrinsic chemical properties, MLN4924 does not cross the blood brain barrier (BBB) (Millenium Pharmaceuticals, personal communication) and we have no doubts that all toxicological studies have already been properly accomplished. Nevertheless, it should also be taken into consideration that some solid tumors frequently produce cerebral metastases, which often disrupt the BBB and might allow the entrance of the compound into the brain. Our results suggest, that some leakiness in the BBB might have severe unwanted side effects in the brain. In the future, further investigations are needed to clarify this possibility.

Altogether, this work represents the first comprehensive evaluation of the expression and function of the Nedd8 conjugation system in the mammalian brain. We think it might open a completely new research topic in the field of molecular biology and biochemistry of the neuron.

7. APPENDIX

7.1. Index of Figures

Table of Contribution:

Figure	*Title*	*Experiments were performed by*
1-11	Figures of the introduction	All drawn by Annette Vogl herself
12	*In situ* hybridization for Ubc12 and Nedd8 in the developing mouse brain.	Annette Vogl
13	*In situ* hybridization in the adult mouse brain highlights the enrichment of Ubc12 and Nedd8 expression in neurons	Annette Vogl
14	Ubc12 expression in a Ubc12-LacZ reporter mouse via LacZ staining	Annette Vogl
15	Intracellular localization of Ubc12 and Nedd8 in young neurons	Annette Vogl
16	Subcellular localization of Ubc12 and Nedd8 in mature neurons	Annette Vogl
17	qRT-PCR analysis of the components of the neddylation pathway in developing neurons	Annette Vogl
18	The Nedd8 pathway changes during the development of primary hippocampal neurons	Annette Vogl
19	The Nedd8 pathway during brain development *in vivo*	Annette Vogl
20	Ubc12-C111S inhibits neddylation	Annette Vogl
21	Validation of shRNA constructs targeting Ubc12 and Nedd8	Annette Vogl
22	Comparison of NAE-inhibitors MLN4924	Annette Vogl
23	NAE-inhibitor MLN4924 inhibits neddylation in developing primary cortical and hippocampal neurons	Annette Vogl
24	NAE-inhibitor MLN4924 inhibits neddylation in mature primary cortical neurons	Annette Vogl
25	Validation of Rbx1 shRNAs	Annette Vogl
26	Neddylation controls polarization and axon development in primary neurons	Annette Vogl
27	Neddylation controls axon growth in primary neurons	Annette Vogl
28	Nedd8 controls axon development independent of Cullins in primary neurons	Annette Vogl
29	Nedd8 effects on axon development are independent of Cullins and specific for the Nedd8 pathway	Annette Vogl
30	Regulation of the cytoskeleton by the Nedd8 pathway	Annette Vogl
31	Regulation of polarization and axon growth by the NAE-inhibitor MLN4924	Annette Vogl
32	Taxol and Cytochalasin D rescue of axon defects induced by MLN4924	Annette Vogl
33	Time lapse imaging of hippocampal neurons transfected with Lifeact-meGFP	Annette Vogl
34	The Nedd8 pathway regulates axon growth during development of the cortex *in vivo*	Annette Vogl
35	The Nedd8 pathway regulates axon growth during development of the hippocampus *in vivo*	Annette Vogl
36	EB3 is neddylated	Annette Vogl
37	Rescue of axon growth by EB3 overexpression in primary neurons	Annette Vogl

38	Dendrite growth is inhibited by MLN4924	Annette Vogl
39	Blocking neddylation via Ubc12-C111S decreases dendrite growth	Annette Vogl
40	Effects of neddylation on dendrite development are independent of cullin E3 RING ligases	Annette Vogl
41	Neddylation regulates dendrite formation and neuronal migration in the upper layers of the developing cortex *in vivo*	Annette Vogl
42	Neddylation controls development of lower layer cortical neurons	Annette Vogl
43	Dendrite formation in the developing hippocampus is impaired by Nedd8 blockade	Annette Vogl
44	Mismigration of hippocampal neurons upon neddylation blockade	Annette Vogl
45-47	Adult neurogenesis experiments: 45: Nedd8 controls dendrite formation of newborn granule neurons in the dentate gyrus of adult mice 46: Inhibition of Nedd8 disturbs migration of newborn granule neurons 47: Blocking the Nedd8 pathway decreases survival of newborn neurons in the dentate gyrus	Retrovirus preparation was performed in collaboration by Dr. Chichung Lie at the Helmholtz-Zentrum München. Injection, tissue processing, immunostainings, microscopy and analysis: Annette Vogl
48	Neddylation critically controls spine development in primary neurons	Annette Vogl
49	Inhibition of neddylation impairs spine formation in cortical neurons	Annette Vogl
50	The Nedd8 pathway controls synaptogenesis and neurotransmission in primary neurons	**(A)** Annette Vogl **(B)** Preparation and transfection of neurons: Annette Vogl; Patch clamp recordings: Gerhard Rammes at the MPI für Psychiatrie.
51	Dynamic behavior of filopodia of neddylation-deficient neurons	Annette Vogl
52	Effects of neddylation on spine formation are independent of cullin E3 ligases	Annette Vogl
53	The Nedd8 pathway regulates early spine development in vivo	Annette Vogl
54	Neddylation controls spine formation *in vivo* in adolescent mice	Annette Vogl
55	Ubc12 and neddylated proteins are present in pre- and post-synaptic compartments in the adult mouse brain	Annette Vogl
56	Validation of the tamoxifen-inducible plasmid system	Annette Vogl
57	Neddylation blockade reduces spine number in mature neurons	Annette Vogl
58	Inhibition of neddylation by MLN4924 treatment results in shrinkage and loss of mature spines	Annette Vogl
59	Neddylation is required for spine maintenance *in vivo*	Annette Vogl
60	Inducible expression of β-Galactosidase does not exert influences on spine stability *in vivo*	Annette Vogl
61	Glutamate stimulation induces Ubc12 accumulation in spines	Annette Vogl
62	Effects of different scaffold proteins on MLN4924-induced spine loss in mature neurons	Annette Vogl

63	PSD-95 rescue experiments *in vitro* and *in vivo* during spine development	Annette Vogl
64	Effect of neddylation on protein stability of PSD-95 in Neuro-2a cells and primary neurons.	Annette Vogl
65	Effect of neddylation on post-synaptic PSD-95 and pre-synaptic Synaptophysin puncta in primary neurons	Annette Vogl
66	Effect of PSD-95 lysine mutants in spine development *in vitro*	Annette Vogl
67	Glutamate-induced increase in synaptic PSD-95 is prevented by MLN4924 pre-treatment	Annette Vogl
68	Generation of Ubc12-LacZ reporter mice	**(A)** ES cell clone was obtained from the GGTC. Blastocyst injections were performed in collaboration with Dr. Ralf Kühn at the Helmholtz-Zentrum München. **(B)-(F)** Annette Vogl
69	Generation of conditional Nae1 KO mice	**(A)** ES cell clones were obtained from Eucomm. Blastocyst injections were performed in collaboration with Dr. Ralf Kühn at the Helmholtz-Zentrum München. **(B)-(E)** Annette Vogl
70	Targeting strategy for conditional overexpression of Ubc12-C111S in the *ROSA26* locus	Annette Vogl
71	Generation of mice conditionally overexpressing Ubc12-C111S in the *ROSA26* locus	**(A)-(D)** Annette Vogl Not shown in the Figure: Blastocyst Injections were performed in collaboration with Dr. Ralf Kühn at the Helmholtz-Zentrum München.

7.2. Index of Tables

7.3. List of Abbreviations

A	Adenine
AAV	Adeno-associated virus
Acet.	Acetylated
AD	Alzheimer disease
ADP	Adenosine diphosphate
AMPA	Amino-3-hydroxy-5-methylisoxazole-4- propionic acid
APC	Anaphase promoting complex
APP	Amyloid precursor protein
Appbp1	β -Amyloid precursor protein binding protein 1 (also called Nae1)
ATP	Adenosine triphosphate
BCA3	Breast cancer associated gene 3
BDNF	Brain-derived neurotrophic factor
BDNF	Brain-derived neurotrophic factor
bHLH tf	Basic Helix –Loop-Helix transcriptin factor
bp	Basepair
BSA	Bovine serum albumin
C	Cysteine
C	Cytosine
C. elegans	*Caenorhabditis elegans*
CA 1/2/3	Cornu ammonis area
CaCl2	Calcium chloride
CaMKII	Calcium/calmodulin-dependent protein kinase II
CAND1	Cullin-associated and neddylation-dissociated 1
c-CBL	Casitas b-lineage lymphoma
Cdc	Cell division cycle
CDH1	WD40 domain-containing proteins cadherin 1
cDNA	Complementary DNA
CIP	Calf intestinal phosphatase
CKO	Conditional knockout
CKO mouse	Conditional knockout mouse
CNS	Central nervous system
CO_2	Carbon dioxide
COP9	Constitutive photomorphogenesis 9
CP	Cortical plate
cpm	Counts per minute
CRL	Cullin RING ligase
CSN	COP9 signalosome
C-terminus	Carboxyl terminus
Cx	Cortex
Da	Dalton
DAB	3,3'-diaminobenzidine

DAPI	4',6-diamidino-2-phenylindole
Dcn1	Defective in Cullin neddylation 1
DG	Dentate gyrus
DG	Dentate gyrus
DH5α	*E.coli* strain DH5α
DIV	Days *in vitro*
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
dNTP	Desoxyribonucleotide triphosphate
DTT	1,4-dithiothreitol
DUB	Deubiquitinating enzymes
Dvl	Disheveled
E	Embryonic day
E.coli	*Escherichia coli*
e.g.	Exempli gratia, for example
EB3	End binding protein 3
EDTA	Ethylendiamintetraacetate
EGF	Epidermal growth factor
eGFP	Enhanced green fluorescent protein
EGTA	Ethylenglycol-bis-(b-aminoethylether)-N,N,N´,N´-tetraacetate
ENU	N-ethyl-N-nitrosourea
EtOH	Ethanol
F-actin	Filamentous actin
FCS	Fetal calf serum
FGF	Fibroblast-growth factor
g	Gramme
g	Gravitational force
G	Guanine
GABA	Gamma-aminobutyric acid
G-actin	Globular actin
GFAP	Glial fibrillary acidic protein
GKAP	Guanylate kinase-associated protein
GRIP1	Glutamate receptor interacting protein 1
GSK-3β	Glycogen synthase kinase-3β
h	Hour
HBSS	Hank's balanced salt solution
HCl	Hydrochloric acid
Hepes	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
het.	Heterozygous
HIFα	Hypoxia inducible factor α
hom.	Homozygous
Hp	Hippocampus
HPRT	Hypoxanthine phosphoribosyltransferase
I	Isoleucine
i.p.	Intraperitoneal (injection)

IAP	Inhibitor of apoptosis
IB	Immuno blot
ICC	Immunocytochemistry
IF	Immunofluorescence
IHC	Immunohistochemistry
IP3	Inositol-triphosphate
IRES	internal ribosome entry site
IκBα	Inhibitor of NFκB
JNK	C-Jun N-terminal kinase
K	Lysine
kb	Kilobasepairs
kDa	Kilodalton
KI	Knockin
Klenow	Large fragment of *E.coli* DNA polymerase I
KO	Knockout
L	Leucine
L	Liter
LacZ	B-Galactosidase gene
LB	Luria broth
LTD	Long-term depression
LTP	Long-term potentiation
LV	Lentivirus
M	Methionine
M	Molar
MAP	Microtubule-associated protein
MAPK	Mitogen-activated kinase
Mdm2	Murine double minute 2
mEPSPs	Miniature excitatory post-synaptic potentials
mg	Milligramme
$MgCl_2$	Magnesium chloride
min	Minute
ml	Milliliter
mRFP	Monomeric red fluorescent protein
mRNA	Messenger ribonucleic acid
MTs	Microtubules
mut	Mutant
MZ	Marginal zone
NaAc	Sodium acetate
NaCl	Sodium chloride
Nae	Nedd8-activating enzyme
Nedd8	neural precursor cell expressed, developmentally down-regulated 8
NFκB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NgCAM	Neuron–glia cell adhesion molecule
NGF	Nerve growth factor
Ngn	Neurogenin

NICD	Notch 1 intracellular domain
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
NP	Neural precursor
NP-40	Nonidet P-40
NT	Neurotrophin
N-terminus	Amino terminus
o.n.	Overnight
OB	Olfactory bulb
OD	Optical density
ORF	Open reading frame
OTU	Ovarian tumor superfamily of ubiquitin isopeptidase
p	P-value (for statistical analysis)
P	Postnatal day
PBS	Phosphate buffered saline
PBS(-T)	Phosphate buffered saline (with Triton X-100)
PCR	Polymerase chain reaction
PD	Parkinson disease
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B (also called Akt kinase)
PKC	Protein kinase C
PSD	Post-synaptic density
PSD-95	Post-synaptic density protein 95
pVHL	von Hippel Lindau protein
qPCR	Quantitative real-time polymerase chain reaction
R	Arginine
Rac1	Ras-related C3 botulinum toxin substrate 1
Rbx	Ring box protein
rcf	Relative centrifugal force
RGC	Radial glial cell
RhoA	Ras homolog gene family member A
RIPA	Radio-immunoprecipitation assay (buffer)
RMS	Rostral migratory stream
RNA	Ribonucleic acid
ROCK	Rho kinase
rpm	Rounds per minute
RT	Room temperature
RT	Reverse transcription
RV	Retrovirus
S	Serine
s	Second
SB	Southern Blot

SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
Sema3A	Semaphorin 3A
SGZ	Subgranular zone
SOC medium	Superoptimal broth with catabolite repression
SPAR	Spine- associated Rap guanosine triphosphatase-activating protein
SUMO	Small ubiquitin-like modifier
SVZ	Subventricular zone
T	Thymine
T X-100	Triton X-100
T-20	Tween 20
TAE	Tris acetate with EDTA
TARPs	Transmembrane AMPA receptor regulatory proteins
TBE	Tris borate with EDTA
TBS(-T)	Tris buffered saline (with Tween)
TE	Tris-EDTA
TGF	Transforming growth factor
Tris	Tris[hydroxymethyl]aminomethane
Trk	Tyrosine receptor kinase
Trk	Tyrosine receptor kinase
Tyr.	Tyrosinated
U	Unit(s)
Ub	Ubiquitin
Uba	Ubiquitin-activating enzyme
Ubc	Ubiquitin-conjugating enzyme
UBL	Ubiquitin-like protein
UCH	Ubiquitin C-terminal hydrolase
UFD	Ubiquitin fold domain
UPS	Ubiquitin-proteasome system
USP	Ubiquitin-specific protease
UTR	Untranslated region (of a mRNA)
UV	Ultraviolet
V	Valine
VZ	Ventricular zone
WB	Western Blot
Wnt proteins	Wingless-type proteins
wt	Wild-type
x	Symbol for crosses between mouse lines

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9. CURRICULUM VITAE

Personal details

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Education

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Publications

Refojo D, Schweizer M, Kuehne C, Ehrenberg S, Thoeringer C, **Vogl AM**, Dedic N, Schumacher M, von Wolff G, Avrabos C, Touma C, Engblom D, Schutz G, Nave KA, Eder M, Wotjak CT, Sillaber I, Holsboer F, Wurst W, Deussing JM (2011) Glutamatergic and Dopaminergic Neurons Mediate Anxiogenic and Anxiolytic Effects of CRHR1. Science 333:1903-1907.

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Meetings and Conferences

Cold Spring Harbor Meeting “Ubiquitin Family”, 2011, USA, Abstract/Poster
12th International Neuroscience Winter Conference, 2009 in Sölden, Austria
International Symposium “Structure and Function of Synapses”, 2009 in Freiburg, Germany
Imaris workshop, 2009 in Freiburg, Germany
Leica for Science Microscope Workshop, 2009 in Munich, Germany
XXVI CINP Congress 2008, Munich, Germany, Abstract/Poster
Neuroscience Meeting, 2007 in San Diego, USA, Abstract/Poster
25th AGNP Meeting, 2007 in Munich, Germany, Abstract/Poster
Interact PhD Symposium, 2007 in Munich, Germany, Abstract/Poster
2nd Interdisciplinary Max Planck PhDnet Workshop, 2007 in Frankfurt am Main, Germany, Abstract/Poster

10. LIST OF PUBLICATIONS

Publications related to the presented project:

Vogl AM, Brockmann M, Czeh B, Maccarone G, Giusti S, Moebus A, Neiman G, Roselli F, Mattusch C, Almeida O, Rammes G, Lee HW, Stein V, Lie DC, Turck C, Wurst W, Deussing JM, Refojo D. *The Nedd8 pathway controls synaptic development and maintenance: critical role of PSD-95 neddylation*, in preparation to be submitted to Science

Vogl AM, Giusti S, Neiman G, Kühn R, Holsboer F, Wurst W, Deussing JM, Refojo D. *Neddylation controls axon development*, in preparation to be submitted to Journal of Neuroscience

Vogl AM, Giusti S, Neiman G, Holsboer F, Wurst W, Deussing JM, Refojo D. *Neuronal cultures from in utero electroporated brain for imaging synaptic development*, in preparation to be submitted Nature Protocols

Publications related to additional projects:

Vogl AM, Graf C, Czeh B, Haeussler J, Wurst W, Holsboer F, Deussing JM, Refojo D. *Antidepressant treatment requires IKKα in glutamatergic neurons*, in preparation to be submitted as a Short Communication to Nature Neuroscience

Giusti S, **Vogl AM**, Deussing JM, Holsboer F, Wurst W, Refojo D. *Bob Sponge, a conditional mutant mouse for sponging miR9 in vivo*, in preparation to be submitted to Nature Methods

Refojo D, Schweizer M, Kuehne C, Ehrenberg S, Thoeringer C, **Vogl AM**, Dedic N, Schumacher M, von Wolff G, Avrabos C, Touma C, Engblom D, Schutz G, Nave KA, Eder M, Wotjak CT, Sillaber I, Holsboer F, Wurst W, Deussing JM (2011) *Glutamatergic and Dopaminergic Neurons Mediate Anxiogenic and Anxiolytic Effects of CRHR1.* Science 333:1903-1907.

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Silberstein S, **Vogl AM**, Bonfiglio JJ, Wurst W, Holsboer F, Arzt E, Deussing JM, Refojo D (2009) *Immunology, Signal Transduction, and Behavior in Hypothalamic-Pituitary-Adrenal Axis-related Genetic Mouse Models.* Annals of the New York Academy of Sciences, 1153: 120–130.

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12. STATEMENT/ERKLÄRUNG

Ehrenwörtliche Versicherung:

Ich versichere hiermit ehrenwörtlich, dass ich die Dissertation mit dem Titel ”Multiple functions of neddylation in neuronal development” selbständig und ohne unerlaubte Hilfe angefertigt habe. Ich habe mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Hilfen und Quellen bedient.

Erklärung:

Hiermit erkläre ich, dass ich mich nicht anderweitig einer Doktorprüfung ohne Erfolg unterzogen habe. Die Dissertation wurde in ihrer jetzigen oder ähnlichen Form bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.