# The impact of Cyclin D1 on proliferating cells in the intact and injured mouse cortex

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**Lionel Nobs**

aus Münchenstein (BL), Schweiz

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Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät
auf Antrag von

Prof. Suzana Atanasoski

Prof. Dr. Heinrich Reichert (Fakultätsverantwortlicher)

Prof. Dr. med. Cordula Nitsch (Korreferentin)

Basel, den 24.4.2012

Prof. Dr. Martin Spiess
(Dekan)

# SUMMARY

Cell proliferation is an essential component of brain formation and homoeostasis, tightly regulated by cell cycle proteins. The members of the cyclin D family are responsible for the initiation of the cell cycle and thus key regulators of the cell cycle. Mammals posess three isoforms of Cyclin D (D1, D2 and D3). It has been shown, that injury to the adult brain leads to a substantial increase of local Cyclin D1 expression. This upregulation has been associated with the apoptosis of neurons, but also with the proliferation of glial cells, which contribute to the glial scar formation and to the inflammatory reaction. Subsequent treatment with pharmacological inhibitors of the Cyclin D1-dependent pathway have shown to decrease injury-induced lesion volume and glial scar formation, suggesting that modulation of cell cycle might be beneficial for the injury outcome.

In this study, I took advantage of mice genetically deficient in the Cyclin D1 gene to study its function in proliferating cells of the intact and injured cortex. I could show that the postnatal proliferation of microglia is completely impaired by the absence of Cyclin D1. In contrast, the proliferation of oligodendrocyte progenitor cells (OPCs) was independent of Cyclin D1 at early postnatal stages and only reduced during adult stages. This finding suggests a switch in the requirement for distinct cell cycle proteins driving proliferation of OPCs during development and in the adult. Using an injury model leading to local neurodegeneration, I could show that the deficiency for Cyclin D1 reduces the size of the lesion three days following insult to the adult brain cortex. In parallel, the injury-induced Proliferation was significantly reduced within the lesion site. A closer examination of the glial cell types within the neurodegenerative area revealed that the proliferation of microglia and OPCs was impaired in the Cyclin D1 knockout animals. Finally, I provided evidence that Cdk4 but not Cdk2 or Cdk6 are required for injury-induced proliferation of OPCs thus indicating Cdk4 most likely to be the interaction partner of Cyclin D1.

# ZUSAMMENFASSUNG

Die Zellproliferation ist ein essentieller Bestandteil der Entwicklung und der Homeostase des Gehirns, welche durch Zellzyklusproteine reguliert werden. Die Mitglieder der Cyclin D-Familie sind für die Initiierung des Zellzyklus verantwortlich und somit Schlüsselregulatoren des Zellzyklus. Säugetiere besitzen drei Isoformen von Cyclin D (D1, D2, D3). Es konnte gezeigt werden, dass eine Hirnverletzung zu einer erheblichen Zunahme an Cyclin D1-Expression führt. Diese Aufregulierung wurde nicht nur mit dem Absterben von Nervenzellen assoziiert, sondern auch mit der Einleitung der Vermehrung (Proliferation) glialer Zellen, welche zur Vernarbung des Gewebes und zur Freisetzung von Entzündungsfaktoren beitragen. Anschliessende Behandlungen mit pharmakologischen Inhibitoren des Cyclin D1-abhängigen Signalwegs haben gezeigt, dass sie zur Reduktion des Läsionsvolumens und der Vernarbung führen, was nahelegt, dass die Modulation des Zellzyklus sich positiv auf den Ausgang einer Verletzung auswirkt.

In dieser Studie habe ich eine Mauslinie verwendet deren *cyclin D1*-Gen entfernt wurde, um die Notwendigkeit von Cyclin D1 in proliferierenden Zellen des intakten und verletzten Kortex zu untersuchen. Dabei konnte ich zeigen, dass der Verlust von Cyclin D1 die postnatale Proliferation der Mikroglia stark beeinträchtigt. Im Gegensatz dazu war die Proliferation von Oligodendrozyten-Vorläuferzellen (OPCs) während der frühen postnatalen Phase Cyclin D1-unabhängig und nur während der adulten Phase reduziert. Dieses Resultat suggeriert einen Wechsel des Bedarfs an spezifischen Zellzyklusproteinen zwischen der Entwicklung und der Erwachsenenphase. Mittels eines Verletzungsmodells, welches zu lokaler Neurodegeneration im Kortex führt, konnte ich am dritten Tag nach der Initiierung der Verletzung zeigen, dass die Grösse der Läsion in der Cyclin D1-Mutante kleiner ist. Parallel dazu war die verletzungsbedingte Proliferation innerhalb der Läsion stark reduziert. Eine

genauere Untersuchung der glialen Zelltypen innerhalb der neurodegenerativen Läsion offenbarte, dass die Proliferation der Mikroglia und OPCs in der Cyclin D1-Mutante beeinträchtigt war. Zudem konnte ich zeigen, dass die verletzungsbedingte Proliferation der OPCs von Cdk4, aber nicht von Cdk2 oder Cdk6, abhängig ist, was darauf hindeutet, dass Cdk4 der Interaktionspartner von Cyclin D1 ist.

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# 1. INTRODUCTION

## 1.1 General introduction

The brain is the center of the nervous system in mammalian animals and one of the most complex organs. Especially the neocortex with its six-layered architecture and compartmentalization into specialized areas which in turn are characterized by a particular connectivity and cellular composition, represents this complexity. Understanding the development and the function of the neocortex remains a major challenge in neuroscience. The developmental processes of the brain are controlled by complex regulatory mechanisms that coordinate proliferation, fate specification and differentiation during neurogenesis and gliogenesis (Dehay and Kennedy, 2007; Rowitch and Kriegstein, 2010).

The development of the central nervous system (CNS) starts with the processes of primary neurulation during embryogenesis. At the end of neurulation, the neural plate and neural tube are composed of a single layer of cells, neuroepithelial cells, which form the neuroepithelium. Neuroepithelial cells, which can be considered stem cells, first undergo symmetric, proliferative divisions, thereby increasing their number (Rakic, 1995). These divisions are followed by asymmetric divisions, each of which generates another neuroepithelial cell and either a neuron or, alternatively, a progenitor cell that will undergo mitosis at a significant distance from the ventricular surface (basal progenitor) and which will generate neurons via a symmetric division (Haubensak et al., 2004). With the generation of neurons, the neuroepithelium transforms into a tissue with numerous cell layers, and the layer that lines the ventricle is referred to as the ventricular zone. Subsequently, the neuroepithelial cells are transformed into radial glial cells which undergo active proliferation and serve as primary multipotent progenitors leading to the initiation of lineages

forming both neurons and glial cells (Anthony et al., 2004; Pinto and Gotz, 2007) (Fig. 1-1). Radial glial cells have the ability to self-renew and to generate intermediate progenitors and terminally differentiated cells until they transform into parenchymal astrocytes, ependymal cells, or continue their stem cell activity (Kriegstein and Alvarez-Buylla, 2009). Lineage tracing experiments showed that radial glia give rise to adult neural stem cells (NSC, also referred to as type B cell) in the subventricular zone (SVZ) (Merkle et al., 2004) (Fig. 1-1).

The mature mammalian brain is characterized by a relatively constant number of glia and neurons. Nevertheless, there is a low rate of constitutive gliogenesis and a more restricted neurogenesis in the adult brain (Rakic, 2002). This constitutive proliferation reflects glial and neuronal turnover, rather than actual brain growth. Neurogenesis in the adult CNS occurs primarily in two germinal

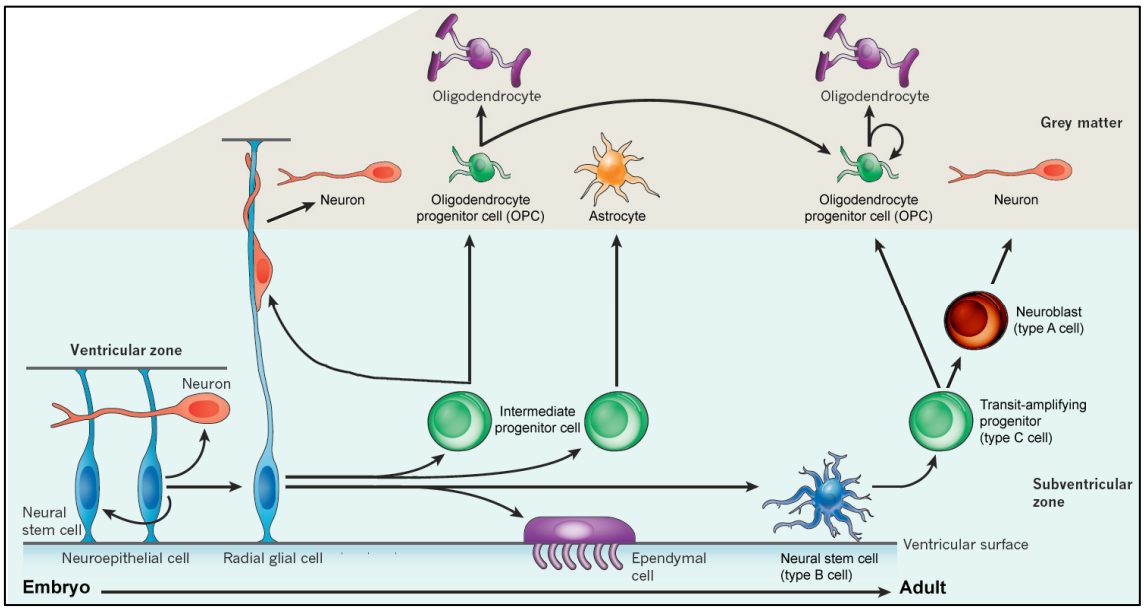



**Fig. 1-1 Neurogenesis and gliogenesis in the mammalian CNS**
Progression from the embryo to the adult is shown from left to right. During early development, neuroepithelial cells in the VZ generate neurons and become transformed into radial glial cells. These produce intermediate progenitor cells that become neurons, oligodendrocyte progenitor cells (OPCs) or astrocytes. The OPCs differentiate into mature oligodendrocytes but they also maintain a self-renewal capacity to produce further oligodendrocytes during adulthood. In addition, radial glia cells also produce ependymal cells. In the adult, radial glial cells persist as neural stem cells which generate transit-amplifying progenitors. These in turn, are able to produce OPCs or migrating neuroblasts which differentiate into new neurons (adapted from Rowitch and Kriegstein., 2010).

zones: the SVZ and the subgranular zone (SGZ) of the dentate gyrus (Taupin and Gage, 2002). NSCs of the SVZ and SGZ give birth to progenitor cells which migrate to their target region where they differentiate into neurons and integrate into the neuronal network (Fig. 1-1). Outside these “typical” germinal niches no constitutive neurogenesis takes place under normal conditions in the parenchyma. In contrast, glial cells are generated in the germinal zones as well as in non-germinal sites, such as in the grey matter where oligodendrocyte progenitor cells (OPCs) exhibit a significant proliferative activity (Dawson et al., 2003; Buffo et al., 2005) (Fig. 1-1).

Injury to the CNS has shown to alter the rate of cell proliferation in the mature brain. NPCs proliferate and their progeny may be recruited to sites of brain injury (Ramaswamy et al., 2005). This injury-proliferation is not exclusive to neuronal progenitor populations, as gliogenesis has also been shown to increase following injury. Activated astrocytes and proliferating microglia undertake active responses directed at isolating and clearing the injured area, while OPCs are induced to proliferate in order to produce new oligodendrocytes (Franklin and Ffrench-Constant, 2008).

It is well known that proliferation is tightly regulated by cell-cycle proteins. Following injury to the CNS, cell cycle proteins are upregulated in neurons, astrocytes and microglia (Di Giovanni et al., 2005; Byrnes and Faden, 2007; Byrnes et al., 2007). Cyclin D is a key cell cycle protein which links extracellular mitogenic signals to the core cell cycle machinery and drives the progression of the cell cycle through the G1 cell cycle checkpoint (Sherr, 1995). Much is known about the involvement of Cyclin D1 in neurogenesis but not for gliogenesis. For example it is known that Cyclin D1 promotes neurogenesis in the developing spinal cord and proliferation of neuronal stem cells in the SVZ and SGZ (Ma et al., 2010; Lukaszewicz and Anderson, 2011). In addition, nonoverlapping expression of Cyclin D1 and D2 has been reported in the developing mouse

forebrain neuroepithelium, suggesting that these isoforms may differentially regulate proliferation and differentiation (Kowalczyk et al., 2004; Glickstein et al., 2007a; Glickstein et al., 2007b).

The overall goal of my thesis was to determine the glial cell types expressing Cyclin D1 and to further investigate its function in proliferating glial cells in the intact and injured cortex of postnatal mice.

## 1.2 G1-phase molecules of the cell cycle machinery

The cell cycle is controlled by complex molecular mechanisms and progression through the cell cycle requires sequential activation of cyclin-dependent kinases (CDKs) and their regulators, the cyclins. The D-type cyclins are part of the cell cycle machinery but in contrast to other cyclins, which are induced periodically during cell cycle progression, the expression of D cyclins is controlled largely by mitogenic or anti-mitogenic signals during G1 phase (Fig. 1-2A). Thus, D-cyclins are regarded as links between the extracellular environment and the core cell cycle machinery (Murray, 2004). When quiescent G0 cells are stimulated by mitogens, D cyclins are expressed and assemble with their catalytic partners CDK4 or CDK6 such that complexes progressively accumulate (Fig 1-2B). These complexes then initiate the phosphorylation and subsequent inactivation of the retinoblastoma tumor suppressor (RB) at mid G1 phase (Sherr, 1993; Sherr and Roberts, 2004). In its unphosphorylatedstate RB blocks the G1/S-phase transition by binding to the transcription factor E2F (E2 promoter binding factor) and repressing it. Phosphorylation of RB releases it from E2F, thus enabling E2F to activate genes whose products are involved in DNA synthesis. E2F also transactivates cyclin E which then enters into a complex with CDK2 further accelerating complete phosphorylation of RB at late G1 phase. In addition, D-type cyclins indirectly affect CDK2 activity by titration of endogenous CDK

inhibitors from cyclin E-CDK2 complexes (Sherr and Roberts, 1999). This shift from cyclin D-CDK4/6 to cyclin E-CDK2 accounts for the loss of dependency on mitogenic factors. Taken together, a wave of transcriptional activity is generated to proceed through the restriction point at G1/S transition and cells that went through the restriction point no longer require mitogenic stimuli to undergo cell division.

Following the G1/S phase transition, RB protein is maintained in the phosphorylated state throughout the S, G2, and M phases by CDK2- and CDK1-cyclin complexes. After cells complete mitosis and enter early G1 or G0, the decline in CDK-cyclin levels and the action of phosphatases lead to dephosphorylation of the RB protein which inhibits E2F activity during early G1 of the next cycle (Sherr, 1993; Sherr and Roberts, 1999; Malumbres and Barbacid, 2005).

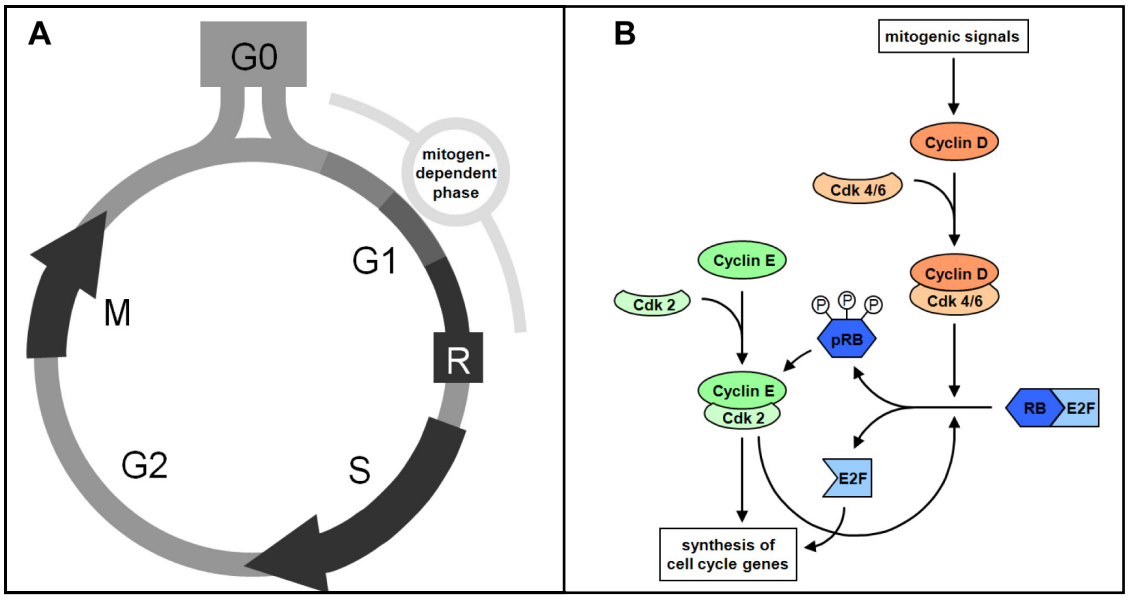



**Figure 1-2: Cyclin D1 is a key protein of the cell cycle**
**A** Schematic representation of the cell cycle. G0, M, G1, S, and G2 refer to the quiescent, mitosis, first gap, DNA synthesis, and second gap phases of the cell cycle, respectively. The decision to replicate is made at the restriction point (R) during the transition from G1 to S phase. The mitogen-dependent phase lasts through G1 until it reaches R. **B** Schematic representation of the processes during mitogen-dependent phase. Mitogenic signals push the cell into a proliferative mode by initiating transcription of D cyclins which associates with CDK4/6. This complex phosphorylates RB which releases E2F leading to synthesis of cell cycle genes.

## 1.3 Expression of G1-phase cell cycle proteins within the CNS

In the mammalian CNS there are three different cyclin D isoforms which drive the progression of the cell cycle through the G1 cell cycle checkpoint (cyclins D1, D2, and D3). Most expression data in the CNS report on Cyclin D1 and Cyclin D2. Mapping of expression of these two proteins from E12.5 up to P60 indicates that these cyclins define separate progenitor pools in embryonic brain (Glickstein et al., 2007a). Analysis in the embryo revealed that Cyclin D1 and Cyclin D2 are expressed in different subsets of radial glial cells (Glickstein et al., 2009). In the adult brain Cyclin D1 is expressed in scattered cells throughout the cortex, the striatum and the hippocampus. Surprisingly cyclin D1 was also found in mature, postmitotic neurons such as postmitotic pyramidal neurons of the CA1 region of the hippocampus and pyramidal neurons in layer III and V of the neocortex (Glickstein et al., 2007b). The authors believe that it is unlikely that the cyclin supports a cell cycle function in these postmitotic cells. Upregulation of cyclin D1 has been associated with neuronal apoptosis in other mature systems. However, because the CA1 labelling seen here was extensive and was found in normal, early adult brain, they propose that cyclin D1 expression there would be less likely to be proapoptotic.

In a subsequent publication Koeller et al. (Koeller et al., 2008) characterized the regions and cell types exhibiting basal Cyclin D1 expression within the adult brain and spinal cord. They detected neuronal Cyclin D1 primarily in large neurons. In the neocortex immunolabelling predominantly marked neurons of the layers II-III and V-VI. Based on the morphology and location of the neurons, the authors proposed that a significant proportion of the neurons were excitatory neurons. This could be clearly shown in the hippocampus: where glutamatergic CA1 field neurons were labeled for Cyclin D1. This was further supported by the

fact that Cyclin D1 was not detected in inhibitory neurons as no double-labeling could be observed with the inhibitory neuron markers GABA, parvalbumin or somatostatin. The authors also suggested that Cyclin D1 is expressed in microglia as they detected immunopositive cells with small dense nuclei within the neocortex.. Additional examinations in the rat showed Cyclin D1 immunopositive neuronal nuclei in the rat cortex and in other brain regions. Interestingely, examinations of human brain tissue revealed that in in comparison to both mouse and rat, no Cyclin D1 labeling was observed in the human neocortex (Koeller et al., 2008).

## 1.4 Function of Cyclin D and its interaction partners within the CNS

Each of the D cyclins may perform unique, cell type-specific functions. To address the functions of Cyclin D1 in development, mice lacking Cyclin D1 were generated and phenotyped (Fantl et al., 1995; Sicinski et al., 1995). The mice were viable and displayed narrow, tissue-specific abnormalities such as reduced body size, retinal hypoplasia and underdeveloped mammary glands (Fantl et al., 1995; Sicinski et al., 1995). In addition to the physical defects, Sicinski et al. reported a neurological abnormality in knockout mice indicated by a leg-clasping reflex. Thereby the mice retract their hind limbs toward the trunk when they are lifted by their tails, in contrast with wild-type and heterozygous littermates, which invariably respond by extending their legs. Although the mice are neurologically impaired, no distinct neuroanatomical abnormality has been identified. Nevertheless, they could not exclude that minor neuroanatomical defects, sufficient to cause these neurological symptoms, could have escaped their attention.

Cyclin D2-deficient mice show abnormalities in the cerebellum (Huard et al., 1999). In addition the female knockouts are sterile due to abnormal gonadal development while male mice have underdeveloped testes (Sicinski et al., 1996). Lastly, cyclin D3-deficient mice showed impaired development of immature T cells (Sicinska et al., 2003).

The function of Cyclin D1 and Cyclin D2 during neurogenesis has been extensively examined. As it is known that the size of the brain is reduced in *Cyclin D1*$^{-/-}$ mice (Fantl et al., 1995; Sicinski et al., 1995) and that Cyclin D1 is expressed in post-mitotic neurons in the forebrain of adult mice (Tamaru et al., 1994; Glickstein et al., 2007a) the cortex thickness was analysed in Cyclin D1-knockout mice (Glickstein et al., 2007b). The authors could observe an overall thinning of the cortex in knockout animals while the distribution pattern and densities of interneuron subtypes showed no differerence (Glickstein et al., 2007b). In contrast, mice deficient for cyclin D2 displayed not only cortical thinning but also a selective reduction of the parvalbumin interneuron subtype. Subsequent investigations revealed that the loss of cyclin D2, but not of cyclin D1, could be associated with reduced proliferation of neural progenitors: while the duration of G1-phase was lengthened, the S-phase was shortened in neural progenitors, leading to a loss of intermediate progenitor cells (Glickstein et al., 2009). Thus Cyclin D2 is important for appropriate expansion of the intermediate progenitor pool (Glickstein et al., 2009). Another study which addressed the function of Cyclin D1 and Cyclin D2 during adult neurogenesis, showed that neuronal proliferation in the hippocampus, was completely inhibited in cyclin D2 mutants, but was unaffected in cyclin D1 mutants (Kowalczyk et al., 2004). Taken altogether, one can state that Cyclin D2 (but not Cyclin D1) plays an important role during neurogenesis.

Compared with Cyclin D1 and Cyclin D2, little is known about the function of Cyclin D3 within the CNS. The fact that double knockout of Cyclin D2 and

Cyclin D1 is not compensated by Cyclin D3 expression in brain (Ciemerych et al., 2002) and that cortical neuroepithelial cells still proliferate in the absence of all D-cyclins (Kozar et al., 2004) suggest that cyclin D3 does not play a significant role in embryonic cortex.

To further dissect the specific functions of the D cyclins, mice lacking two of the three cyclin Ds were analyzed. In the tissues of these double-knockout mice, the remaining intact D cyclin became ubiquitously expressed and afforded nearly normal development of organs (Ciemerych et al., 2002). These observations strongly supported the notion that the functions of the D Cyclins are largely exchangeable. However, in some tissues the remaining Cyclin D could not fully rescue the defect or was not even upregulated. A further study, in which a knock-in strain of mice expressed Cyclin D2 in place of Cyclin D1 showed that the ectopically expressed Cyclin D2 was able to nearly completely substitute for cyclin D1 (Carthon et al., 2005). But also in this case the rescue of cyclin D1-null phenotype was incomplete, indicating non-redundant functions of these proteins. One interpretation of these findings is that while the two cyclins can perform similar functions in most Cyclin D1-dependent compartments, Cyclin D2 is less efficient than Cyclin D1 in some of them.

It is proposed that the acquisition of multiple D cyclins during evolution may have allowed mammalian cells to drive optimal proliferation of the diverse array of cell types. Thus, how a progenitor divides is likely to be directed by the cooperation of external and intrinsic regulators to integrate cell divisions with differentiation.

Concerning the interaction partners of Cyclin D1, generation of mice deficient for Cdk2 (Berthet et al., 2003; Ortega et al., 2003), Cdk4 (Rane et al., 1999; Tsutsui et al., 1999) and Cdk6 (Malumbres et al., 2004) hav been previously reported. Phenotyping of these mouse lines showed tissue-specific abnormalities such as reduced postnatal proliferation of pancreatic β-cells (Rane et al., 1999;

Tsutsui et al., 1999; Martin et al., 2005) or impaired hematopoiesis (Malumbres et al., 2004).

Most of the studies in the intact CNS that investigate on the function of Cdk2, Cdk4 or Cdk6 are related to neurogenesis but not to gliogenesis. As an exception to it, Caillava et al showed that during CNS development loss of Cdk2 does not affect OPC cell cycle, oligodendrocyte cell numbers, or myelination (Caillava et al., 2011). On the "neurogenesis side", Jablonska et al. could show that knockout of Cdk2 significantly affects proliferation of NPCs in the SVZ (Jablonska et al., 2007). Other studies on Cdk4 and Cdk6 showed that Cdk6 but not Cdk4 is essential for NPC proliferation within the neurogenic niches. Specifically, Cdk6 deficiency prevents the expansion of NPCs by lengthening their G1 phase duration and thereby reducing proliferation. The authors suggest that Cdk6 is one intrinsic key molecular regulator of this neurogenesis in the adult. Taken together, it has been shown that the cyclin-dependent kinases Cdk2 and Cdk6 play important roles in neurogenesis.

## 1.5 Glial cell lineages in the adult cortex

Brain tissue is composed of cells that derive from two major cell lineages: neuronal cells and glial cells. In the mammalian brain, glial cells are estimated to occupy half of the brain space and outnumber neurons by about ten to one (Alberts, 1989; Kettenmann and Ransom, 1995), reflecting the importance of these cells. The name glia, which is Greek for glue, is based on the perceived function of these cells as structural cohesive elements within the brain. Gliogenesis, the development and maturation of glia, occurs during the later stages of cortical development that follow neurogenesis.

Glial progenitors, which are generated from radial glial cells and SVZ glioblasts, migrate through the brain parenchyma where they eventually

differentiate into different types of macroglial cells including astrocytes and oligodendrocytes. Microglia, the resident macrophages of the brain, arise in the bone marrow from hematopoietic stem cells which first differentiate into monocytes before travelling to the brain, where they settle and fully differentiate.

### *1.5.1 Astrocytes*

Astrocytes perform different tasks such as structural support for neurons, formation of the blood brain barrier, provision of nutrients to the nervous tissue and maintenance of extracellular ion balance (Barres, 2008). They are stellate-shaped cells with a highly ramified cytoskeleton and small cell body. During development radial glia and perinatal SVZ glioblasts both give birth to cortical astrocytes (Jessen and Richardson, 2001), which themselves quickly exhaust their proliferation capacity in the postnatal brain (Burns et al., 2009). The astrocytes within the grey matter are termed “protoplasmic astrocytes”. They possess many fine processes that contact blood vessels, forming so called “perivascular” endfeet, and also multiple contacts with neurons. Protoplasmic astrocytes are organized in non-overlapping spatial domains, with overlap of only the most peripheral processes of neighbouring astrocytes (Bushong et al., 2002). The cytoskeleton and process morphology of immature astrocytes are primarily made up of the intermediate filaments vimentin and nestin (Eliasson et al., 1999). As astrocytes mature, vimentin and nestin are gradually replaced by another intermediate filament, glial fibrillary acidic protein (GFAP) (Eliasson et al., 1999). Therefore, GFAP is a characteristic protein in mature astrocytes of the adult brain. Although astrocytes have been shown to express GFAP, they do not always label positive for GFAP. Several explanations for this phenomenon have been put forth. One hypothesis is that GFAP expression may be so low within a subset of cells that it is virtually undetectable (Walz, 2000). Support for this

argument comes from models of brain trauma and injury. Trauma-induced brain injury that leads to reactive gliosis is characterized by an increase in GFAP immunoreactivity without increasing cell proliferation, suggesting that existing cells that did not previously appear to express GFAP may, under these circumstances, do so (Walz, 2000). Another explanation for GFAP negative astrocyte-like cells is that immunohistological detection methods may only recognize GFAP that is incorporated into the astrocyte cytoskeleton and fail to stain the soluble subunits, thereby providing a false negative for the presence of GFAP (Stichel et al., 1991). Some studies have used the calcium binding protein S100b, which is expressed in mature astrocytes (Raponi et al., 2007). However, S100b only labels the cell body, and, therefore, is not optimal for identifying complete cellular morphology.

### *1.5.2 Oligodendrocytes*

Oligodendrocytes are specialized cells important for myelination which leads to insulation of axons and thereby to fast axonal signalling. The morphology of oligodendrocytes reveals an elaborate array of branched projections and a small cell body (Pfeiffer et al., 1993). An individual oligodendrocyte can myelinate numerous axons. The myelin sheath itself is not continuous down a single axon as there are gaps between the myelin sheaths. These gaps, termed Nodes of Ranvier, are dense in sodium voltage-gated ion channels which allow regeneration of action potentials in a saltatory manner (Waxman and Ritchie, 1993; Rosenbluth, 2009).

The heterogeneity of oligodendrocytes is well known. Classical morphological studies done with Golgi technique suggested that the oligodendrocyte population includes four morphological subtypes in the white matter (del Rio-Hortega, 1928). Thus, oligodendrocytes derive from OPCs that have experienced a series

of developmental changes before acquiring a mature phenotype (Pfeiffer et al., 1993). Specific markers characterize the successive stages of oligodendrocyte maturation. First, expression of Olig2 (a basic helix–loop–helix transcription factor required for oligodendrocyte lineage specification) within adult multipotent progenitor cells promotes development into an OPC (Ligon et al., 2006).

Typically, adult OPCs express the proteoglycan nerve/glial antigen 2 (NG2) and the alpha subtype of the platelet-derived growth factor receptor (PDGFRα). Adult OPCs are capable to further develop into postmitotic, premyelinating APC+ oligodendrocytes which in turn differentiate into mature myelinating oligodendrocytes expressing both APC and the myelin basic protein MBP (Bhat et al., 1996; McTigue et al., 2001) (Fig. 1-3). Most of the markers mentioned above are expressed consecutively, but Olig2 is expressed throughout the entire - oligodendroglial lineage (Ligon et al., 2004; Ligon et al., 2006). While 99% of the adult NG2 cells express Olig2, about 17% of the Olig2 cell population expresses NG2 (Ligon et al., 2006). Moreover, analysis of mice lacking Olig2 function demonstrate a failure of NG2 cell development at embryonic and perinatal stages (Ligon et al., 2006). Taken together, Olig2 is required for proper NG2 cell development.

Oligodendrocyte development occurs in three distinct waves. In the first wave, oligodendrocyte precursor cells (OPCs) appear in the medial ganglionic eminence (MGE) and anterior entopeduncular area (AEP) at around E12.5 (Kessaris et al., 2006). They arrive in the cortex at around E16, and mature into myelinating oligodendrocytes postnatally (Richardson et al., 2006). A second wave of OPCs originates from the lateral ganglionic eminence (LGE) and caudal ganglionic eminences at around E14.5 (Kessaris et al., 2006). Finally, a third wave of OPCs has been observed to emerge in the dorsal telencephalon during postnatal stages (Kessaris et al., 2006).

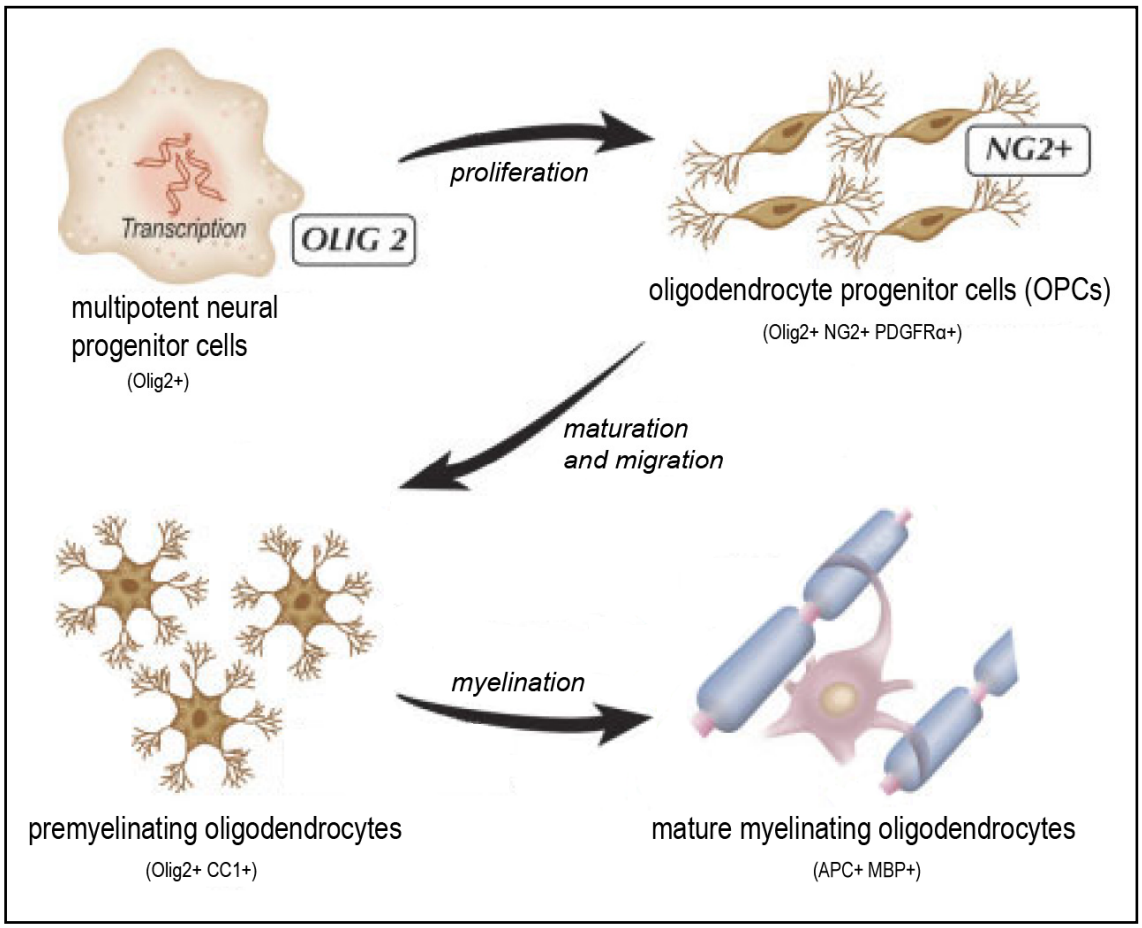



**Figure 1-3: Olig2 and NG2 function in adult development**
Olig2 is expressed in adult multipotent progenitor cells promoting development into oligodendrocyte lineage and thus differentiation to NG2+ oligodendrocyte progenitor cell (OPC), the most prevalent cycling cell in the adult brain. These in turn will further differentiate into premyelinating APC+ oligodendrocytes capable to develop into mature myelinating oligodendrocytes which express MBP (adapted from Ligon et al., 2006a).

The study of adult oligodendrocyte progenitor cells (OPCs) started to rise with the development of antibodies against NG2 and so they came to be known as "NG2 cells". They were first identified in the rat optic nerve and later in other parts of the adult mammalian CNS (Ffrench-Constant and Raff, 1986). Adult OPCs are more or less uniformly distributed in the CNS, comprising ~5% of all cells in the adult rodent CNS (Pringle et al., 1992; Nishiyama et al., 1996; Nishiyama et al., 2009). It is hypothesized that they are glial precursors fulfilling a homeostatic role in the adult CNS, replacing oligodendrocytes and possibly

astrocytes that might die as a result of injury or disease or through normal wear and tear of the tissue.

Adult OPCs represent the main proliferating cell population in the adult brain and continue to generate new oligodendrocytes during adulthood for an extended period, though at a steadily decreasing rate (Levison et al., 1999; Nishiyama et al., 2002; Dawson et al., 2003). By following the fate of dividing NG2 cells using “Cre-lox” technology in adult transgenic mice it could be shown that at 8 months after birth new myelinating oligodendrocytes are still being formed (Dimou et al., 2008; Rivers et al., 2008; Psachoulia et al., 2009). Moreover, cumulative BrdU labelling experiments in adult animals revealed that within the corpus callosum (white matter) and the cerebral cortex (grey matter) approximately 50% of all NG2cells are actively engaged in the cell cycle (Psachoulia et al., 2009). The authors suggested that there are distinct subsets of adult OPCs, a mitotically active population and a separate quiescent population (Fig. 1-4).

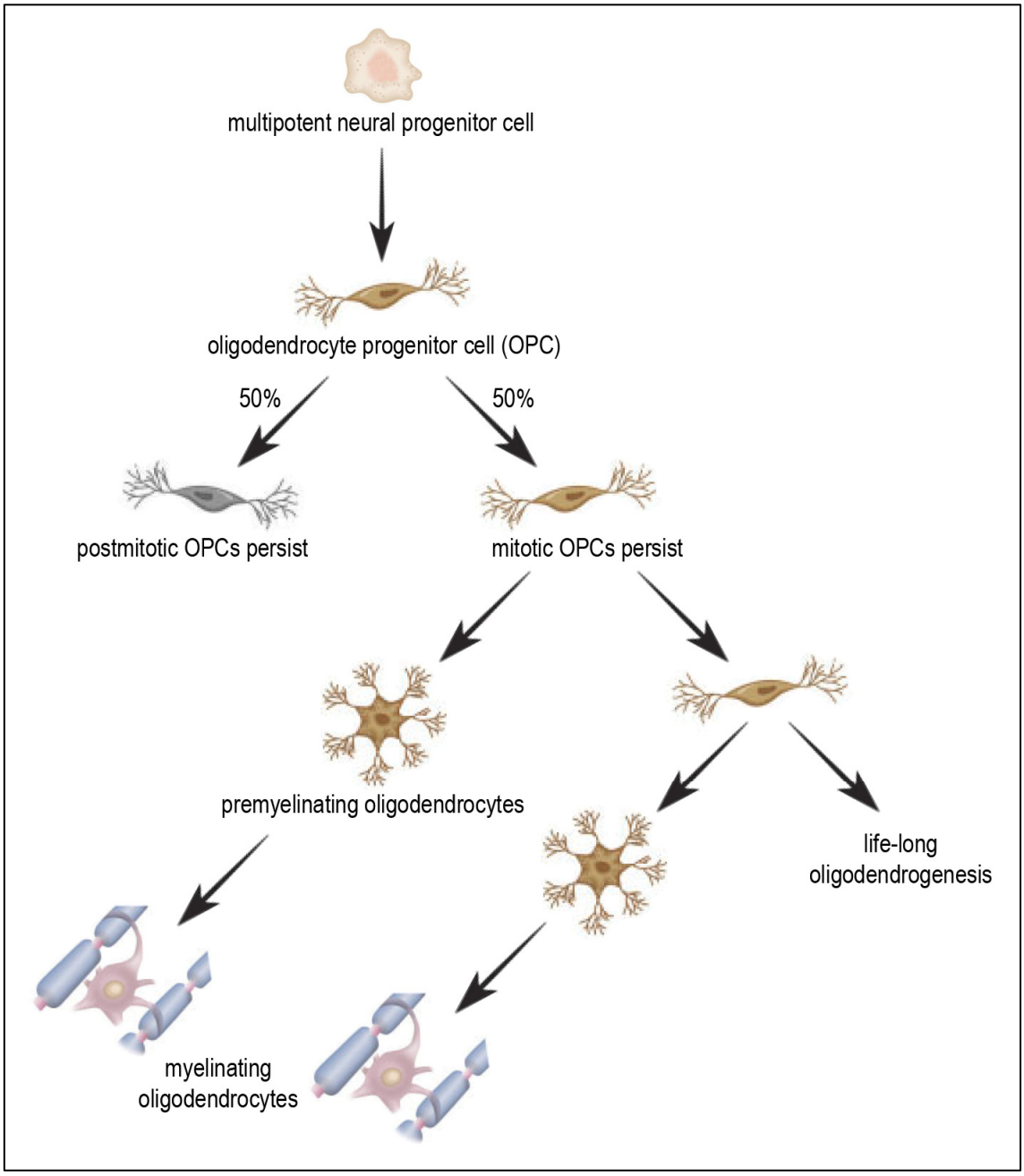



**Figure 1-4: The birth and behaviour of NG2 glia**
Adult forebrain NG2 cells (OPCs) are derived from multipotent neural progenitor cells. Approximately half of all NG2 cells represent a quiescent NG2 cell population which appears at early postnatal phase and persists throughout life. The function of these cells is unknown. The other half of the NG2 cell population remains in cycle throughout life. One major function of the proliferating OPCs is to generate new oligodendrocytes throughout life (adapted from Psachoulia et al., 2009).

### *1.5.3 Microglia*

Microglia were first described by del Rio-Hortega in the early 20th century (del Rio-Hortega, 1932). They belong to the mononuclear phagocyte lineage and are the primary immune defence cells residing in the CNS, representing 5-20% of the adult CNS cells (Gehrmann et al., 1995; Aloisi, 2001).

It is commonly believed that microglia, which derive from bone marrow cells, migrate as microglial precursors from the blood circulation into the CNS during early development and that they may continue to invade the CNS over the course of life, particularly after injury (Aloisi, 2001; Graeber and Streit, 2010). It has been shown that the steady-state turnover of microglia in the intact adult CNS relies on slowly proliferating resident microglia but also on precursor cells recruited from the circulating monocyte pool (Lawson et al., 1992). Once residing within the CNS, microglia develop a ramified morphology with a small cell body and active processes but become quiescent (Cuadros and Navascues, 1998; Prinz and Mildner, 2011). In this resting state, adult microglia continuously sense the environment and are involved in the local immune surveillance (Nimmerjahn et al., 2005).

## 1.6 Injury to the adult brain

### *1.6.1 Primary and secondary injury mechanisms*

CNS injuries cause cell death and neurological dysfunction through both direct physical disruption of tissue termed primary injury, as well as through delayed cellular mechanisms that cause progressive white and grey matter damage which is termed secondary injury (Loane and Faden, 2010).

Primary injury events encompass the immediate and irreversible tissue damage localized in areas that absorb mechanical energy following the initial

traumatic insult to the brain (Loane and Faden, 2010). It affects neurons, glia and blood vessels as a result of shearing, focal contusions and hematomas (Saatman et al., 2008).

Secondary injury develops latently including reversible cellular mechanisms that evolve and expand over a period of hours, days and weeks following the primary injury evoked by the initial trauma (Graham et al., 2000; Loane and Faden, 2010). The secondary injury cascades are responsible for a significant component of the neurodegeneration and thus are thought to account for the development of many of the neurological impairments observed after brain injury (McIntosh et al., 1996; Loane and Faden, 2010). Some of the more important secondary injury mechanisms involve release of neurotransmitters (such as excitatory amino acids), intrinsic neuronal cell death pathways, initiation of inflammatory and immune processes (microglial activation), and secondary neurotoxicity (Graham et al., 2000; Loane and Faden, 2010). Another principal secondary injury mechanism is the activation of the cell cycle (Byrnes and Faden, 2007; Byrnes et al., 2007).

### *1.6.2 Astrocytic response following injury*

Following brain injury astrocytes become activated thereby extending their processes and becoming hypertrophic. They also upregulate synthesis of the glial fibrillary acidic protein (GFAP) (Fitch and Silver, 2008) and begin to proliferate, leading to hyperplasia of astrocytes (Amat et al., 1996; Norton, 1999). These reactive astrocytes, migrate to the lesion border where they form a dense web with their plasma membrane extensions (a process called astrogliosis), culminating in the formation of a glial scar (Fawcett and Asher, 1999; Fitch and Silver, 2008) (Fig. 1-5). The glial scar surrounds the lesion and seals the injury site to prevent cytotoxic spread. At the same time it inhibits the regeneration of

axons from uninjured parts of the brain into or through the lesion (Davies et al., 1999). Classically, reactive glial cells have been identified as the main inhibitor to regeneration, such as axon re-growth or cell replacement, following brain injury (Buffo et al., 2010). In regard to the regeneration and functional recovery of injured brain regions this inhibitory effect is seen as detrimental. But on the other hand, such an inhibitory action would help to confine the extension of the damage from the lesion to healthy parts of the brain. Indeed, mature functional networks depend on the specificity of their connection and architecture, a specificity that can only be acquired after a long and complex developmental process. Thus, astrocytes and other cells forming the glial scar limit further destabilization of the remaining neural networks by preventing random growth of axons and the formation of aberrant connections with the damaged area seal the injury site to prevent cytotoxic spread at the same time inhibiting neuronal regeneration (Harel and Strittmatter, 2006; Sofroniew and Vinters, 2010).

### *1.6.3 Proliferation of OPCs following injury*

Norton had postulated that it is likely that a population of quiescent oligodendrocyte progenitors cells exists throughout the parenchyma ready to provide new cells for repair when needed (Norton, 1999). He based his conclusion on studies that show that cells that remyelinate lesions in adult brain are endogenous progenitors (Gensert and Goldman, 1997) and that they are not recruited over great distances (Franklin et al., 1997). Later on, it could be shown that NG2 cells undergo proliferation in response to different insult to the CNS, such as stab injury (Watanabe et al., 2002; Hampton et al., 2004; Lytle et al., 2009) (Fig. 1-5). Moreover, studies by Buffo et al. demonstrated that Olig2+ glial progenitor cells constituted the largest pool of dividing cells around stab wound

lesions, and that the majority of the proliferating NG2-positive glia were Olig2-positive (Buffo et al., 2005).

### *1.6.4 Activation and proliferation of microglia following injury*

Quiescent in the healthy CNS, microglia are highly sensitive to pathologic changes and are the first cell type to react after injury to the adult cerebral cortex (Kim and de Vellis, 2005; Nimmerjahn et al., 2005; Hanisch and Kettenmann, 2007). Thereby, microglia switch their phenotype from resting microglia which

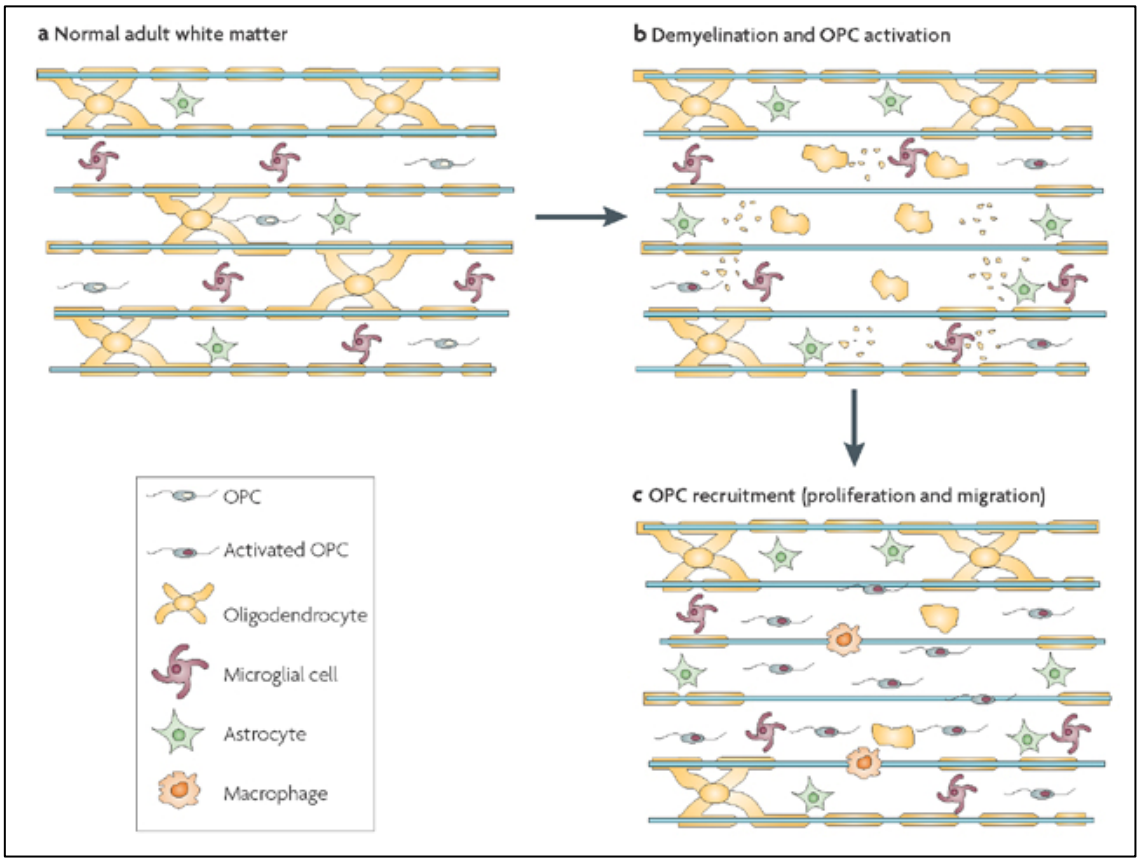



**Figure 1-5: Model of injury mechanism following demyelination to the white matter**
**a** Normal adult white matter contains astrocytes, microglia and oligodendrocyte precursor cells (OPCs), in addition to myelinating oligodendrocytes. **b** Following demyelination (in which oligodendrocytes and myelin are lost) the microglia and astrocytes become activated, which in turn leads to the activation of any OPCs in the vicinity. **c** The activated OPCs respond to mitogens and pro-migratory factors that are generated predominantly by reactive astrocytes and inflammatory cells. The proliferation and migration of the OPCs results in the demyelinated area becoming populated by an abundance of OPCs. Macrophages also start to remove the myelin debris. (adapted from Franklin and ffrench-Constant, 2008).

display ramified cellular morphologies to more activated forms displaying hypertrophic or bushy morphologies (Soltys et al., 2001). Activated microglia migrate to the lesion site, proliferate and become able to prime naïve T cells (Aloisi et al., 2000; Vilhardt, 2005) (Fig. 1-5). In parallel, they secrete pro-inflammatory cytokines, chemokines, neurotrophic and cytotoxic factors and modulate the local immune response (Hanisch, 2002; Ambrosini and Aloisi, 2004; Di Giovanni et al., 2005; Byrnes et al., 2007; Hanisch and Kettenmann, 2007). Moreover, activated microglia transform into phagocytosing macrophages and along with macrophages derived from the circulatory system act to clear cell debris from the lesion site (Amor et al., 2010). Following a stab wound injury the number of proliferating microglia reaches a maximum at 4 days postinjury (Amat et al., 1996).

## 1.7 Commonly used lesion models

A number of animal models have been developed over the last few decades that mimic different aspects of traumatic brain injury in the human with varying degrees of accuracy (Gennarelli, 1994; Morales et al., 2005; Wang and Ma, 2010). As the immediate cell death resulting from the initial impact on the brain tissue is irreversible, treatments focus on interruption or inhibition of the secondary injury cascades expanding this primary injury. The use of animal models is essential for better understanding of the secondary injury processes and for the development of novel therapies. Some of the most widely employed lesion models are fluid percussion injury (FPI), controlled cortical impact (CCI), cryogenic injury, stab wound injury and injection injury. The first and the second models mentioned mimic a traumatic brain injury: while FPI models produce brain injury by rapidly injecting fluid volumes onto the intact dural surface through a craniotomy, the CCI models utilize a pneumatic pistol to deform

laterally the exposed dura and provide controlled impact. The advantage of the fluid percussion model resides primarily in its simplicity and its ability to produce significant disturbances in the brain, but it allows little control over injury parameters often causing collateral injury and death (Lighthall et al., 1989). The controlled cortical impact model has been shown to produce a more precise injury (Dixon et al., 1991). But for both models, conclusions regarding axonal pathology must take account of the superimposed effects of contusion and haemorrhage that are usually present. The method of cryogenic injury is generally produced by applying a cold rod to the exposed dura or skull (Siren et al., 2000) leading to a focal brain lesion. The major advantages of this method are the clearly circumscribed lesion which is highly reproducible in size, location and pathophysiological processes of the secondary lesion expansion at the cortical impact site. But it leads to profound brain edema and blood brain barrier leakage. The stab wound injury represents a penetrating trauma model designed to generate focal cortical injury by application of a microdissecting knife. The advantage of this model is the high reproducibility. The disadvantages are the large mechanical damage to the tissue and the rather small affected area.

## 1.8 The ibotenic acid (IBO) model

To study the injury-induced proliferation of cells *in vivo* we decided to choose an injury-model with a limited, localized neuronal injury. We performed stereotactic injections of ibotenic acid (IBO) into the brain tissue of WT and knockout mice. Injection of IBO leads to a compact, well-demarcated lesion affecting only neurons (Inglis and Semba, 1997). The excitotoxin IBO is an agonist of glutamate (Zorumski et al., 1989) which efficiently binds to NMDA receptors. Thereby it is overstimulating them (Marret et al., 1996) leading to excessive Ca2+ influx into the neuron. Moreover, it activates metabotropic glutamate

receptors mediating the release of Ca2+ from intracellular stores. Together, this results in elevated intracellular Ca2+ concentrations which acutely induces apoptosis (and necrosis).

The advantages of this method are (1) the well-defined target area without the potential influence of continued neuronal apoptosis, (2) the larger affected area compared to a stab injury and (3) the less disrupted tissue compared to a mechanically-induced wound.

In this well-established animal model (Maetzler et al., 2004; Maetzler et al., 2010), neurodegeneration immediately starts after toxin injection, and a proliferative cellular reaction is observed in the lesion during the first week after IBO injection.

# 2. AIM OF THESIS

The determination of cell extrinsic and intrinsic factors controlling proliferation and differentiation of progenitor cells in the postnatal brain is crucial to elucidate fundamental mechanisms of postnatal neurogenesis and gliogenesis. The proliferation of cells is tightly regulated by cell-cycle proteins, most notably the D-type cyclins play an important role in driving the progression of the cell cycle through the G1 phase (Sherr, 1995). Much is known about the involvement of Cyclin D1 in neurogenesis but not for gliogenesis. Gliogenesis is generally delayed compared with neurogenesis with a wave of gliogenesis in early postnatal stages and a widespread proliferative activity of some glial progenitors that persists in the adult CNS parenchyma. Furthermore, the existence of fast and slowly proliferating subpopulations of glial cells was demonstrated before in the adult brain (Dimou et al., 2008; Psachoulia et al., 2009). In addition, it is well known that following injury to the CNS, cell cycle proteins are upregulated in neurons, astrocytes and microglia (Di Giovanni et al., 2005; Byrnes and Faden, 2007; Byrnes et al., 2007).

To answer the question whether the cell cycle protein cyclin D1 is crucial for driving proliferation in glial cell types of the postnatal cortex *in vivo* I used different proliferation paradigms: I examined fast-proliferating glial cells during early postnatal development and slow-proliferating glial cells during adult stages by determining their proliferation rate and cell density. In addition, I used an injury paradigm to induce acute proliferation in the adult cortex which enabled me to analyze fast-proliferating cells in the adult cortex. Functional requirement of Cyclin D1 was assessed by analyzing proliferation rates and cell density of glial cells, as well as lesion size in mice deficient for Cyclin D1. As Cyclin D is only the regulatory subunit of an active holoenzyme formed with Cdk4 and Cdk6, one could assume that knockout of the binding partner of Cyclin D1 might

have similar effects as seen in the *Cyclin D1*$^{-/-}$ mice, allowing me to determine the potential interaction partner of Cyclin D1. Therefore I also applied the injury model in mice deficient for *Cdk4* and *Cdk6* and assessed the cell proliferation to compare it with *Cyclin D1* knockout animals.

# 3. MATERIALS AND METHODS

## 3.1. Mice

Animal experiments were approved by the veterinary office of the Canton of Basel-Stadt. The *Cyclin D1-/-* mice used in this study originate from the lab of Clive Dickson. They were generated by replacing most of the first exon of the Cyclin D1 gene with sequences encoding neomycin resistance (Fantl et al., 1995). Cdk2- (Ortega et al., 2003), cdk4- (Rane et al., 1999) and cdk6-deficient mice (Malumbres et al., 2004) have been previously described. Adult WT and knockout mice were bred and maintained in the animal facility of the Zentrum für Lehre und Forschung (ZLF), Kantonsspital Basel on a 12/12-hour light dark schedule. The animals had free access to food and drinking water. In accordance with the Swiss Law of Animal Protection, the breeding colony as well as the operative procedures detailed below were approved by and were under the constant control of the official veterinarian of the City of Basel (license No. 2171)

For the injury paradigm 4-5 month-old mice of either sex were used. Furthermore uninjured animals were examined at 8 months of age. To obtain mice from postnatal day 3 (P3) male and female mice heterozygous for cyclin D1 were time-mated.

## 3.2 Primary antibodies

The primary antibodies used in this study were as follows: anti-Cyclin D1 (NeoMarkers, RM-9104, rabbit, 1:300), anti-NeuN (Millipore, MAB377, mouse, 1:500); anti-Olig2 (Chemicon, AB9610, rabbit, 1:500), anti-S100 (Sigma, S-2532, mouse, 1:300), anti-GFAP (Cell Signaling Tech., 3670, mouse 1:500), anti-NG2 (Millipore, AB5320, rabbit, 1:100), anti-APC (CC1, Calbiochem, clone

OP80, mouse, 1:300), anti-MBP (Chemicon, MAB386, rat, 1:300), anti-Iba1 (Abcam, ab5076, goat, 1:300), anti-Ki-67 (NeoMarkers, RM-9106, 1:300), anti-BrdU (Novus Biologicals, NB500-169, rat, 1:100).

Neuronal nuclei (NeuN) is an antigen used widely in research to identify postmitotic neurons throughout the central and peripheral nervous system (Mullen et al., 1992). The monoclonal antibody proved to bind an antigen expressed only in neuronal nuclei and to a lesser extent the cytoplasm of neuronal cells. In the adult cortex Olig2 is a robust and specific lineage marker of oligodendroglial cells in the mouse CNS, expressed in OPCs, premyelinating and myelinating oligodendrocytes (Ligon et al., 2004). S100β is a late marker of astrocyte development characterizing a mature stage of the astrocyte (Raponi et al., 2007). GFAP (glial fibrillary acidic protein) is upregulated in activated astrocytes following cortical injury (Fitch and Silver, 2008). NG2 is a chondroitin sulfate proteoglycan and transmembrane protein which is expressed by a range of cell types within and outside the nervous system. Within the the developing and adult CNS expression of NG2 is used as a marker for oligodendrocyte progenitor cells (OPCs) (Nishiyama et al., 2009). APC (adenomatous polyposis coli) is a tumor suppressor protein that is enriched in the somata of mature oligodendrocytes (Bhat et al., 1996). Thus anti-APC labels mature oligodendrocyte cell bodies but not myelin sheaths. Iba1 (ionized calcium binding adaptor molecule 1) is a protein that is expressed in macrophages/microglia. Within brain tissue, the Iba1 gene is specifically expressed in microglia (Ito et al., 1998). The Ki-67 protein is a marker for measuring cell proliferation, being present during all active phases of the cell cycle (G1, S, G2, and mitosis), but absent in G0 (Scholzen and Gerdes, 2000). Myelin basic protein (MBP) is a protein expressed in the myelin membrane and therefore a marker for mature myelinating oligodendrocytes.

## 3.3 Secondary antibodies

Secondary antibodies used were Alexa488- (Molecular Probes), Cy2-, Cy3- or Cy5-conjugated antibodies (all Jackson ImmunoResearch; 1:500).

## 3.4 Determination of genotype

DNA was extracted from mouse tail or toe by the alkalinelysis (HotSHOT) method (Truett et al., 2000) and analyzed by PCR to distinguish cyclin D1 wild-type and mutant alleles. Following primers were used for the PCR: MD1 1535 5'-ACC AGC TCC TGT GCT GCG AA-3' and MD1 1848 5'-ACC GAG TCC TAG CAA CGC AC-3' for WT animals (amplicon size 313 bp), neo forward 5'-GGA GAG GCT ATT CGG CTA TGA C-3' and neo reverse 5'-CGC ATT GCA TCA GCC ATG ATG G-3' for *Cyclin D1*$^{-/-}$ animals (amplicon size 457 bp). In addition, immunohistochemistry was carried out to confirm the results obtained by PCR.

## 3.5 Surgical procedure

Animals were anesthetized with a combined treatment of ketamine (Ketasol-100, 80 mg/kg sc), climazolam (Climasol, 5 mg/kg ip), and atropine (Atropinum sulf, 0.05 mg/kg ip), while buprenorphin (Temgesic, 0.1 mg/kg sc) was administered preoperatively for analgesia. After the fur on the skull had been shaved, the skin was cleaned with 70% ethanol and the animal placed into a stereotaxic head frame (David Kopf Instruments, Model 500) (Fig. 3-1a).

To place the animal in the apparatus, one ear bar was fixed in the apparatus and the animal's head gently position to lead its ear canal onto the ear bar (Fig. 3-

1c). The animal's head was kept in place and the second ear bar slowly positioned to complete the fixation. The animal's tongue was pulled out (this helps to prevent breathing problems) and hold aside while the incisor adapter (Fig. 3-1d) was slowly moved into the mouth until the animal's incisors 'fit' in the opening of the adapter. The adaptor was slightly pulled back and fixed in place. The last point of fixation, the nose clamp, was used with very low pressure on the animal's nose. Lubricant eye ointment (Vitamin A, Blausch & Lomb Swiss AG) was applied to prevent corneal drying during the surgery. The skull was exposed and the coordinates of bregma were measured. Surgery was done under the use of a dissecting microscope (Leica M651) (Fig. 3-1e). Unilateral lesions of the prefrontal cortex were performed by injecting ibotenic acid (Sigma-Aldrich, 0.5 μg in 0.2 ul 0.9% NaCl) in the left hemisphere using a microsyringe (Hamilton, model 7001 SN, 26s gauge) (Fig. 3-1b). Lesion coordinates were determined from the mouse brain atlas of Franklin and Paxinos (1997) and were as follows: anteroposterior (AP) +2.4 mm from bregma; mediolateral (ML) +1.5 mm from midsagittal vein and dorsoventral (DV) -2.3 mm from cranial bone. The skull overlying the target coordinates was drilled using a hand-held drill (KaVo, model EWL 10A) (Fig. 3-1f).This was carefully done by applying a slight pressure downward and stopping as soon the blood vessels in the dura became clearly visible. Using a hook a small incision was cut in the dura. Before infusions were made, the syringe was lowered 0.3 mm past the injection site and kept at lower depth for 20 seconds to increase spread of drug diffusion. The syringe was then raised to the injection site, and the ibotenic acid was infused over 3 min. The needle was left in place for another 20 min before being slowly withdrawn and the wound closed with a non-absorbable polypropylene suture (Ethicon, suture needle Prolene #8648G). For postoperative care the mice were kept under a hood in a cage equipped with a heat blanket (Fig. 3-1g) which was connected to a temperature control unit (Letica, model HB 121/2) (Fig. 3-1h) set at 37°C. All

animals were monitored carefully for at least 4 hours after surgery and then daily. Furthermore the analgesic meloxicam (Metacam, 0.001 mg/kg ip) was administered once 2 hours following the surgical intervention. Animals were also provided with soft food. Sham animals underwent the same procedure as injured mice but were injected with saline solution instead of the neurotoxin ibotenic acid.

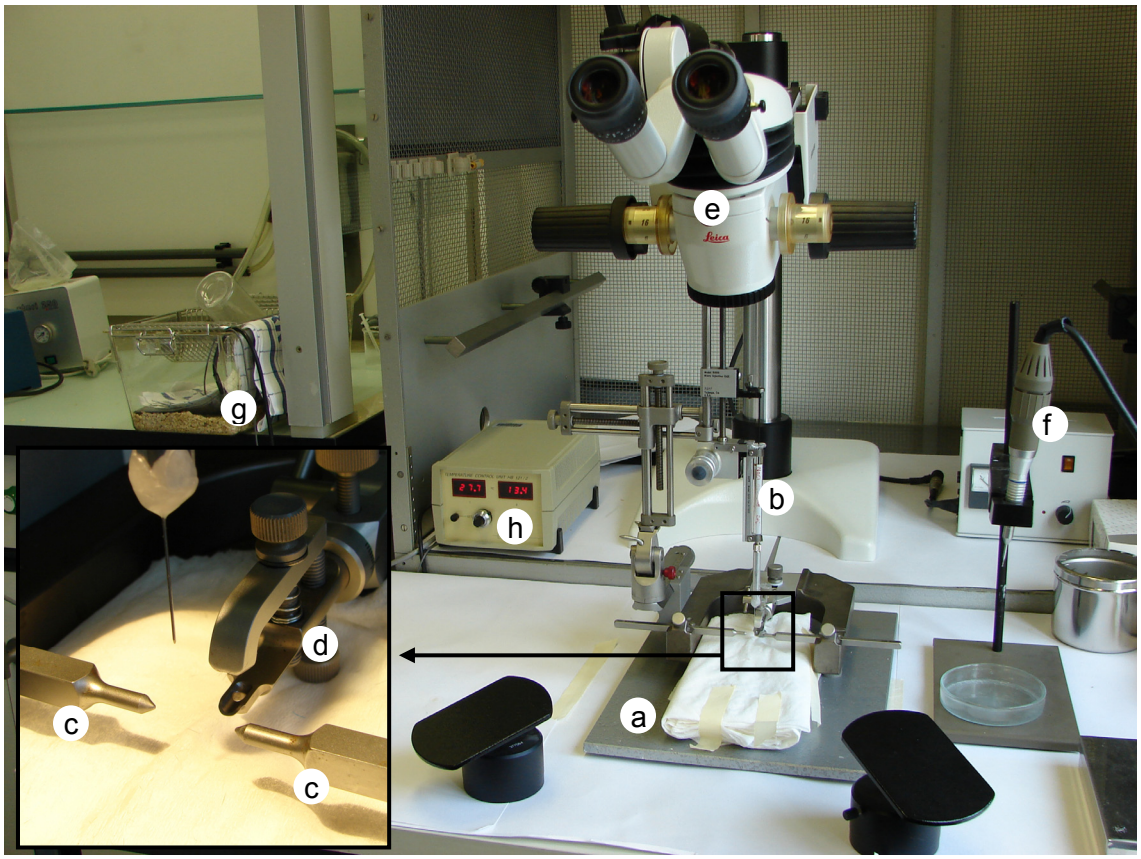



**Figure 3-1: Setup and procedure for stereotaxic injections**
The setup for the surgical procedure included a stereotaxic instrument (a) into which a microsyringe was placed (b). To keep the mouse within the stereotaxic apparatus the head was hold in place by ear bars (c) and an incisor adapter (d). Surgery was done under the use of a dissecting microscope (e). A hand-held drill was used to perforate the skull (f) and the microsyringe lowered to perform the injection. After the surgery the mice were kept in a cage equipped with a heat blanket (g) which was connected to a temperature control unit.

## 3.6 Tissue preparation

### *3.6.1 Transcardial perfusion*

After a recovery period of 1, 3 or 30 days adult injured animals were perfused for fixation and the brains extracted. Perfusion of the animals was performed by transcardial perfusion using a gravity system. Therefore, 4% paraformaldehyde (PFA; in 0.1 M phosphate buffer pH 7.4) was freshly prepared, filtered and poured into a bottle with rubber cap (Ecotainer, B. Braun Medical) which permitted connection to a tube. Together with a bottle containing physiological saline (Ecotainer NaCl 0.9%, B. Braun Medical) both were set 1.6 m above work area and connected to a tubing system (Braun Medical, V4508) which allowed to join both tubes of the bottles to one end at which a 23 gauge needle (Terumo, Luer Nr. 14, 23G) was connected. In addition work was performed on a bench with air suction to avoid inhalation of toxic formaldehyde gases (Fig. 3-2A).

Mice were deeply anesthetized with the pentobarbital Vetanarcol (Veterinaria AG; diluted 1:4 in NaCl 0.9%, used 0.04 g/kg ip) and placed in the supine position (lying on the back with face upward) onto a supportive element that could collect excess fluids during the perfusion. To ensure a stable positioning of the mouse its extremities were fixed with tape onto the supportive element (Fig. 3-2B). Tail and toe pinch reflex were used to assess depth of anesthesia before proceeding with the next step. The thoracal and ventral skin surface of the animal was wetted with 70% ethanol and superficially removed to expose the thoracic membrane. This was done using surgical scissors (Fig. 3-2C) with which an incision was made into the skin over the xiphoid process. While slightly pulling the skin at the incision with blunt foreceps (Fig. 3-2C) the skin covering the thorax was cut away with the scissors. Next, a small hole was cut into the peritoneal membrane just below the zyphoid process. Now, one could grasp the cartilage of the zyphoid process with blunt foreceps and cut the diaphragm from

on lateral aspect to the other to open the thoracic cavity. Carefully, the ribs were cut longitudinally through both sides of the rib cage up to the level of the clavicles while avoiding lung and heart. The ventral part of the rib cage was folded back and a clamp attached to the zyphoid process to keep the thorax open

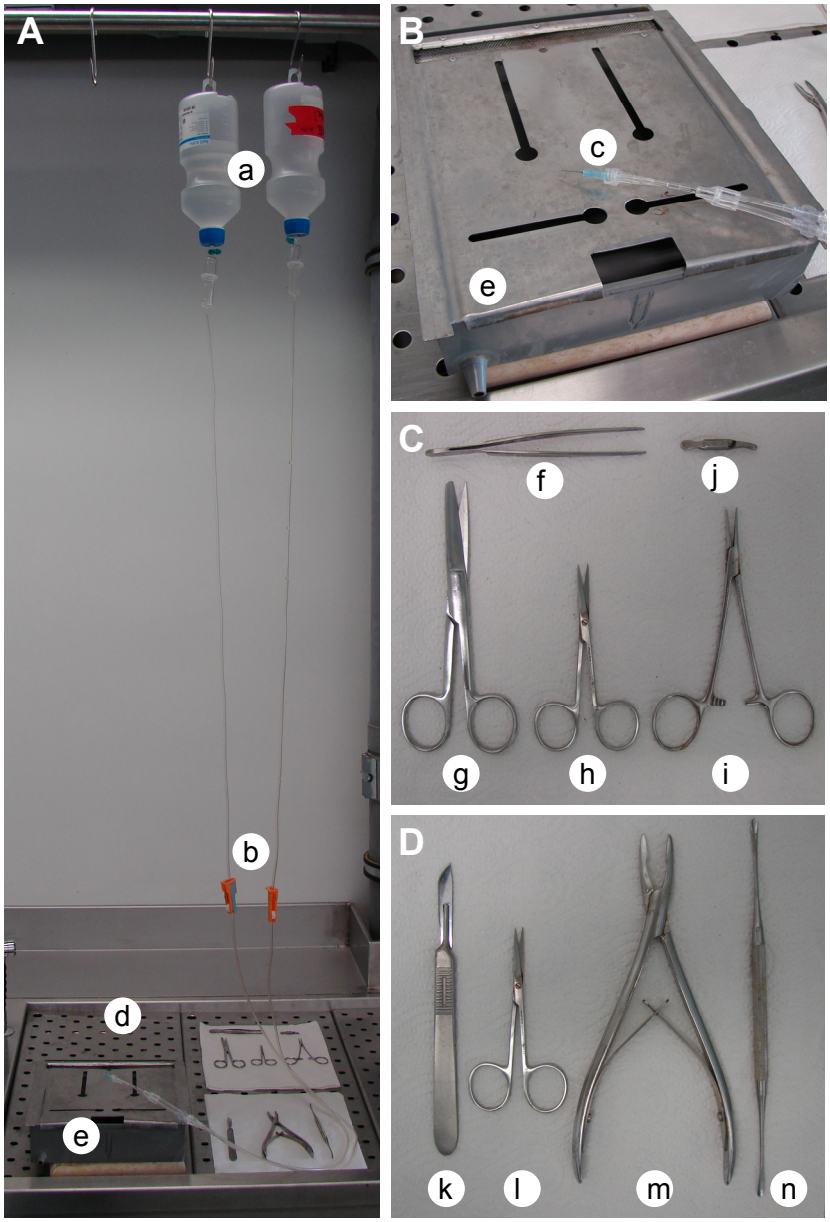



**Figure 3-2: Setup and instruments for trans-cardial perfusion and tissue preparation**
**A, B** The setup for the trans-cardial perfusions in-cluded a gravity system which was composed of two bottles (a) each connected by a tubing system (b) which allowed to join both tubes of the bottles to one end at which a 23 gaugle needle (c) was attached. The perfusion was performed on an air suction bench (d). During the perfusion the mouse was fixed with tape onto supportive element that could collect excess fluids (e). **C** Following surgical instruments were used to prepare the mouse for the perfusion: blunt foreceps (f), surgical scissors (g), small scissors (h), clamp (i) and bulldog clamp (j). **D** For the extraction of the brain following instru-instruments were used: scalpel (k), small scissors (l) rongeur (m) and spatula (n).

exposing the heart. The beating heart was gently hold with blunt foreceps and using the small, pointed scissors a small incision was made at the apex of the left atrium. Quickly the 23 gauge needle was inserted in the atrium and clamped to apex of the heart using a small bulldog clamp (Fig. 3-2C). The right atrium was immediately cut with pointed scissors to allow exit of blood. In addition, a clamp (Fig. 3-2C) was applied onto the whole abdomen thereby shutting the descending aorta, such that only the upper part of the body would be perfused and thus the fixative not distributed within the whole circulatory system. Now the transcardial perfusion was performed first with physiological saline to wash out blood from the circulatory system. This was done until the fluid exciting the right atrium was entirely clear (~1 min). Perfusion was then switched from the saline to the PFA and perfusion continued for 20 min at a flow rate of 3-4 ml/min. One could observe movements of the head resulting from the aldehyde–crosslinking of nerves and muscles.

For brain tissue preparation of non-injured animals the same procedure was applied as for injured mice, except for P3 mice. For these neonatal mice, the transcardial perfusion was not done by using the gravity system but by manual injection using 20ml-syringes. Thereby, P3 mice were injected 4 ml 0.9% NaCl and 20 ml 4% PFA at a flow rate of 3-4 ml/min.

### *3.6.2 Brain extraction and sectioning*

At the completion of the paraformaldehyde perfusion the surgical instruments were removed and the mice decapitated with scalpel blade (Fig. 3-2D). Using the scalpel the scalp was incised in the mid-sagittal line starting near the nasal bone and running caudally to the occipital bone to expose the skull. Using a small, pointed scissors the posterior part of the skull was cut starting from the posterior portion into the occipital plate of the skull. While holding the head of the mice

firmly on a cutting board the skull plates were then removed by using a rongeur (Fig. 3-2D). This was done by sliding the bottom jaw of the rongeurs under the skull plates, squeezing the jaws of the rongeurs together and rolling the wrist to oneself to remove the skull plates from the brain. While doing this it was important to maintain pressure with the lower jaw of the rongeurs against the inside surface of the skull and away from the brain not to damage it. Once all the skull plates were displaced, a spatula (Fig. 3-2D) could be slid between the ventral surface of the brain and the bottom skull plates. By moving the spatula laterally from side-to-side cranial nerves were separated from the brain such that the brain could be removed. It was immediately weighed and subsequently the brain hemispheres were separated with a scalpel blade. The hemispheres of adult mice were now immersed in the PFA for another 1 h. In the case of P3 mice, the post-fixation was done overnight.

Using a vibratome (Microm GmbH, model HM 650V) the brain tissue was cut sagittaly in 30-µm sections which were collected in ice cold 0.1 M PBS in a 24-well cell culture plate (#353047, BD Falcon). Culture plates containing the brain sections were kept at 4°C until they were used for immunohistochemistry.

## 3.7 Proliferation analysis

For in vivo labelling of fast proliferating cells in injured adult mice and non-injured P3 mice, the synthetic nucleoside 5-bromo-2'-deoxyuridine (BrdU, Roche Diagnostics) was injected (100 mg/kg ip) 2 h before perfusion. To label fast and slow proliferating cells in non-injured adult animals BrdU was provided for 15 days in the drinking water (1 mg/ml) before perfusion. Therefore fresh BrdU was prepared every 3 days and used to replace the the drinking water in the bottles. In addition, the bottles were wrapped in aluminium foil to protect the BrdU from light.

## 3.8 Immunohistochemistry

For immunohistochemistry free-floating sections were selected and transferred to a 12-well cell culture plate (#3513, Corning Inc.) containing PBS before subsequent processing. First, the sections were solubilised and blocked 1h in 0.1 M PBS containing 0.2% Triton X-100 and 10% normal serum (horse or goat serum). Next, the sections were incubated with primary antibodies at 4°C overnight. Then, the sections were subjected to three washing steps in PBS for 5 min at RT and before being incubated for 1 h at RT with secondary antibody. After another three washing steps in PBS sections were immersed in DAPI (0.2 ug / ml PBS) to label nuclei. Finally, the sections were mounted on glass slides, briefly dried and coverslipped with permanent mounting medium (Vectamount, Vector Laboratories).

For detection of BrdU incorporation, free-floating sections were first subjected to the method of "heat induced epitope retrieval" (HIER). Therefore the sections were immersed in 10 mM citrate buffer pH 6.0 and the multiwell plate was placed in a steamer (Pascal, DAKO Cytomation) where it was kept for 5 min at 95°C. After cooling down to room temperature DNA was denatured by incubating the sections in 2N HCl for 20 min at 37°C in a hybridization oven (Shel Lab, model 1012). Subsequently, the sections were processed with the standard protocol above-mentioned.

## 3.9 Detection of apoptosis by TUNEL staining

Extensive DNA degradation is a characteristic event which occurs in the late stages of apoptosis. Cleavage of the DNA may yield double-stranded DNA fragments which can be detected by enzymatic labelling of the free 3'-OH termini. The terminal deoxynucleotidyl transferase (TdT) is able to label blunt

ends of doublestranded DNA breaks with modified nucleotides. This end-labelling method to detect end-stage of programmed cell death has been termed TdT-mediated X-dUTP nick end labelling (TUNEL) and the protocol for in situ detection on histological sections has been previously described (Gavrieli et al., 1992).

Sections were blocked and solubilised for 1 h at RT in TUNEL blocking buffer (0.1 M PBS containing 0.01% Triton X-100, 0.1% BSA and 1% normal goat serum). After 10 min of equilibration at RT in TdT-buffer (30 mM Tris-HCl pH 7.2, 140mM sodium cacodylate, 1mM cobalt chloride) the sections were incubated for 1 h at 37°C with 200ul TUNEL-mix consisting of the following: 1.6 ul TdT enzyme (Roche Diagnostics, #11767305001), 1.2 ul 50 nmol biotin-dUTP (Roche Diagnostics, # 11093070910), 1.2 ul 1mM ATP, 200ul TdT-buffer. The reaction was terminated by immersing the sections in 2x SSC buffer (300 mM NaCl, 30 mM sodium citrate, pH 8.0). Next, the sections were washed 3x for 5min in PBS before being incubated for 1 h at RT with the secondary antibody (streptavidin-Alexa Fluor® 488 conjugate 1:300, Molecular Probes). Further processing was done as mentioned in the immunohistochemistry protocol above following the incubation with secondary antibodies.

Cells only positively stained by TUNEL and containing clear chromatin condensation were referred as apoptotic cells. Necrotic cells may also become TUNEL positive but their staining is diffuse without chromatin condensation.

## 3.10 Nissl staining

Nissl staining is a classic nucleic acid-staining method traditionally used on nervous tissue sections. The method refers to staining of the cell body, and in particular endoplasmic reticulum. This is done by using a basic dye (aniline, thionine, or cresyl violet) to stain the negatively charged RNA blue which in turn

highlights the soma and dendrites of neurons. Therefore, sections were mounted onto glass slides in 1xPBS and dried overnight. Slides were first immersed for 10 min in 0.4% acetic acid, stained for 4 min in cresyl violet solution (0.2% cresyl violet acetate, 0.02% sodium acetate-3-hydrate, 0.2% acetic acid, pH 3.5), washed for 1 min in 0.4% acetic acid and shortly rinsed with ddH2O. Sections were dehydrated in ascending ethanol (50% ethanol, 70% ethanol), decoloured for 15 min in 4% acetic acid in 96% ethanol, followed by 1 min in 100% ethanol and finally cleared in xylene before cover-slipped with Eukitt (Kindler, Germany).

## 3.11 Microscopy

Sections stained with cresyl violet were evaluated using a light microscope (Nikon, Eclipse E800) equipped with a ProgRes C14plus camera (Jenoptik) and pictures taken using the image capture software ProgRes Capture Pro 2.5.
All other images were collected on a fluorescence microscope with a motorized stage (Imager Z1, Carl Zeiss) equipped with a Axiocam MRm (monochrome CCD) camera and with an X-Cite 120 illuminator (EXFO). Images were collected and analyzed with Axio Vision Image Analysis Software (Improvision, 4.8.1). Images were optimized for size, color, and contrast using Photoshop CS4 (Adobe) for Windows.

## 3.12 Statistical analysis

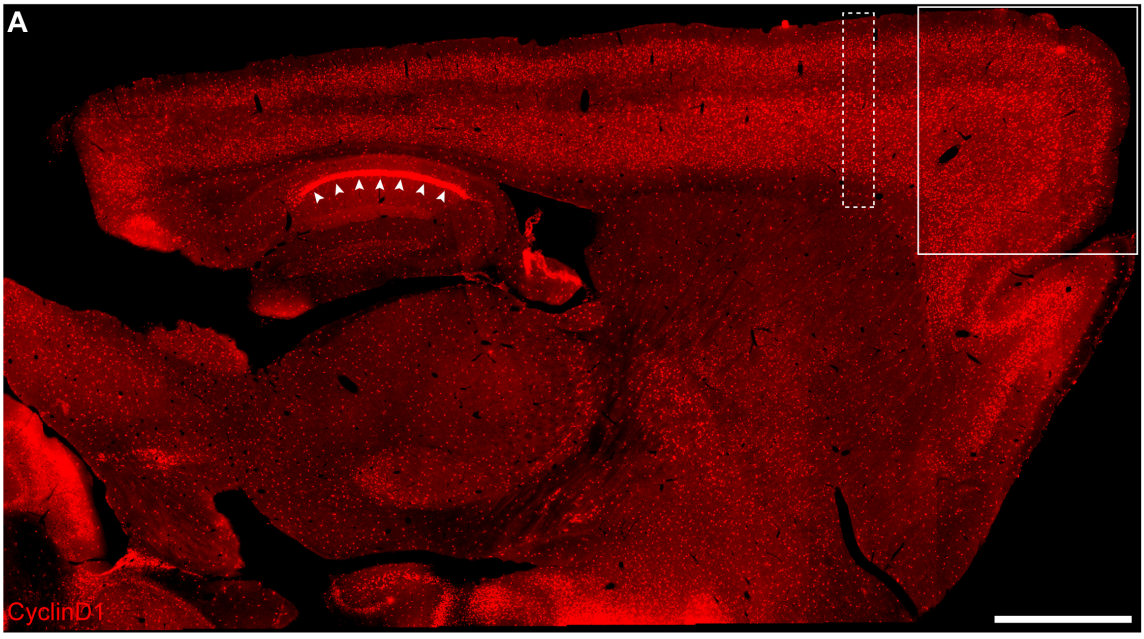
All statistical analysis were performed in at least three animals of each genotype. For quantitative analysis from intact prefrontal cortex, cell counting was performed on the whole prefrontal cortex (2.2 – 2.9 $mm^2$ area) and three sections

per animal quantified. The average was calculated for each animal before statistical testing was performed. For quantitative analysis within cortical lesions, cell counting was performed within the neurodegenerative area leaving out necrotic zones. Depending on the size of the lesion quantifications were done in 1 – 3 sections per animal. The average was calculated for each animal before statistical testing was performed. All quantitative data are presented as mean ± standard error of mean (SEM) and have been analyzed by two-tailed Student's *t* test. Statistical tests were performed using Microsoft Excel 2003 for Windows.

# 4. RESULTS

## 4.1 Expression of Cyclin D1

Immunoreactivity to Cyclin D1 was predominantly nuclear and could be detected in all cortical regions. As previously reported by Glickstein et al. (Glickstein et al., 2007a; Glickstein et al., 2007b), the signal was more robust in rostral than in caudal neocortex (Fig. 4-1A). I could also confirm the predominant expression of Cyclin D1 in neurons of the layers II-III and V-VI of the neocortex (Fig. 4-1B) and the elevated expression level in CA1 field neurons of the hippocampal formation (Fig. 4-1A) which was described by Koeller et al. (Koeller et al., 2008). The prefrontal cortex showed scattered cells positive for Cyclin D1 with different levels of expression (Fig. 4-1C) while the negative control which was done on sections of *Cyclin D1*$^{-/-}$ mice was devoid of labelling for Cyclin D1 (Fig. 4-1D).



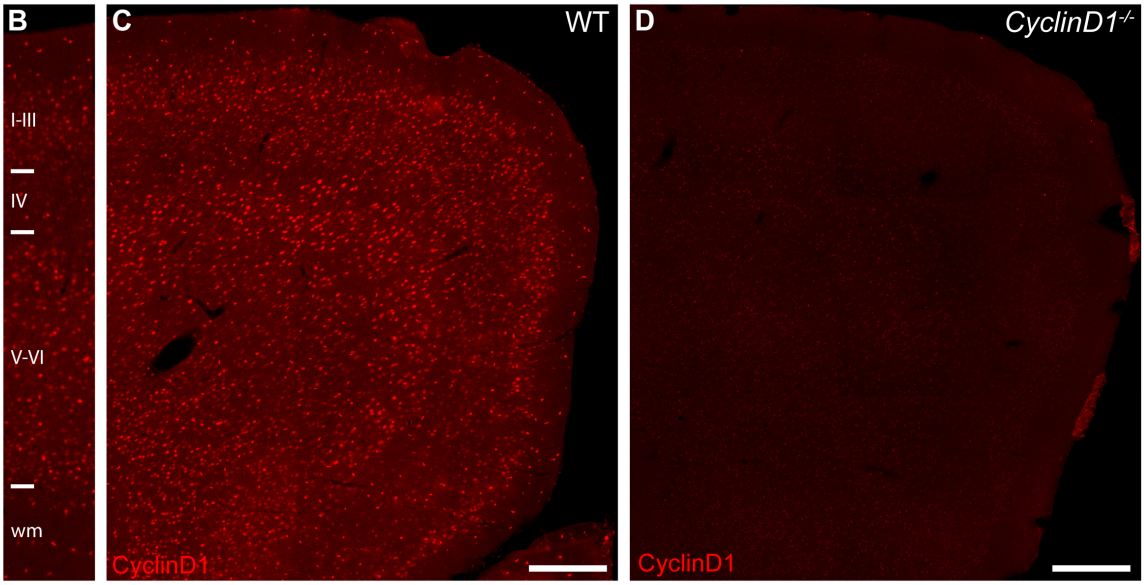



**Figure 4-1: Cyclin D1 expression in the brain of adult mice**
**A** Whole sagittal section of the brain labelled for Cyclin D1. Cells expressing Cyclin D1 are scattered throughout the brain. Elevated expression is found in CA1 field neurons (arrow heads). Scale bar 1000 µm. **B** Magnification of the dashed box in (A) depicting Cyclin D1 expression in the layers II-III and V-VI of the cortex. **C** Magnification of the boxed region in (A) showing Cyclin D1 expression in the prefrontal cortex. **D** No Cyclin D1 expression was detected in the brain of knockout animals. Scale bars (A, C, D) 250 µm.

## 4.2 Characterization of the cortical lesion

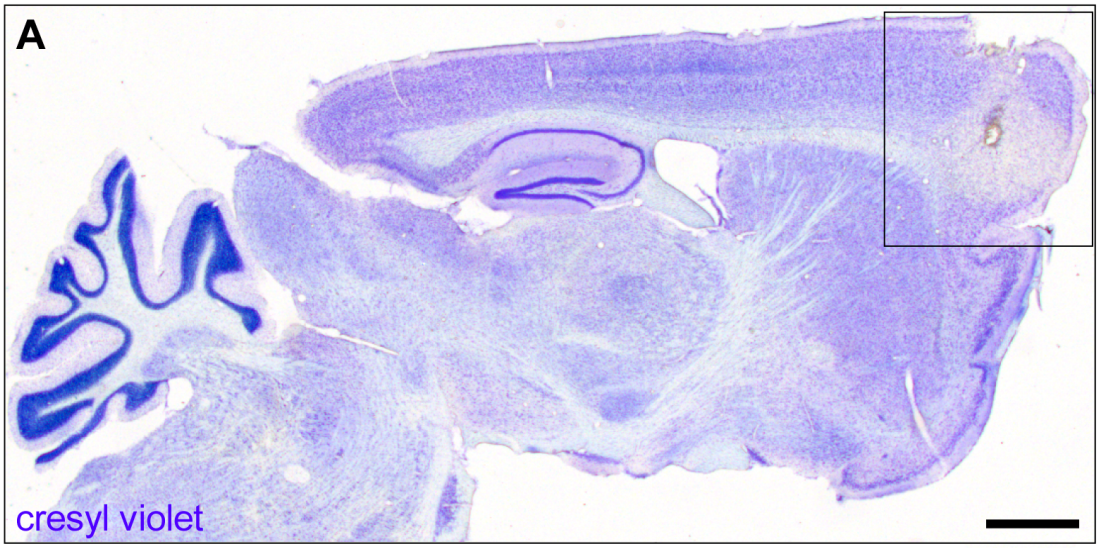
Pilot studies had to be performed in order to establish the injury model: the appropriate coordinates as well as the volume and the concentration of the ibotenic acid needed to be determined. The prefrontal cortex proved to be the best region of the cortex to generate reproducible cortical injuries. Furthermore, a stepwise approach by reducing both, volume and concentration, was performed to reach the desired size of the lesion. It proved that best results were obtained by injecting 0.2 µl of 0.25 % ibotenic acid (in physiological saline).

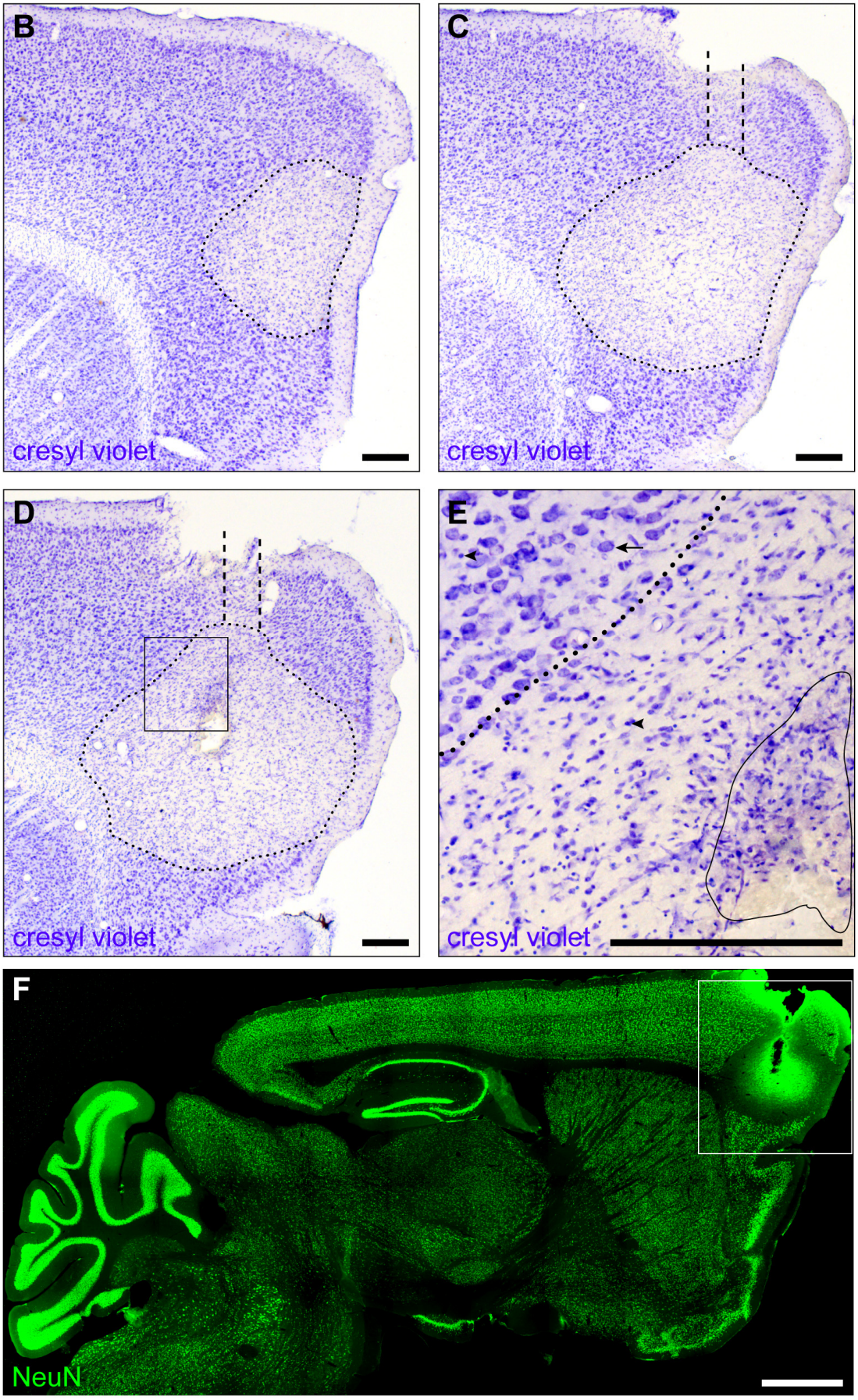
In a first step, I evaluated the extent of damage to the brain following the injection of IBO into the prefrontal cortex. Therefore, I performed histological analysis by using Nissl staining and light microscopy (Fig 4-2A). I could detect

the lesion as a characteristic morphologic abnormality composed of disrupted tissue along the needle track and a pale area displaying the loss of neurons. This neurodegenerative area resulted in a spherical neuron-depleted zone of 1 – 1.5 mm diameter which could be observed 3 days postinjection (dpi) (Fig 4-2B-D).

Furthermore, a zone of necrosis in the center of the lesion site where the needle tip had been positioned, as well as a thin rim of necrotic neurons and focal haemorrhage adjacent to the needle tract were visible. This might be accountable by the mechanical damage produced by the needle injury alone as previously reported (Petito et al., 1982). For most of the lesions the border between neuron-depleted area and healthy tissue was well definished and the morphology of the lesions comparable to previous studies (Maetzler et al., 2004; Maetzler et al., 2010) (Fig 4-2E).

In a second step I performed immunohistochemical staining with NeuN and showed that at 3 dpi, the lesion area was defined by the absence of NeuN labelling identical to the one observed with cresyl violet (Figure 4-2F). This validated NeuN as a “lesion marker” for immunohistochemical analysis.




B
cresyl violet
C
cresyl violet
D
cresyl violet
E
cresyl violet
F
NeuN

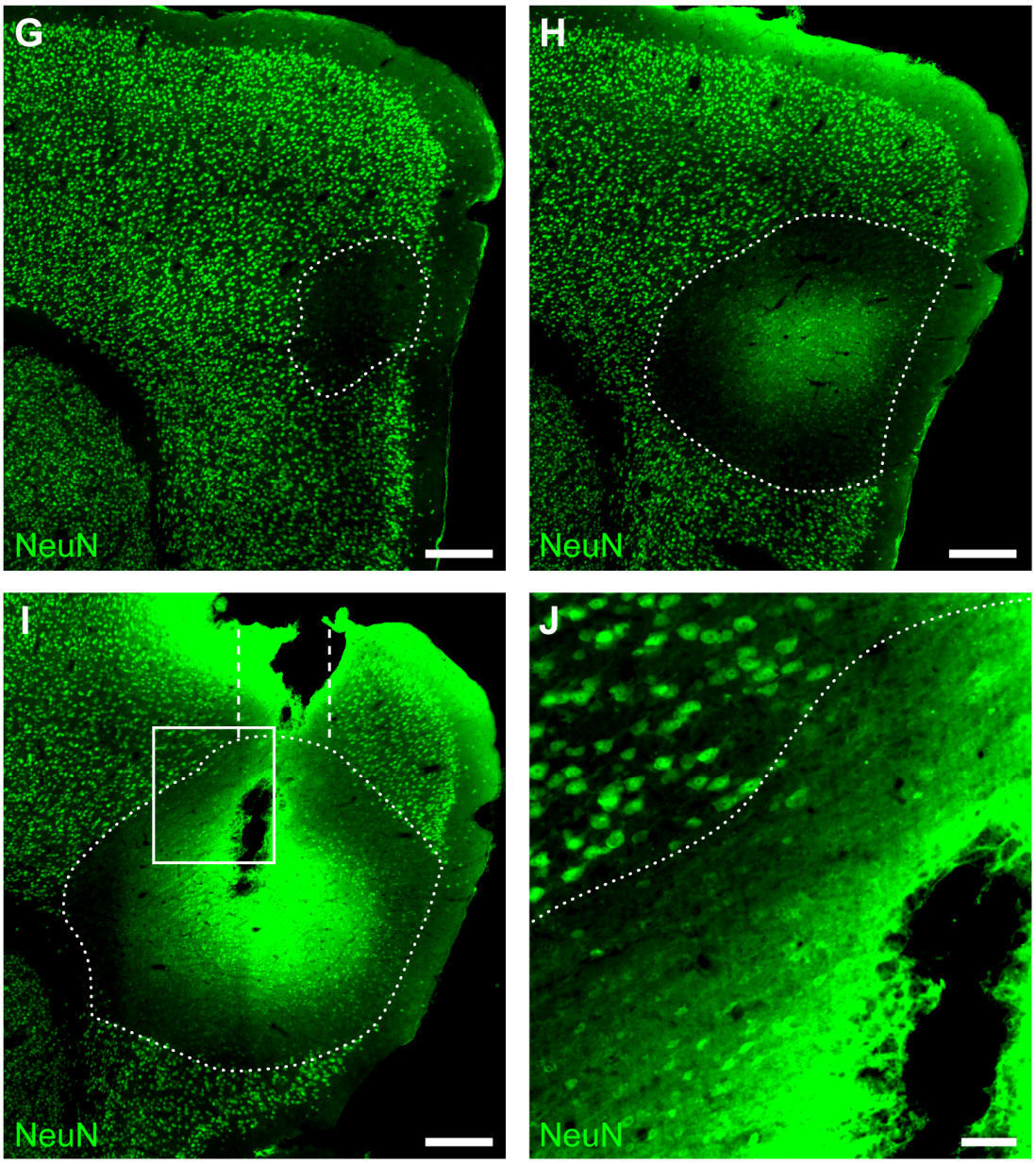


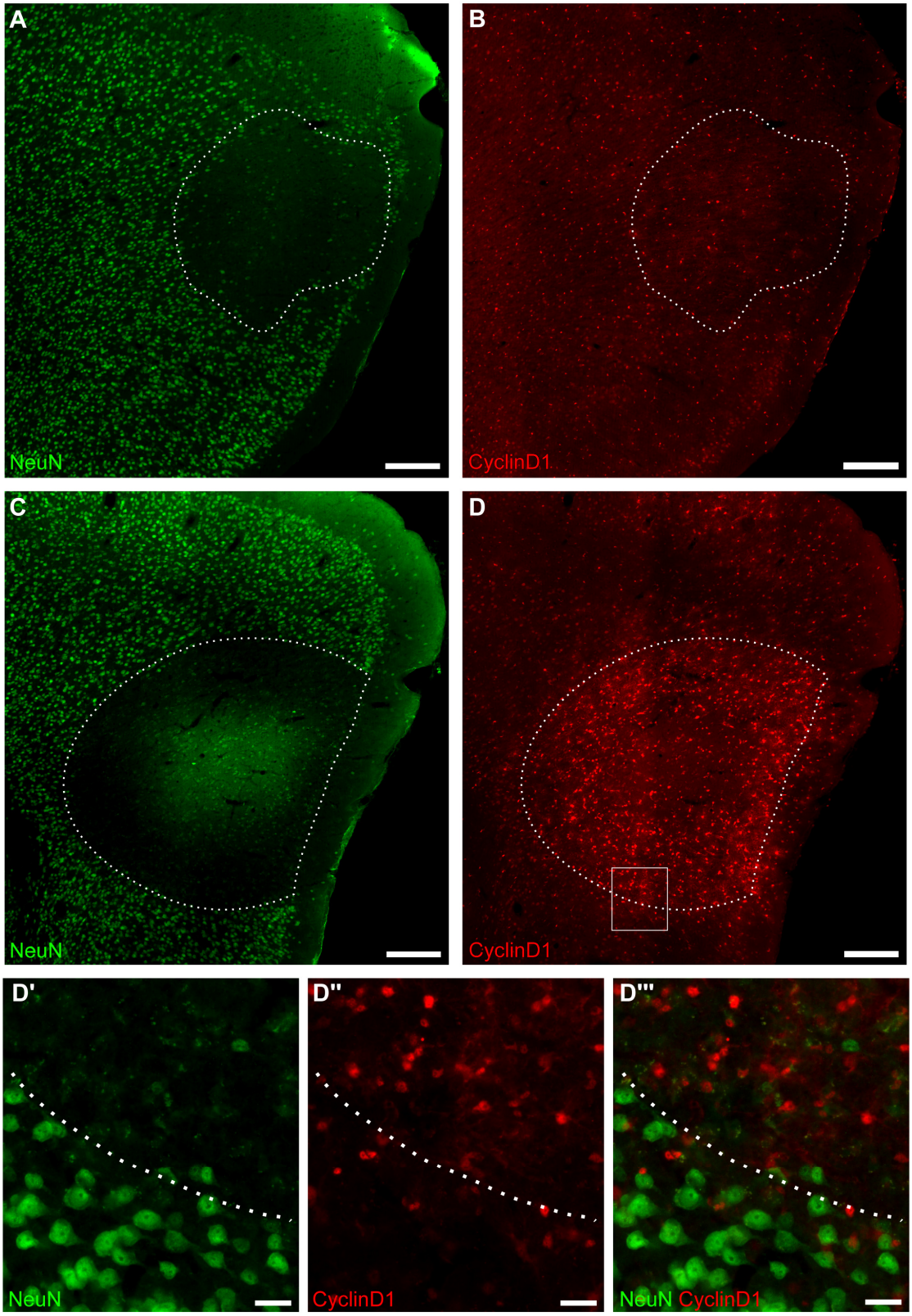

**Figure 4-2: Characterization of the lesion induced by the injection of ibotenic acid**
**A, B, C, D, E** Ibotenate-induced lesions in the adult wild-type mouse visualized with cresyl violet staining on sagittal sections 3 days post injection. The injury results in a large loss of neurons in the cortex at the injection site. **A** Whole sagittal section of the brain at the central level of the lesion. **B, C, D** Magnifications of the lesion site on serial sections (from lateral to medial). The lesion is circumscribed by a dotted line, while the needle tract is denoted by dashed lines. **E** Enlarged view of the boxed region in panel D identifies the border of the lesion. An intact neuron is indicated with an arrow outside of the lesion, while two of the small, dark stained microglial cells which are present in and around the lesion, are pointed out with arrowheads. The necrotic centre where tissue elements are destroyed by compression or even loss is circumscribed. **F, G, H, I, J** Visualization of the lesions with anti-NeuN staining **F** Whole sagittal section of the brain at the central lateral level of the lesion. **G, H, I** Magnifications of the lesion site at successive levels from lateral to medial of the hemisphere. The lesion is circumscribed by a dotted line, while the needle tract is denoted by dashed lines. **I** corresponds to the boxed region in (F). **J** Enlarged view of the boxed region in panel I. The dotted line demarcates the border of the lesion with NeuN+ neurons above the line and the NeuN- neurodegenerative area below the line. Residual staining within the neurodegenerative area is due to non-specific binding of secondary antibodies. Scale bars 1 mm (A, F); 250 µm (B, C, D, G, H, I), 50 µm (E, J).

## 4.3 Expression of Cyclin D1 following injury

Next, I examined the expression of Cyclin D1 following injury to the prefrontal cortex. To determine expression of Cyclin D1 early after injury I examined the injury one day postinjury (1 dpi). Then I analyzed the lesion at 3 dpi allowing the full development of the neurotoxic effect of the ibotenic acid. For the third time point, I chose to wait for 30 days, in order for Cyclin D1 expression and inflammation reaction to reach normal levels.

Analysis of the injury site at 1 dpi showed clear neurodegeneration as observed at 3 dpi (Fig 4-3A). As for Cyclin D1 expression one could not observe any increase (Fig. 4-3B). At 3 dpi the area depleted of neurons was slightly enlarged as compared to the lesion observed at 1 dpi (Fig. 4-3C). The level of Cyclin D1 expression was markedly increased inside of the neurodegenerative area. Nevertheless, there was a focal decrease of Cyclin D1 expression within the very center of the neurodegenerative lesion. This effect was due to the necrosis and the mechanical damage to the tissue by the insertion of the needle. At high magnification, one can clearly observe an increase in the number of cells expressing Cyclin D1 within the lesion, while outside of the neurodegenerative area, this number is notably low (Fig 4-3D'-D'''). At 30 dpi the neurodegenerative area was still clearly recognisable (Fig 4-3E) while the Cyclin D1 level dropped back to original level.

In addition, I performed control experiments with sham-operated mice that were injected with physiological saline to mimic the mechanical injury caused by the needle and the intracortical injection of fluid. Within the neurodegenerative area caused by the sham-operation I could observe a slight increase in cells expressing Cyclin D1. This non-significant upregulation of Cyclin D1 could be attributed to the mechanical damage by the needle injury alone.


A
NeuN
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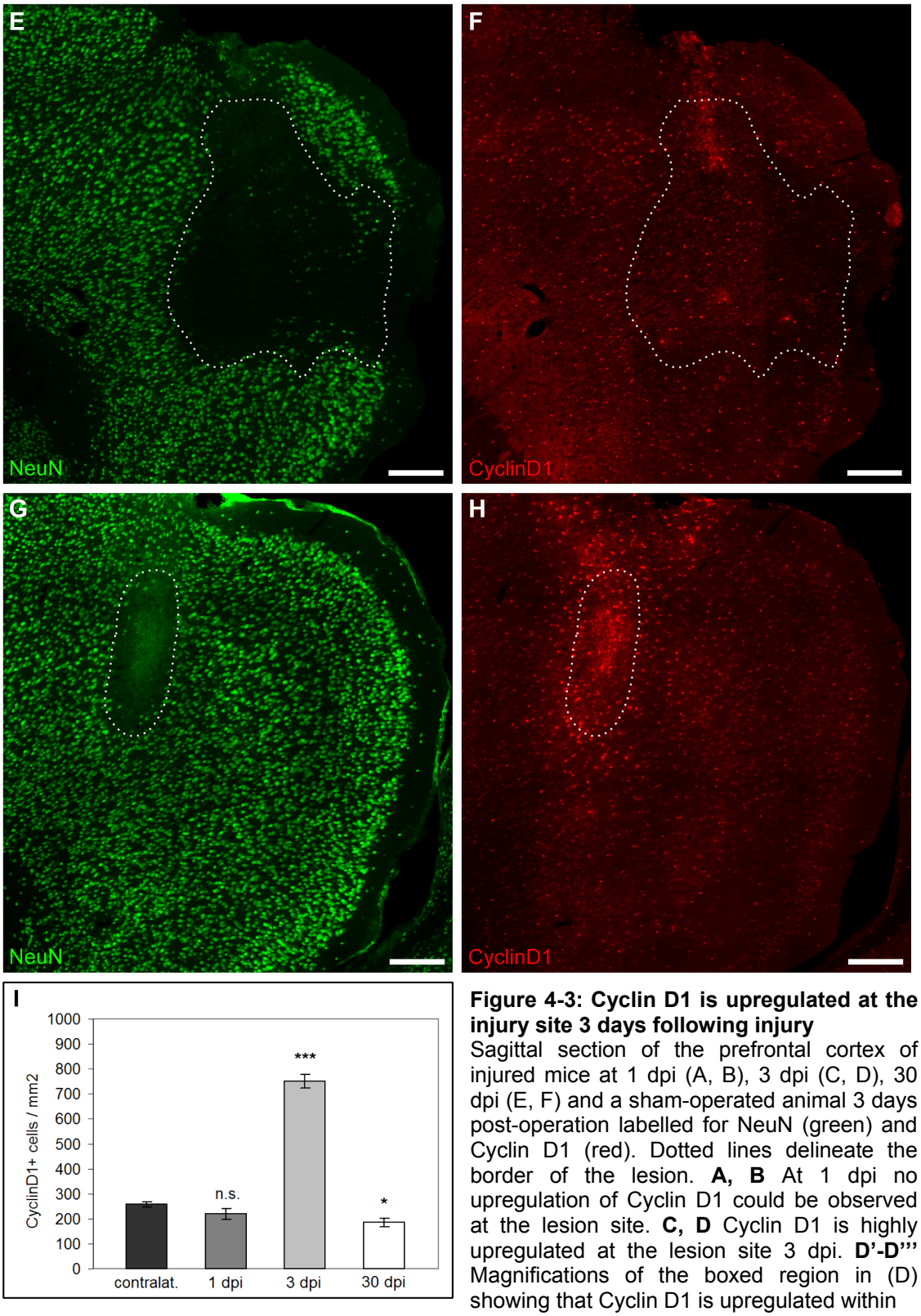



**Figure 4-3: Cyclin D1 is upregulated at the injury site 3 days following injury**
Sagittal section of the prefrontal cortex of injured mice at 1 dpi (A, B), 3 dpi (C, D), 30 dpi (E, F) and a sham-operated animal 3 days post-operation labelled for NeuN (green) and Cyclin D1 (red). Dotted lines delineate the border of the lesion. **A, B** At 1 dpi no upregulation of Cyclin D1 could be observed at the lesion site. **C, D** Cyclin D1 is highly upregulated at the lesion site 3 dpi. **D'-D'''** Magnifications of the boxed region in (D) showing that Cyclin D1 is upregulated within the lesion site. The border of the injury is well demarcated such that one can clearly see that the number of Cyclin D1 expressing cells increases within the lesion. **E, F** At 30 dpi Cyclin D1 expression has decreased. **G, H** Sham-operated animal showing a slight upregulation of Cyclin D1 3 dpi within the needle track. **I** Histogram representing the absolute number of cells expressing Cyclin D1 per analyzed field within the contralateral prefrontal cortex and within the lesion site 1 dpi, 3 dpi and 30 dpi. Results are expressed as means ± SEM (error bars).

Quantitative analysis of immunohistochemical data revealed a three-fold increase in the number of Cyclin D1 expressing cells in lesions 3 dpi, as compared to the uninjured, contralateral hemisphere (3 dpi: 750 cells/mm2 ± 27 cells/mm2; contralateral: 258 cells/mm2 ± 11 cells/mm2; p=1.4x10-7)(Fig. 4-3I). Lesions of 1 dpi show no significant change in numbers (1 dpi: 219 cells/mm2 ± 22 cells/mm2), whereas lesions 30 dpi show even a reduction of Cyclin D1 expressing cells (186 cells/mm2 ± 17 cells/mm2).

Taken together, Cyclin D1 expression was highly upregulated three days after injection of ibotenic acid and thus demonstrating this point in time to be most interesting for further investigations.

## 4.4 Proliferation following injury

Next, I determined the presence of fast proliferating cells following neurotoxic injury. In parallel to the analysis of Cyclin D1 proliferation was measured at 1, 3 and 30 dpi.

For *in vivo* labeling of proliferating cells, the synthetic nucleoside 5-bromo-2'-deoxyuridine (BrdU) was intraperitoneally injected 2 h prior to sacrifice. Within these two hours the BrdU incorporated in all cells that underwent S phase. As the cells are exposed only for a short period of time to the BrdU this ensured that only fast proliferating cells incorporate the nucleoside. I reasoned that I would detect fast proliferating cells at 1 and 3 dpi, while at 30 dpi the number of such cells reacting to the injury would have dropped to zero.

Qualitative analysis of BrdU immunohistochemistry at 1 dpi revealed only single cells positive for BrdU (Fig. 4-4A) whereas at 3 dpi a large number of proliferating cells could be detected within the neurodegenerative area and the surrounding tissue (Fig. 4-4B). As expected, at 30 dpi only very few proliferating

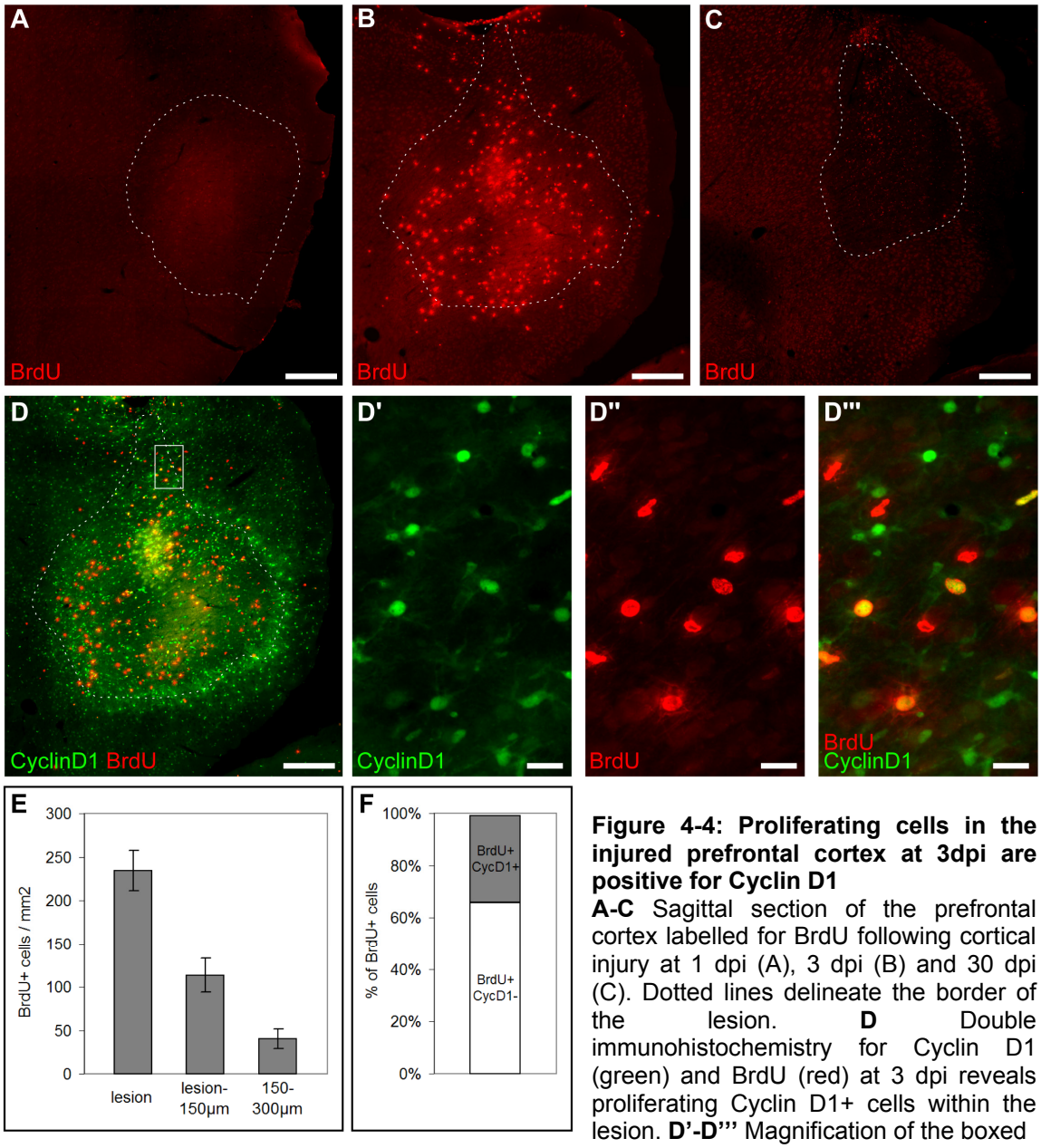



**Figure 4-4: Proliferating cells in the injured prefrontal cortex at 3dpi are positive for Cyclin D1**
**A-C** Sagittal section of the prefrontal cortex labelled for BrdU following cortical injury at 1 dpi (A), 3 dpi (B) and 30 dpi (C). Dotted lines delineate the border of the lesion. **D** Double immunohistochemistry for Cyclin D1 (green) and BrdU (red) at 3 dpi reveals proliferating Cyclin D1+ cells within the lesion. **D'-D'''** Magnification of the boxed region in (D) showing coexpression of BrdU (red) and Cyclin D1 (green). A-D scale bars 250 µm; D'-D''' scale bars 20 µm. **E** Histogram representing the absolute number of BrdU+ cells per area within the lesion and the surrounding tissue, including an area within 150 µm distance to the border of the lesion and a second area within 150 – 300 µm distance to the border of the lesion. Results are expressed as means ± SEM (error bars) **F** Histogram representing the fraction of Cyclin D1-expressing cells within the population of BrdU+ cells.

cells could be detected within the lesion (Fig. 4-4C). To ensure that the immunohistochemistry for BrdU worked out properly, the BrdU signal was controlled in the subventricular zone and in the rostral migratory stream. In both

regions endogenous proliferation typically takes place and thus BrdU+ cells can always be detected there.

Quantification of BrdU+ cells at 3 dpi revealed a high number of fast proliferating cells within the lesion (234 cells/mm$^2$ ± 23 cells/mm$^2$). Outside of the lesion, the number rapidly declined with increasing distance to the injury border (within 150 μm distance to the injury border: 154 cells/mm$^2$ ± 16 cells/mm$^2$; within 150 – 300 μm distance to the injury border: 114 cells/mm$^2$ ± 20 cells/mm$^2$) (Fig. 4-4E).

Given that the number of Cyclin D1-positive cells is strongly increased within the lesion at 3 dpi I examined whether they belong to the observed fast proliferating cell population. Indeed, double-immunohistochemistry of BrdU with Cyclin D1 revealed that approximately a third of the fast-proliferating cells within the lesion area are positive for Cyclin D1 (31%) (Fig 4-4D, D'-D'''). Reciprocally, a 10% of the Cyclin D1+ cells were also positive for BrdU.

## 4.5 Cyclin D1 is not expressed in apoptotic cells following cortical injury

I investigated on the potential contribution of Cyclin D1 to apoptosis following cortical injury at 3 dpi. TUNEL labelling of injured cortex at this time point revealed markedly increased numbers of intense TUNEL-labelled cells within the lesion (Fig. 4-6A). TUNEL-positive cells showed round and shrunken morphology with a condensed nucleus typical of apoptosis (Fig. 4-5A'). Double-labelling experiments combining TUNEL and Cyclin D1 immunohistochemistry revealed no Cyclin D1 expression within apoptotic cells (Fig. 4-5B, B'). Thus, at 3 dpi Cyclin D1 is not involved in apoptosis within the neurotoxic lesion.

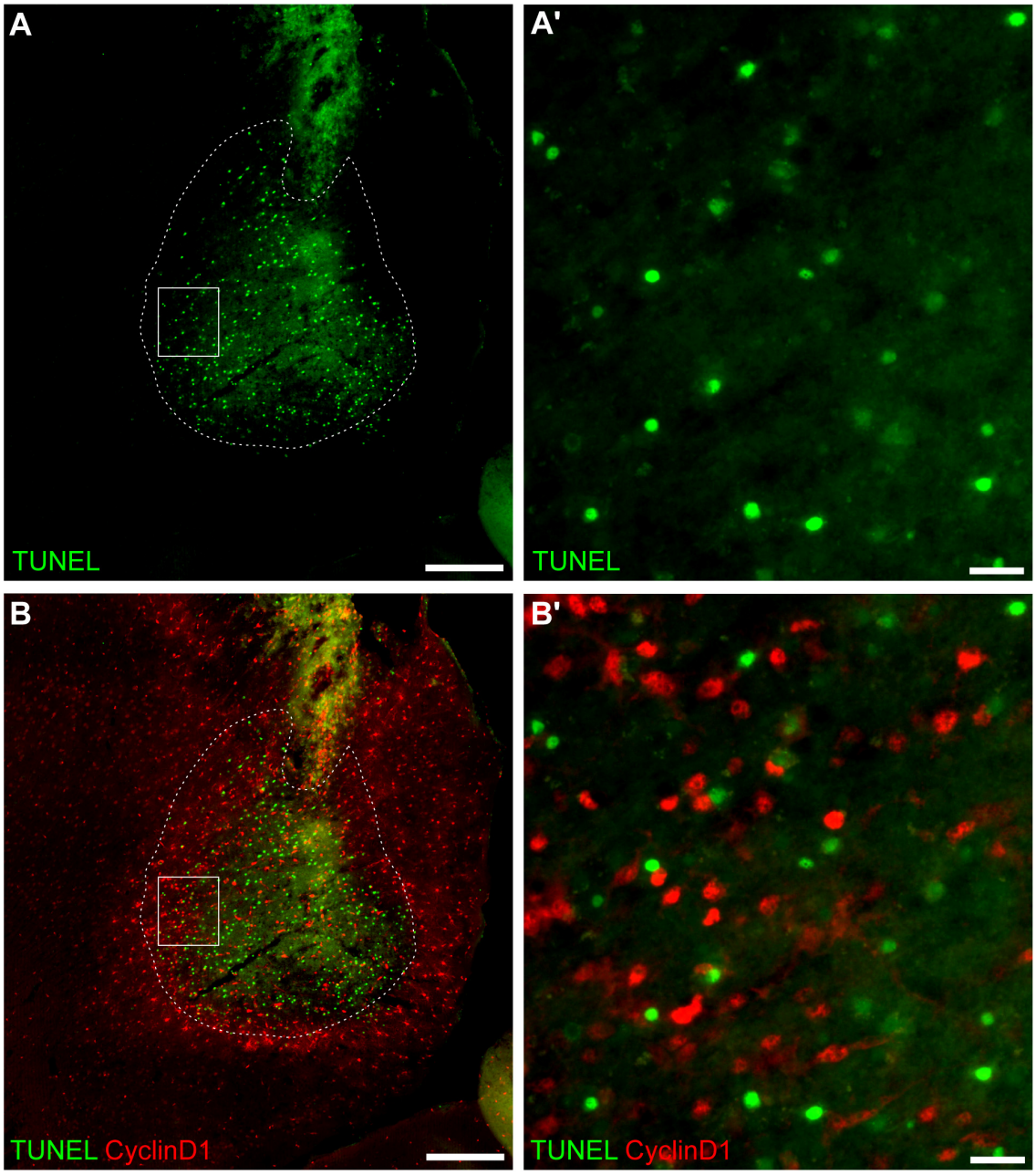



**Figure 4-5: Apoptosis following injury to the prefrontal cortex**
**A** Apoptotic cells positive for TUNEL staining are detected 3 days following cortical injury. **A'** Eight-fold magnifications of the boxed area in (A) showing TUNEL positive cells with rounded and shrunken morphology typical for apoptotic cells. **B** Double-immunohistochemistry for TUNEL (green) and Cyclin D1 (red) reveals no double staining within the lesion. **B'** Magnifications reveals that none of the Cyclin D1 cells are apoptotic. Dotted lines delineate the border of the lesion. Scale bars A, B 250 µm, A', B' 20 µm.

## 4.6 Reaction of glial cell types following cortical injury

Injury to the brain induces cell cycle activation in glial cells, which can result either in the apoptosis of post-mitotic glial cells, or in the proliferation and activation of glial cells such as astroglia and microglia. Thus I analysed and quantified the population of microglia, astrocytes and oligodendroglial cells within the intact cortex and following neurotoxic injury.

### *4.6.1 Microglia*

As microglia are the first glial cell type to react to brain injury, I examined the microglia marker Iba1 3 days following cortical injury. I performed immunostainings with anti-Iba1. In the intact prefrontal cortex of adult mice I could observe a regular distribution of ramified microglia (Fig 4-6A). These resting microglia are composed of a small cellular body and long branching processes (Fig 4-6A'). Analysis of the cortical injury at 3 dpi revealed a significant and sustained increase of microglia within the lesion (Fig 4-6B), which show a hypertrophic phenotype (Fig. 4-6B'). Only microglial cells in the immediate vicinity of the lesion were activated, whereas cells farther away did not respond (Fig 4-6B). Quantification of microglial cells showed almost a 3-fold increase in number of activated microglia within the lesion at 3 dpi, as compared to the number of ramified microglia in the intact cortex (intact cortex: 268 cells/mm$^2$ ± 1 cells/mm$^2$; lesioned cortex: 757 cells/mm$^2$ ± 91 cells/mm$^2$; p=0.0016) (Fig. 4-6G).

### *4.6.2 Mature Astrocytes*

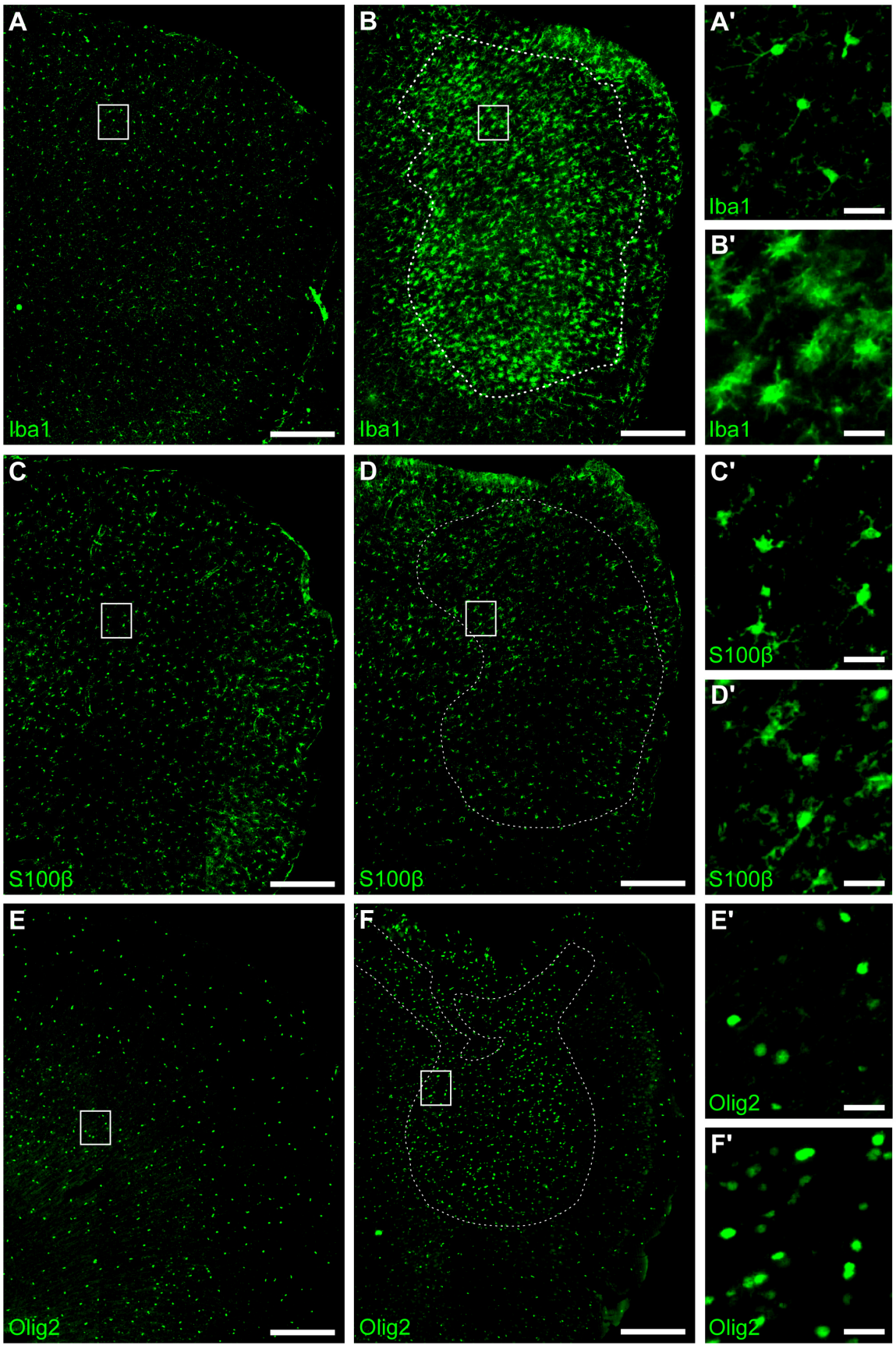
Staining for astrocytes using anti-S100β antibody in the intact cortex revealed an even distribution pattern, similar to the one observed for microglia (Fig. 4-6A).

On the morphological level S100β+ astrocytes showed a strongly stained cell body and star shaped processes (Fig. 4-6A'). Cortical injury did not lead to a increase in cellular density of S100β+ astrocytes but rather to a reduction of cell number which was statistically non-significant (intact cortex: 352 cells/mm$^2$ ± 67 cells/mm$^2$; lesioned cortex: 292 cells/mm$^2$ ± 9 cells/mm$^2$; p=0.47) (Fig. 4-6H). In addition, the astrocytes within the lesion showed a decrease in S100β-signal intensity.

### *4.6.3 Oligodendroglial cells*

In the intact cortex of adult mice an important number of cells expressed Olig2 (Fig. 4-6E). Compared to the other glial cell types, Olig2+ cells are clearly more abundant in the intact cortex. At 3 dpi, I observed almost a two-fold increase in the number of Olig2-immunoreactive cells within the neurodegenerative area as compared to the intact contralateral cortex (Fig. 4-6F). (intact cortex: 732 cells/mm$^2$ ± 59 cells/mm$^2$; lesioned cortex: 1381 cells/mm$^2$ ± 68 cells/mm$^2$; p=0.00001) (Fig. 4-6I). This increase of Olig2 is in line with observations after stab wound injuries (Buffo et al., 2005). In addition to the increase in density, Olig2-cells were spread out across the injury border, up to 400 μm from the lesion (Fig. 4-6F).

Taken together, the results of the three glial cell types within the lesion at 3 dpi could be summarized as follows: mature astrocytes did not change in number while microglia and oligodendroglial cells displayed an important increase.


A
Iba1
B
Iba1
A'
Iba1
B'
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C
S100β
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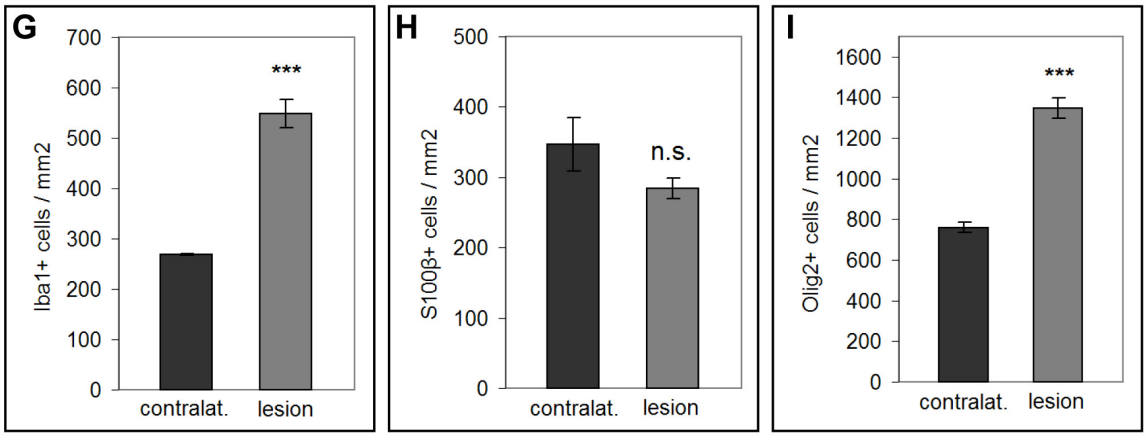


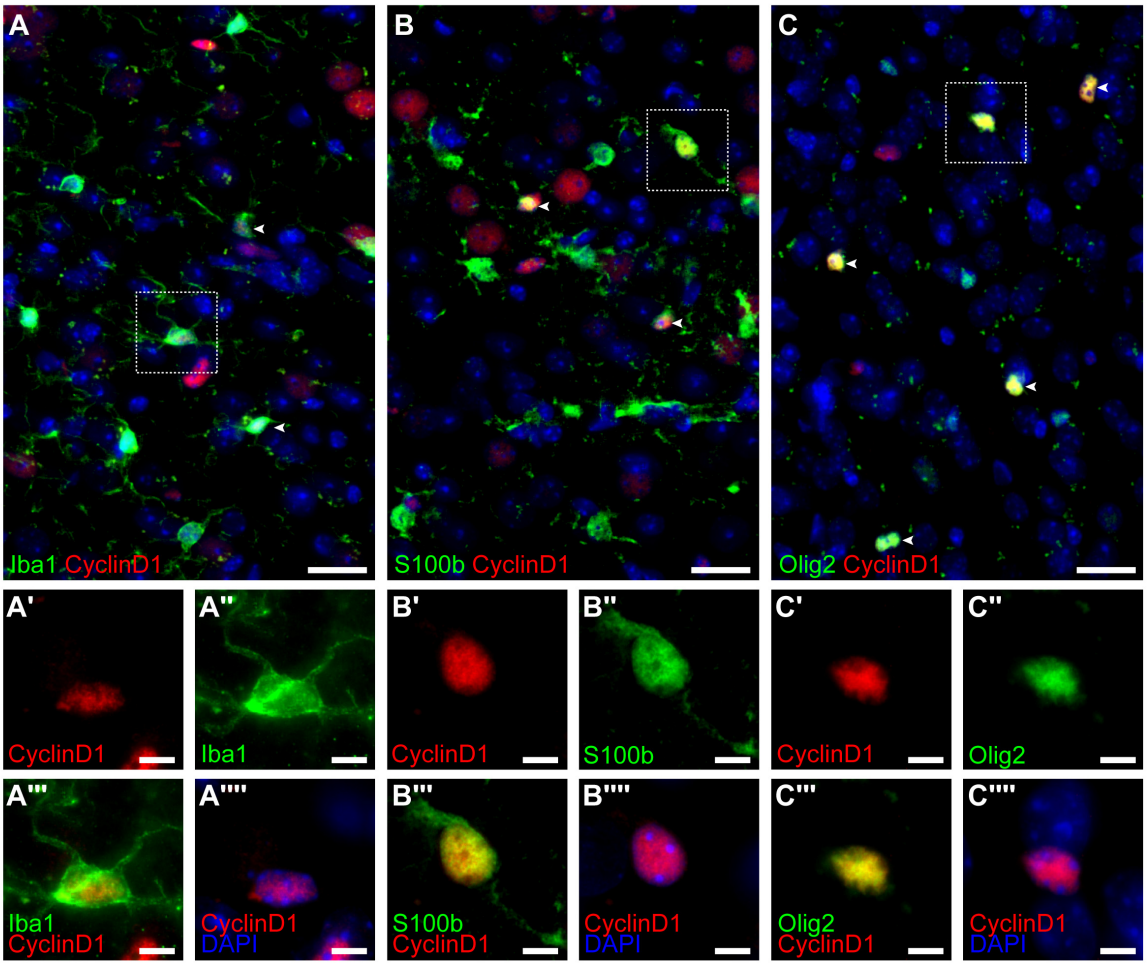

**Figure 4-6: Microglia, astrocytes and oligodendroglial cells in intact prefrontal cortex and following cortical injury at 3 dpi**
Sagittal sections of intact and injured cortex labelled for microglia with Iba1 (A, B), for mature astrocytes S100β (C, D) and for oligodendroglial cells with Olig2 (E, F). Dotted lines delineate the border of the lesion. Cells in the boxed areas are represented with six-fold magnification (A', B', C', D', E', F'). **A, B** Compared to the intact cortex (A) one can clearly recognize the inflammatory response of microglia within the at the lesion (B). While the in the microglia in the intact cortex are of ramified phenotype with thin protrusions (A') the ones within the neurodegenerative area show activated and hypertrophic character. **C, D** There is no obvious difference, when examining mature astrocytes within the intact (C) and injured (D) cortex. In both condition the astrocytes show stellate morphology (C' D'). E, F Cells of the oligodendroglial lineage show a clear increase in number within and around the lesion site (F) as compared to the intact cortex (E). The increase can be clearly seen at higher magnification (E', F'). **G, H, I** Histograms comparing the absolute number of microglia (G), S100β cells (H) and oligodendroglial cells (I) within the intact cortex and the within the neurodegenerative are of the injured cortex. There is a clear increase in microglia and oligodendroglial cells following injury to the cortex. Results are expressed as means ± SEM (error bars) and were analyzed by a *t* test. Scale bars 250 µm (A, B, C, D, E, F), 25 µm (A', B', C', D', E', F').

## 4.7 Quantitative analysis of glial cell types in the adult intact cortex

Next, I determined the presence of Cyclin D1 in glial cell types. Therefore, I performed double immunohistochemistry for Cyclin D1 with the three glial markers mentioned above.

In the intact cortex, Iba1-stained microglia showed weak nuclear Cyclin D1 expression (Fig. 4-7A), while S100β+ astrocytes (Fig. 4-7B) and Olig2+ oligodendroglial cells displayed clear expression of nuclear Cyclin D1. I further assessed the proportions of the glial cell types within the Cyclin D1-cell

population. Microglia composed about one fourth (Iba1+: 24% ± 1%) and astrocytes one third (S100β+: 33% ± 2%) of the Cyclin D1 population.



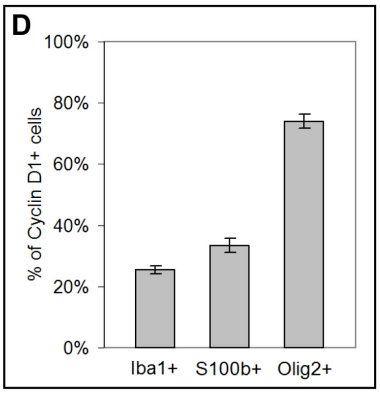




**Figure 4-7: Expression of glial markers within the Cyclin D1 cell population in the intact cortex**
Co-immunohistochemistry for Iba1, S100 β, Olig2 (all green) and Cyclin D1 (red), while nuclei are stained with DAPI (blue). **A** Partial population of Iba1-positive microglia (green) show coexpression of Cyclin D1. Magnification (A'- A'''') reveals that Cyclin D1 is expressed within the nucleus, stained for DAPI. **B** Some of the astrocytes labelled with S100β show coexpression of Cyclin D1. Magnification (B'-B'''') reveals that Cyclin D1 is expressed within the nucleus. **C** Olig2+ oligodendroglial cells show coexpression of Cyclin D1. Magnification (C'-C'''') reveals that Cyclin D1 is coexpressed within Olig2 in the nucleus. Scale bars A, B, C 20 µm; magnifications 5 µm. **D** Histogram representing the relative expression glial cell type markers within the Cyclin D1 population in the contralateral cortex showing that Olig2 is mainly coexpressed within the Cyclin D1 cell population. Results are expressed as means ± SEM (error bars).

In contrast, the oligodendroglial cells made up the largest proportion (74% ± 2%) (Fig. 4-7D). Thus in the intact brain most of the Cyclin D1-cells belong to the oligodendroglial lineage.

## 4.8 Quantitative analysis of glial cell types following cortical injury in the adult

To figure out whether cortical injury modulates Cyclin D1 expression within glial cells I performed double immunohistochemistry on injured brain section analogous to the one in intact cortical tissue and analyzed the cell population within the neurodegenerative area.

Three days following injury the nuclear expression of Cyclin D1 was markedly increased within activated, hypertrophic microglia (Fig. 4-8A, C’’’’). Oligodendroglial cells too showed intense expression of Cyclin D1 within the nuclei (Fig. 4-8C, C’’’’). In contrast, activated astrocytes showed not only increased nuclear Cyclin D1 expression but also weak cytoplasmic expression, observable in the processes (Fig. 4-8B, B’, B’’’’). As in the intact cortex, oligodendroglial cells composed the largest proportion of Cyclin D1-cells (Iba1+: 16% ± 1%; S100β+: 18% ± 3%; Olig2+: 73% ± 3%) (Fig. 4-8D).

## 4.9 Reactive astrocytes surrounding the lesion express Cyclin D1

To highlight reactive astrocytes in the injured cortex at 3 dpi I labelled sections for GFAP. The antibody used did not highlight astroglia in the intact cortex but only reactive astrocytes. I could show that at 3 dpi most of the reactive astrocytes surround the lesion site (Fig. 4-9A), with a few cells located within the neurode-

generative area (Fig. 4-9A). Reactive astrocytes could be detected up to 600 µm distance from the border of the lesion.

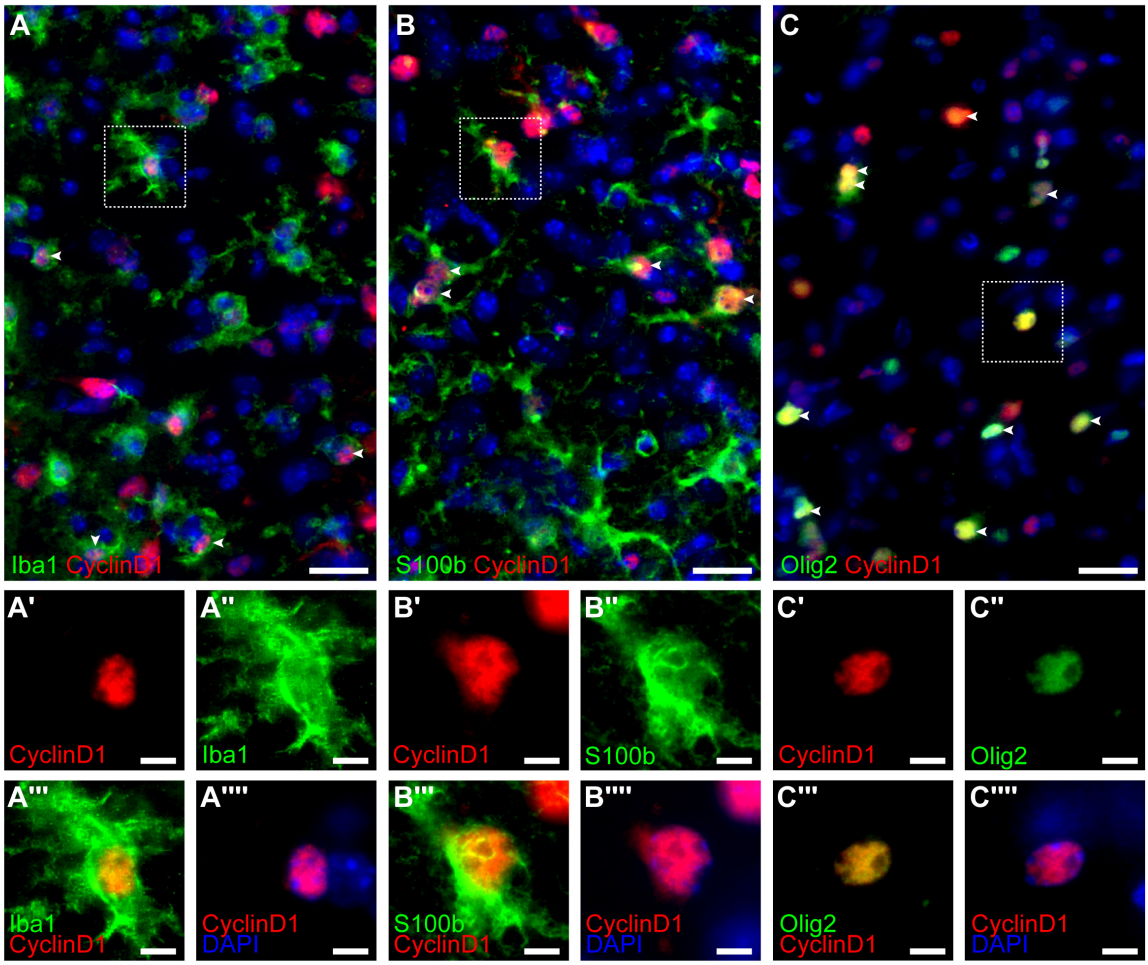



**Figure 4-8: Identification of Cyclin D1-immuno-reactive cells after cortical injury.**
Co-immunohistochemistry for Iba1, S100 β, Olig2 (all green) and Cyclin D1 (red), while nuclei are stained with DAPI (blue). **A** Activated Iba1-positive microglia with hypertrophic morphology show in part coexpression of Cyclin D1. Magnification (A'-A'''') shows that Cyclin D1 is prominently expressed within the nucleus. **B** Activated astrocytes labelled with S100β show coexpression of Cyclin D1. Cyclin D1 is strongly expressed within the nucleus (B'-B''''). **C** Oligodendroglial cells positive for Olig2 show coexpression of Cyclin D1. Magnification (C'-C'''') reveals that Cyclin D1 is coexpressed within Olig2 in the nucleus. Scale bars A, B, C 20 µm; magnifications 5 µm. **D** Histogram representing the relative expression of glial cell type markers within the Cyclin D1 population within the cortical lesion at 3 dpi showing that Olig2 is the most expressed marker in Cyclin D1 cells following injury. Results are expressed as means ± SEM (error bars).

Preliminary quantification of GFAP+ reactive astrocytes within 300 µm distance to the border of the lesion revealed that they were found in a comparable density to the one of mature astrocytes (461 cells/mm$^2$ ± 24 cells/mm$^2$; n=1). In addition, I could show that reactive astrocytes coexpressed Cyclin D1 (Fig. 4-9B, B'-B''''). The relative fraction of GFAP+ reactive astrocytes expressing Cyclin D1 was comparable to the one of S100β+ mature astrocytes of the intact prefrontal cortex (GFAP+: 19%; S100β+: 23%).

Thus, following neurotoxic insult to the prefrontal cortex astrocytes form a glial scar with a fraction expressing Cyclin D1.

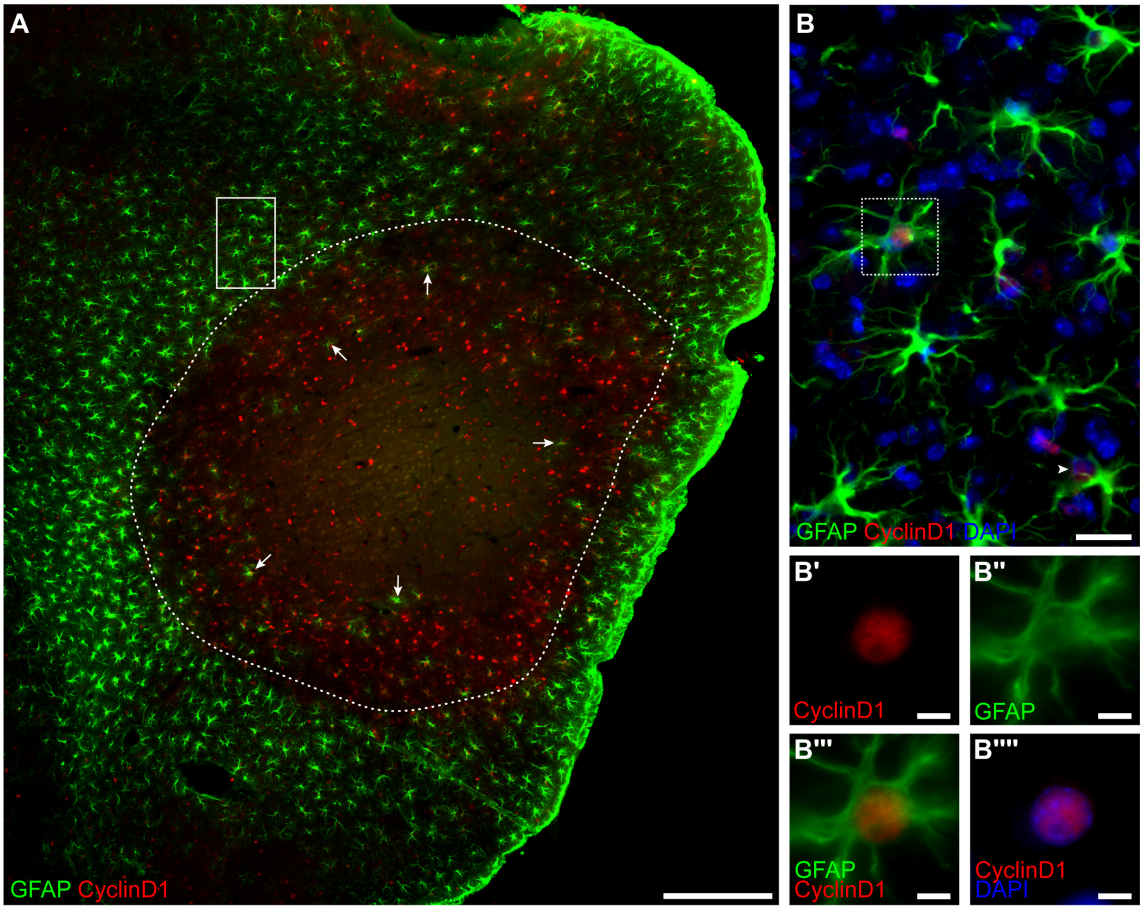



**Figure 4-9: Reactive astrocytes surround the neurodegenerative area at 3pi and show partial expression of Cyclin D1 at 3dpi**
**A** Sagittal section of injured cortex labelled for reactive astrocytes with GFAP. Dotted line delineates the border of the lesion. Very few GFAP+ astrocytes could be detected within the neurodegenerative area (arrows). **B** Magnification of the boxed region in (A) showing reactive astrocytes expressing Cyclin D1 (arrowheads). Cyclin D1 is weakly expressed within the nucleus (B'-B'''').Scale bars 250 µm (A), 25 µm (B), 5 µm (B'-B'''').

## 4.10 Absence of Cyclin D1 reduces the lesion area following cortical injury

To determine whether knockout of cyclin D1 gene can decrease the area of the lesion induced by neurotoxic insult to the brain, NeuN staining was done at 3 dpi and the lesion compared between wild-type and *Cyclin D1*$^{-/-}$ mice. As I was only interested in the area affected by the action of the ibotenic acid but not by the mechanical damage, I measured the size of the neuron-depleted area and subtracted the zone showing necrosis and / or mechanical damage due to needle

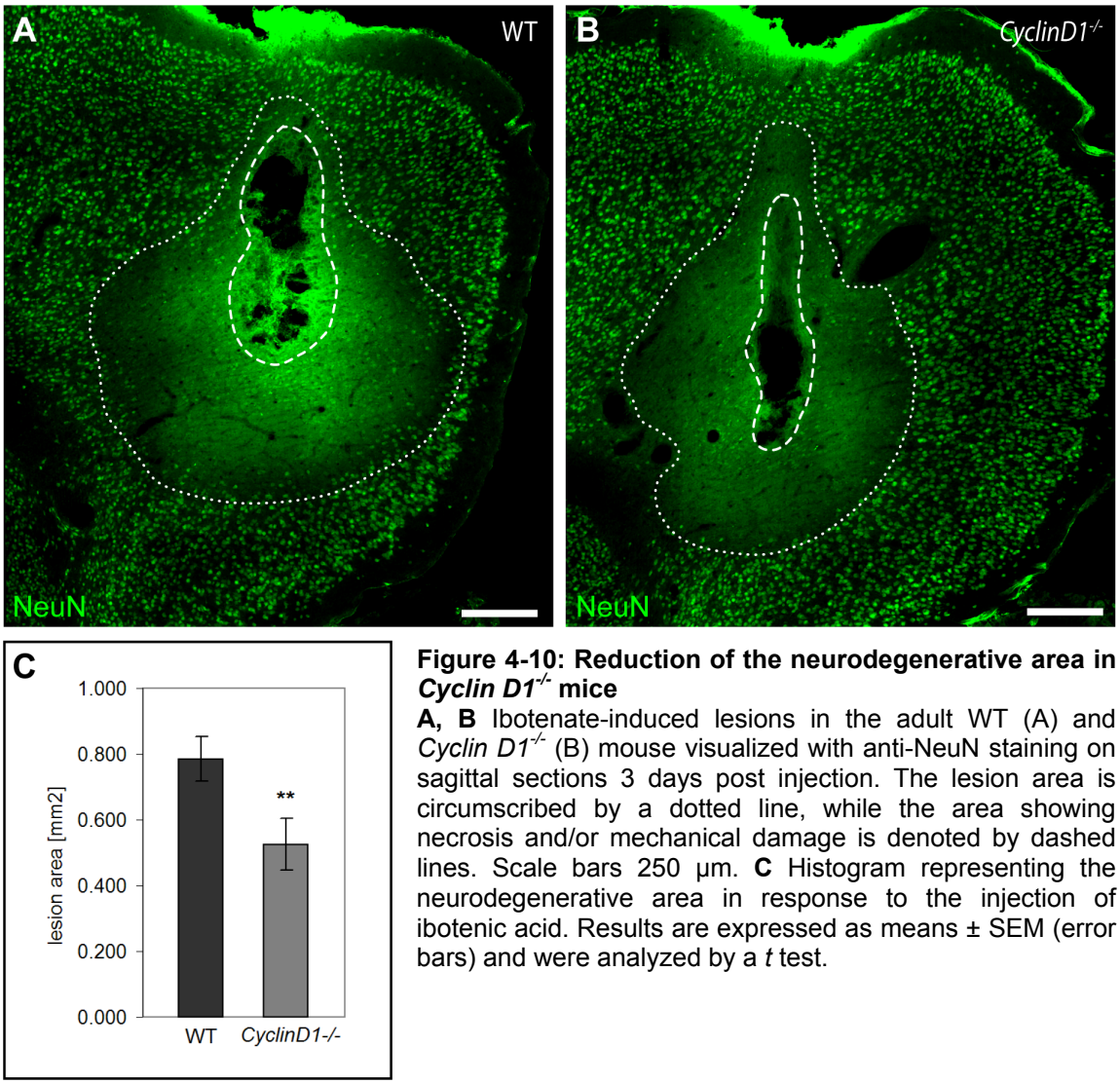



**Figure 4-10: Reduction of the neurodegenerative area in *Cyclin D1*$^{-/-}$ mice**
**A, B** Ibotenate-induced lesions in the adult WT (A) and *Cyclin D1*$^{-/-}$ (B) mouse visualized with anti-NeuN staining on sagittal sections 3 days post injection. The lesion area is circumscribed by a dotted line, while the area showing necrosis and/or mechanical damage is denoted by dashed lines. Scale bars 250 µm. **C** Histogram representing the neurodegenerative area in response to the injection of ibotenic acid. Results are expressed as means ± SEM (error bars) and were analyzed by a *t* test.

insertion (Fig. 4-10A, B). The mean size of this neurodegenerative area revealed tracted the zone showing necrosis and / or mechanical damage due to needle insertion (Fig. 4-10A, B). The mean size of this neurodegenerative area revealed to be reduced by 33% in *Cyclin D1*$^{-/-}$ animals (WT 0.785 mm2 ± 0.205 mm2 of neurodegenerative area, n = 9; *Cyclin D1*$^{-/-}$ 0.525 mm2 ± 0.068 mm2, n = 9; P = 0.007) (Fig. 4-10C).

## 4.11 Deficiency for Cyclin D1 does not affect reactive astrocytes

To assess whether the absence of Cyclin D1 has an impact on glial scar formation 3 days following neurotoxic injury I compared the glial scar of WT and *Cyclin D1*$^{-/-}$ mice. I could not observe any obvious difference (Fig. 4-11A, A', B, B'). To certify this qualitative result I determined the density of reactive astrocytes surrounding the lesion. I first had to define the area of interest. As shown before, reactive astrocytes were located within 300 – 600 µm distance to the border of the lesion. Thus I decided to consider GFAP+ cells within 300 µm distance to the border of the neurodegenerative area and to discard cells within the needle tract. I could not detect any difference in the density of reactive astrocytes between wild-type and knockout animals (Cyclin D1$^{-/-}$: 470 cells / mm$^2$ ± 32 cells/mm$^2$; WT 483 cells/mm$^2$ ± 14 cells/mm$^2$) (Fig. 4-11C).

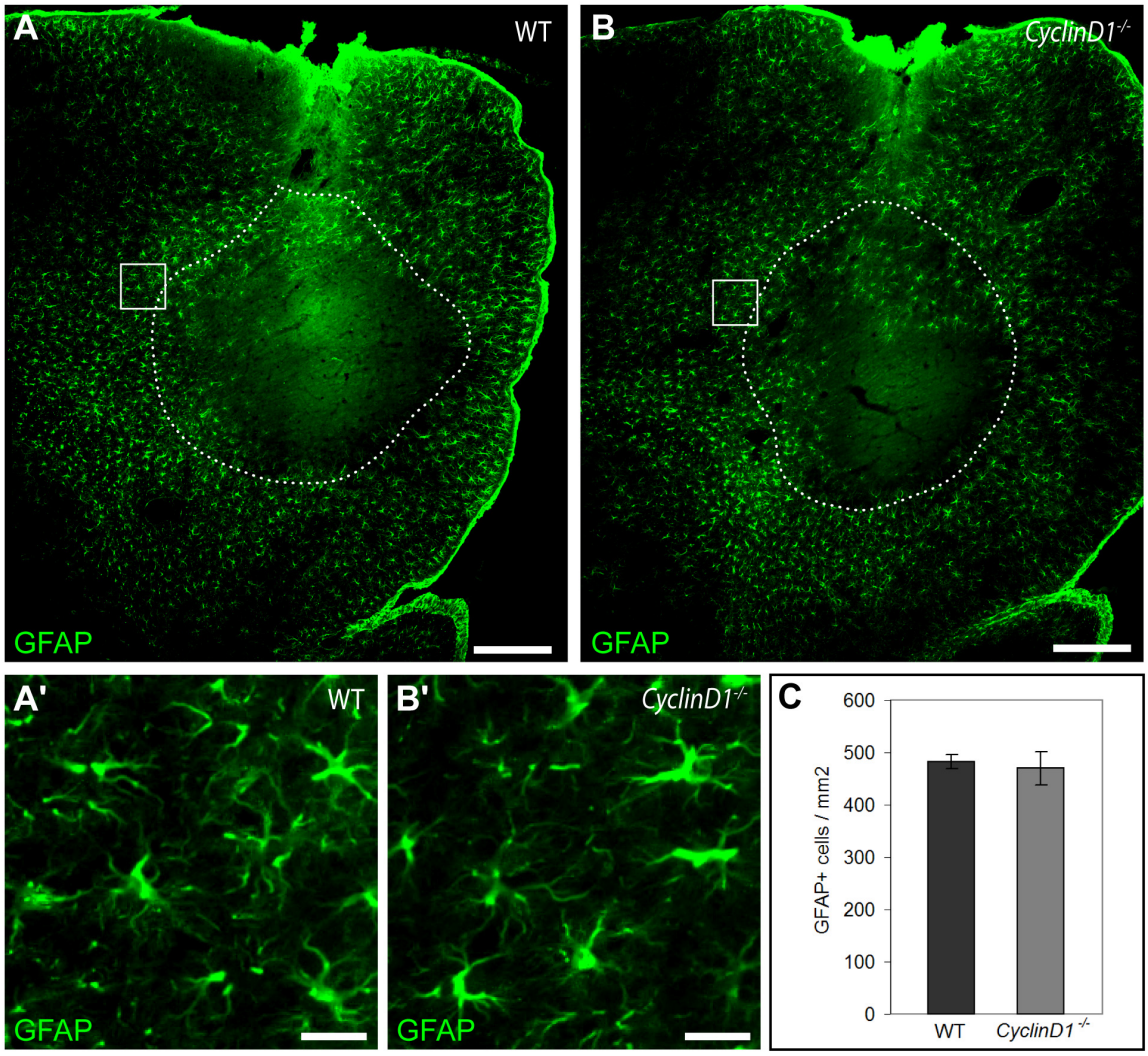



**Figure 4-11: Equal number of reactive astrocytes within the glial scar of WT and *Cyclin D1*$^{-/-}$ mice 3 days following cortical injury**

**A, B** Reactive astrocytes labelled for GFAP following cortical injury at 3pi surrounding the lesion in adult WT (A) and *Cyclin D1*$^{-/-}$ (B) mice. **A', B'** Magnification of the boxed region in (A) and (B) showing GFAP+ cells within the glial scar. **A, B** scale bars 250 µm; **A', B'** scale bars 25 µm. **C** Histogram representing the number of GFAP+ cells per analyzed area within 300µm distance to the border of the lesion. Results are expressed as means ± SEM (error bars).

## 4.12 Loss of Cyclin D1 impairs injury-induced proliferation in the adult brain

As shown in WT animals, Cyclin D1 is expressed in an important proportion of fast-proliferating cells within the cortical lesion. Thus I investigated the function of Cyclin D1 in cell proliferation by comparing proliferation in adult WT and

*Cyclin D1*$^{-/-}$ mice in response to the neurotoxic injury. Therefore, I first quantified fast-proliferating cells (BrdU+) within the lesion area. In a second step, I assessed the impact on general proliferation by quantifying cells positive for the cell cycle marker Ki-67.

Qualitative analysis of BrdU (Fig 4-12A, B) and Ki-67 (Fig 4-12D, E) three days following injury shows a reduced number of proliferating cells within the lesion of *Cyclin D1*$^{-/-}$ mice, as compared to WT mice. This observation was confirmed by quantification; revealing a significant decrease in the total number of proliferating cells following injury of the prefrontal cortex. The number of

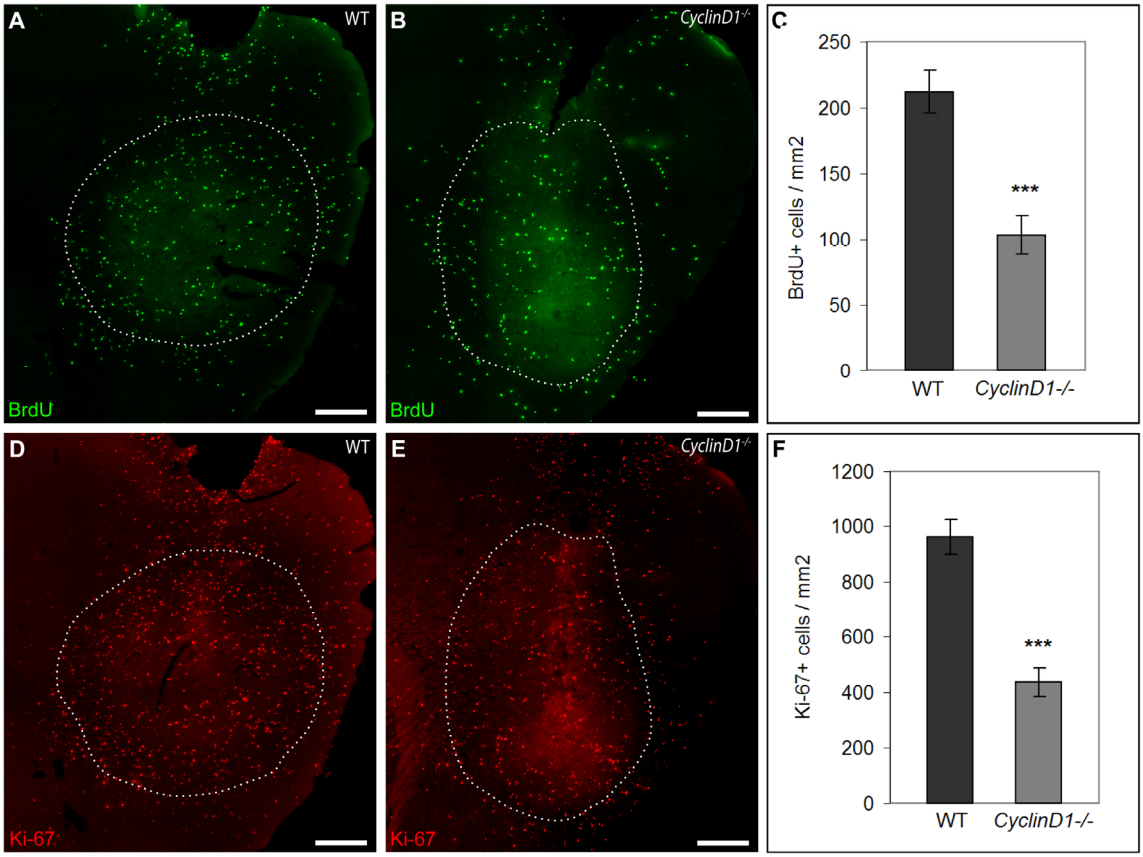



**Figure 4-12: Injury-induced proliferation is reduced in the adult brain of *Cyclin D1*$^{-/-}$ mice.** Immunohistochemistry was done for quantitative assessment of cell proliferation at 3 dpi. **A, B D, E** Proliferating cells were labelled with anti-BrdU or Ki-67 at the lesion site at 3 dpi of adult WT (A, D) and *Cyclin D1*$^{-/-}$ mice (B, E), which show a reduced number of proliferating cells. Dotted lines delineate the border of the lesion. Scale bars 250 µm. **C, F** Histograms representing the number of BrdU respectively Ki-67 labelled cells per analyzed area. Results are expressed as means ± SEM (error bars; ***, $P < 0.001$) and were analyzed by a *t* test.

that incorporated BrdU within the lesion of *Cyclin D1*$^{-/-}$ mutants, was decreased by 49% (Cyclin D1$^{-/-}$: 114 cells/mm$^2$ ± 9 cells/mm$^2$; WT 224 cells/mm$^2$ ± 11 cells/mm$^2$; p=0.005) while the number of cells positive for Ki-67 was decreased by 55% (Cyclin D1$^{-/-}$: 437 cells/mm$^2$ ± 51 cells/mm$^2$; WT 961 cells/mm$^2$ ± 62 cells/mm$^2$; p=0.0002) (Fig. 4-13C). Thus, knockout of Cyclin D1 diminished injury-induced cell proliferation following insult to the cortex.

## 4.13 Glia shows differential requirement for Cyclin D1

As Cyclin D1 is expressed in microglia, astrocytes and oligodendroglial cells, I tried to figure out whether the knockout of Cyclin D1 has an impact on the respective glial cell type within the intact and injured cortex of *Cyclin D1*$^{-/-}$ mice.

Hence, I first compared the total cell number for the different glial cell types within the intact cortex of adult WT and *Cyclin D1*$^{-/-}$ mice. The distribution pattern and the morphology of all three cell types were not altered in Cyclin D1 knockout mice. For the S100β I could not detect any obvious difference when comparing the staining of WT and knockout mice (Fig. 4-14C, D), while Iba1+ (Fig. 4-14A, B) and Olig2+ cells (Fig. 4-14E, F) seemed to be slightly reduced. However, quantitation showed that the number of ramified microglia decreased by 38% (Cyclin D1-/-: 167 cells/mm2 ± 5 cells/mm2; WT 268 cells/mm2 ± 1 cells/mm2; p=0.002) and the one of oligodendroglial cells by 39% (Cyclin D1-/-: 443 cells/mm2 ± 26 cells/mm2; WT 732 cells/mm2 ± 59 cells/mm2; p=0.003) (Fig. 4-14G).

In a second step I examined the three cell types at 3 dpi. Knockout of Cyclin D1 significantly reduced hypertrophic microglia (Cyclin D1$^{-/-}$: 470 cells/mm$^2$ ± 64 cells/mm$^2$; WT 757 cells/mm$^2$ ± 1 cells/mm$^2$; p=0.002), oligodendroglial cells (Cyclin D1$^{-/-}$: 1031 cells/mm$^2$ ±54 cells/mm$^2$; WT 1381 cells/mm$^2$ ± 68

cells/mm$^2$; p=0.0013) and also activated astrocytes (Cyclin D1$^{-/-}$: 156 cells/mm$^2$ ±5 cells/mm$^2$; WT 292 cells/mm$^2$ ± 9 cells/mm$^2$; p=0.0013) (Fig. 4-14H).

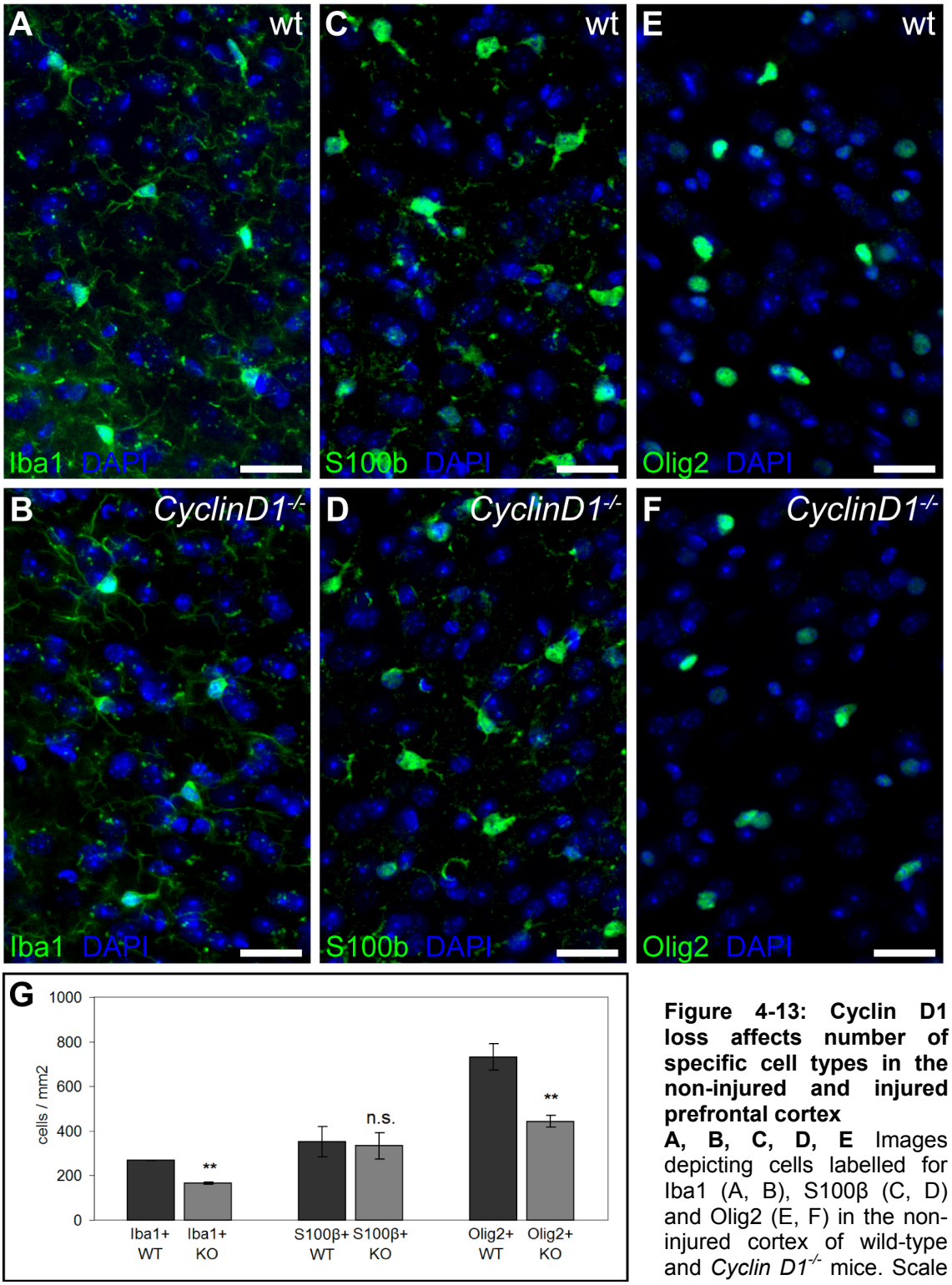
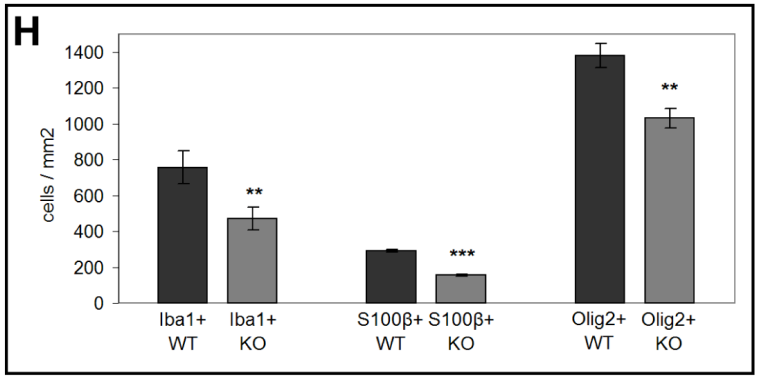



**Figure 4-13: Cyclin D1 loss affects number of specific cell types in the non-injured and injured prefrontal cortex**
**A, B, C, D, E** Images depicting cells labelled for Iba1 (A, B), S100β (C, D) and Olig2 (E, F) in the non-injured cortex of wild-type and *Cyclin D1*$^{-/-}$ mice. Scale




bars 25 µm. **G, F** Histograms representing the number of Iba1-, S100β- and Olig2-cells per analyzed area within the intact cortex (G) and within the neurodegenerative area of the injured cortex (H). Results are expressed as means ± SEM (error bars; n.s. = not significant; **, P < 0.01; ***, P < 0.001) and were analyzed by a *t* test.

## 4.14 Microglia but not oligodendroglial cells are reduced in newborn *Cyclin D1*$^{-/-}$ mice

Next I enquired whether the reduction of microglia and oligodendroglial cells observed in the intact cortex of adult *Cyclin D1*$^{-/-}$ mice was due to the loss of the cell cycle protein during adulthood, or whether it already had an effect during early development, which could be observed at early postnatal stages. While microglia enter the CNS from the blood circulation early in development (Aloisi, 2001), proliferation and maturation of the third wave of oligodendrocytes predominantly occurs in the first week after birth (Kessaris et al., 2006). Thus we decided to examine the expression of Iba1 and Olig2 during this period and choose the postnatal day 3 (P3) as point in time.

At this early stage, the number of Iba1+ cells observed in the cortex of WT was much less abundant than in the adult and their distribution within the cortex was uneven (Fig 4-15A). Compared to this, Olig2+ cells were densely packed and showed an equal distribution (Fig 4-15D). Quantitation showed that in the knockout the number of microglia was reduced by 30% (Cyclin D1$^{-/-}$: 35 cells/mm$^2$ ± 2 cells/mm$^2$; WT 25 cells/mm$^2$ ± 2 cells/mm$^2$; p=0.0025) (Fig 4-15C), whereas the Olig2+ cells were about the same number (Cyclin D1$^{-/-}$: 1296 cells/mm$^2$ ± 69 cells/mm$^2$; WT 1226 cells/mm$^2$ ± 30 cells/mm$^2$) (Fig 4-15F).

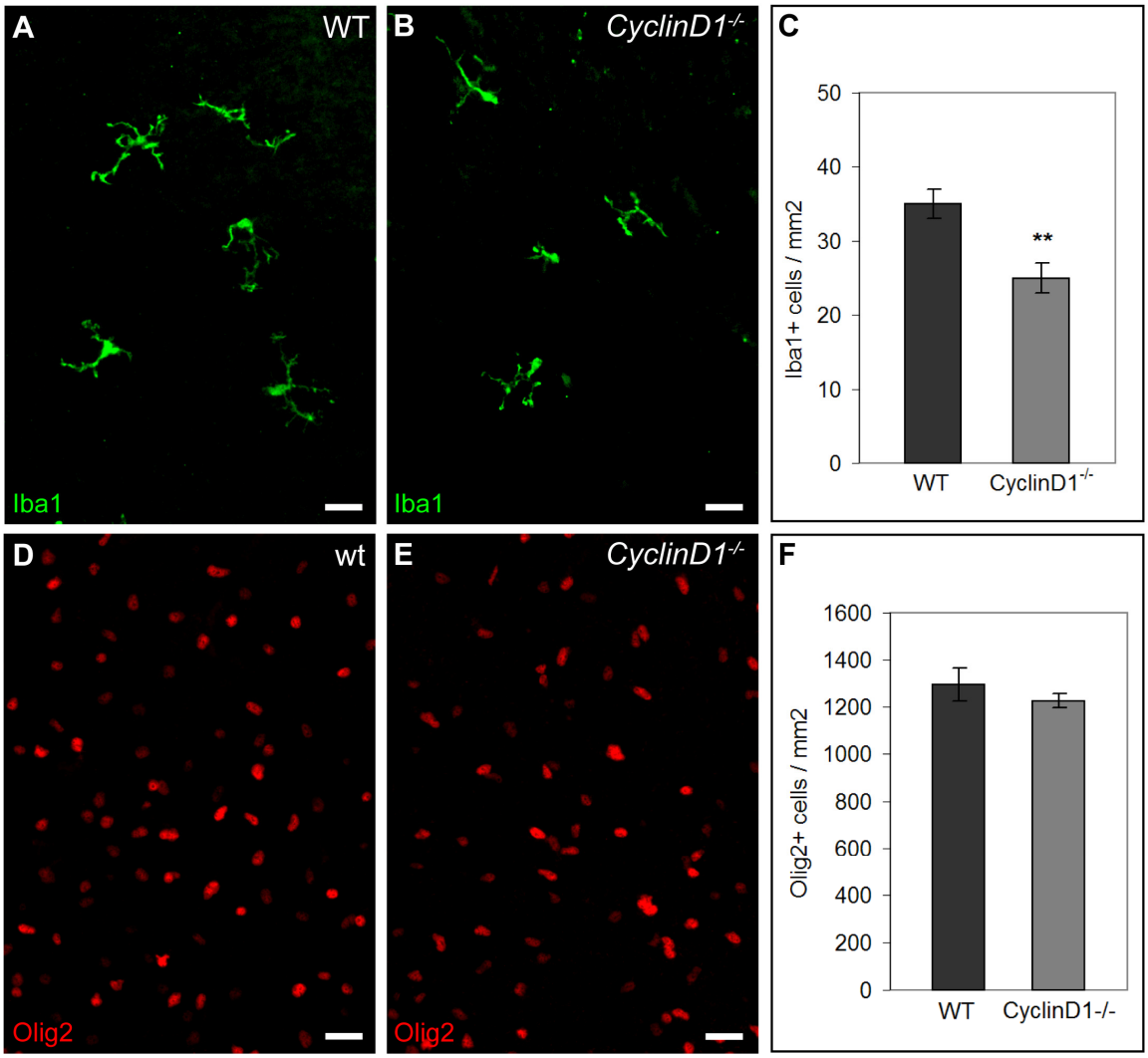



**Figure 4-14: Cyclin D1 loss affects number of Iba1+ but not Olig2+ cells at P3**
**A, B** Iba1+ cells in the cortex of P3 WT (A) and *Cyclin D1*$^{-/-}$ (B) mice. **D, E** Olig2+ cells in the cortex of P3 WT (D) and *Cyclin D1*$^{-/-}$ (E) mice. mice. Scale bars 25 µm. **C, F** Histograms representing the number of Iba1+ (C) respectively Olig2+ (F) cells per analyzed area. Results are expressed as means ± SEM (error bars; **, P < 0.01) and were analyzed by a *t* test.

## 4.15 Adult *Cyclin D1*$^{-/-}$ mice possess less mature oligodendrocytes

Given that the number of oligodendroglial cells is equal in WT and *Cyclin D1*$^{-/-}$ mice at early perinatal stage, but reduced in adult stages, we further asked whether this effect is increasing with age of the animal. The adult animals we

used before they were four to five month old. Thus we decided to determine the number of oligodendroglial cells at later stages, in eight-month-old animals.

The number of Olig2+ cells in the cortex of 8 month old knockout mice was reduced by 38% as compared to WT animals (Cyclin $D1^{-/-}$: 393 cells/$mm^2$ ± 33 cells/$mm^2$; WT 636 cells/$mm^2$ ± 21 cells/$mm^2$; p=0.006) (Fig 4-16A,B, C). This decline was consistent to the one observed in 4-5 month aged animals.

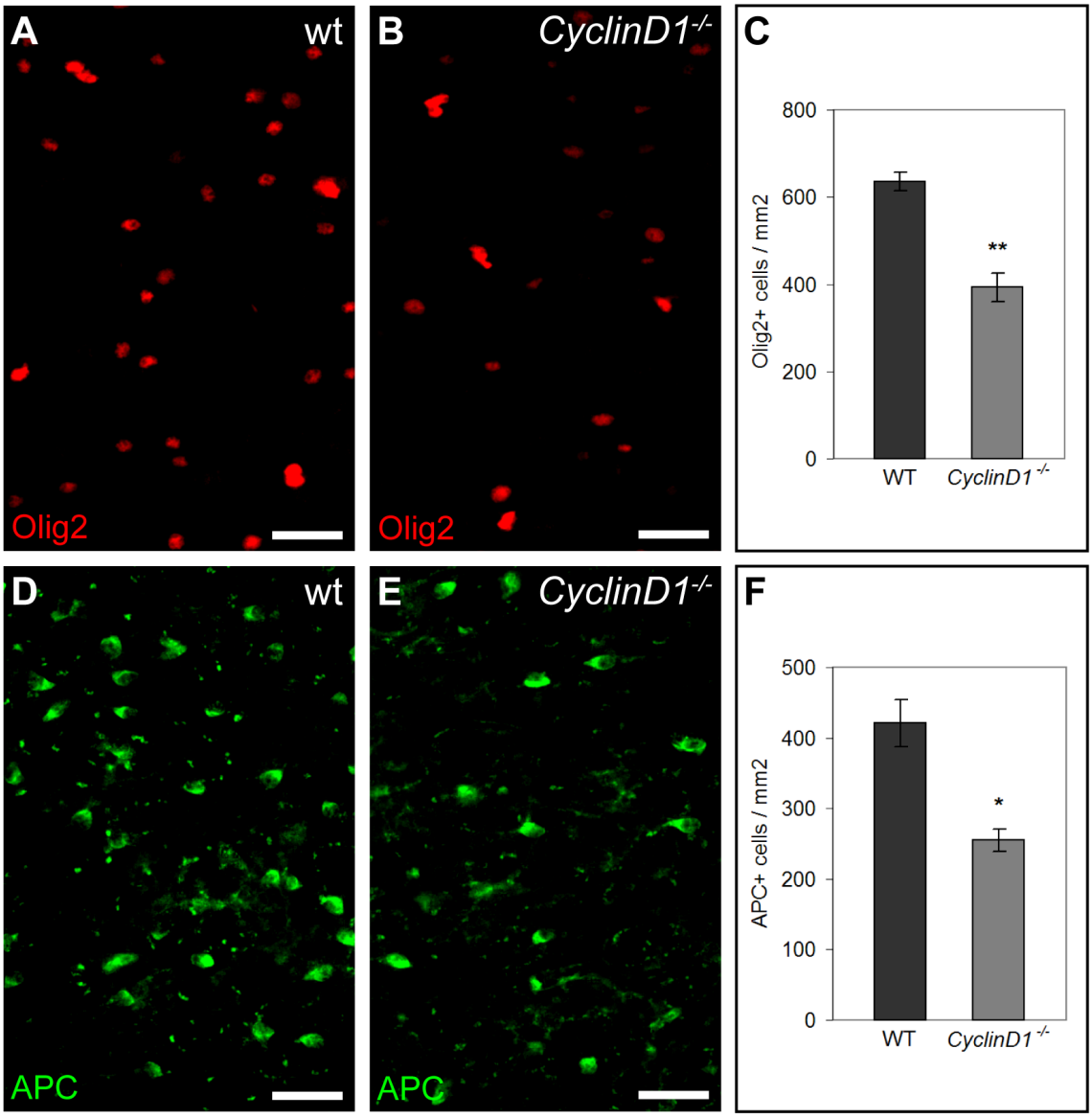



**Figure 4-15: Cyclin D1 loss affects number of Olig2+ and APC+ cells at 8 month**
**A, B** Olig2+ cells in the cortex of 8 month aged WT (A) and *Cyclin D1*$^{-/-}$ (B) mice. **D, E** APC+ cells in the cortex of 8 month aged WT (D) and *Cyclin D1*$^{-/-}$ (E) mice. mice. Scale bars 25 µm. **C, F** Histograms representing the number of Olig2+ (C) respectively APC+ (F) cells per analyzed area. Results are expressed as means ± SEM (error bars; ***, P < 0.001) and were analyzed by a *t* test.

As the number of oligodendroglial cells was reduced in the Cyclin D1-deficient animals we decided to investigate whether it resulted in a decline of mature oligodendrocytes. Therefore, I used an antibody against APC with which mature oligodendrocytes could be labelled. As expected, the number of APC+ cells was reduced by 39% (Cyclin $D1^{-/-}$: 255 cells/$mm^2$ ± 16 cells/$mm^2$; WT 421 cells/$mm^2$ ± 33 cells/$mm^2$; p=0.04) (Fig 4-16D, E, F). Thus, I concluded that the absence of Cyclin D1 leads to a reduction of mature oligodendrocytes.

A logic consequence of the reduction in oligodendroglial cells – as seen for the markers Olig2, NG2 and APC – would be a reduction in the end-product of

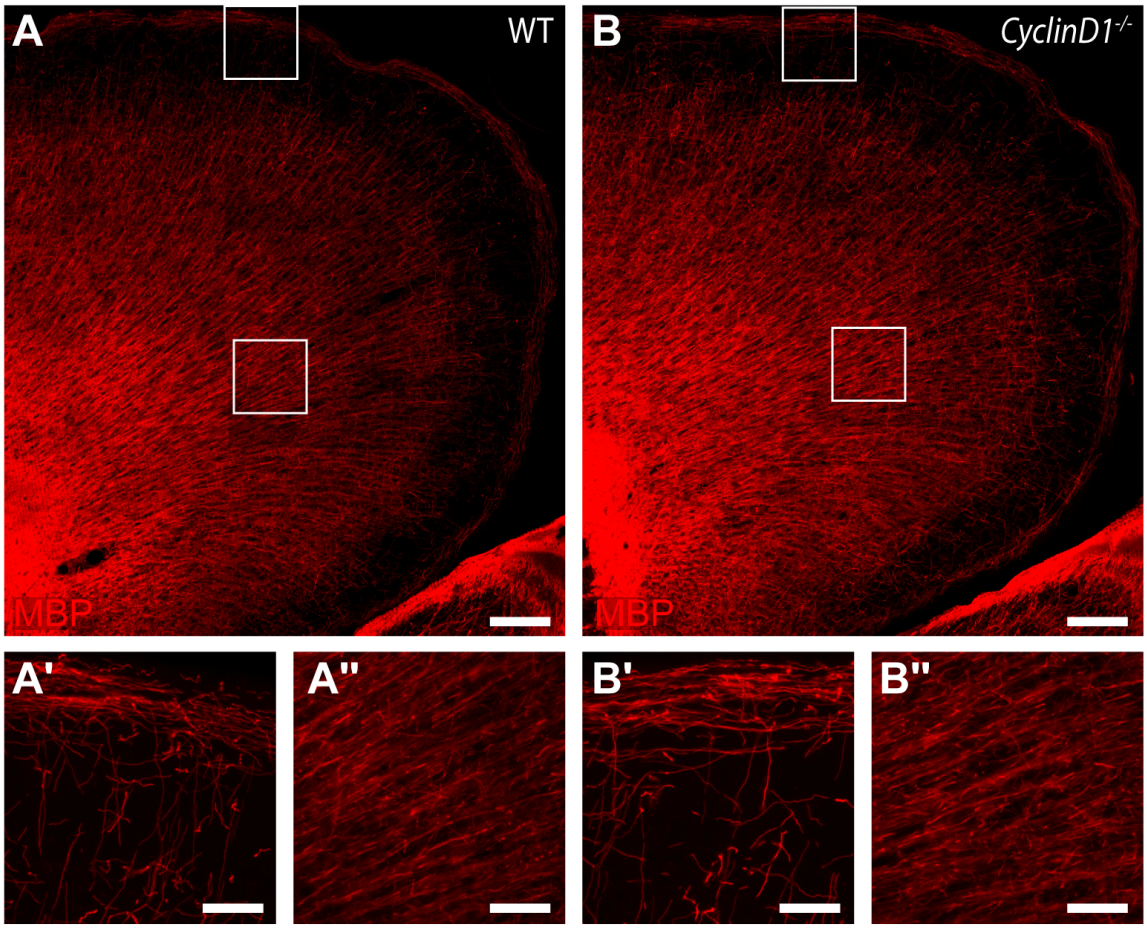



**Figure 4-16: Myelin staining in the WT and *Cyclin D1-/-* mice.**
**A, B** MBP staining within the intact adult cortex of WT (A) and *Cyclin $D1^{-/-}$* (C) mice did not show any obvious difference. Further magnifications of the upper cortical layers (A', B') and of the prefrontal cortex (A'', B'') could not reveal any discrepancy. Scale bars 250 µm (A, B) and 50 µm (A', A'', B', B'').

the oligodendroglial lineage, namely myelinating oligodendrocytes. Therefore, I stained brain sections for myelin basic protein (MBP) which labels myelin membranes of differentiated oligodendrocytes. As the effect might increase over time, I analysed the staining in 8-month-aged WT and Cyclin D1-knockout mice.

Qualitative analysis of the prefrontal cortex of WT and knockout animals did not show any difference in staining pattern (Fig. 4-16A, B). The density of the myelin within the prefrontal cortex was so high that it was not possible to discriminate single myelinated nerve fibers (Fig. 4-16A'', B''). Hence it was also not possible to perform any quantitative analysis of the immunohistochemistry.

## 4.16 Absence of Cyclin D1 does not affect density of mature neurons

To know whether Cyclin D1 also has an impact on neurons in the adult cortex, I analysed the NeuN staining in the intact cortex of 8-month-old WT and Cyclin D1-deficent animals (Fig. 4-17A, B). Comparison of the cortex between WT and KO animals showed a reduction of the cortical thickness (Fig. 4-17A', B') which amounted to 11% (Cyclin $D1^{-/-}$: 1.11 mm ± 0.02 mm; WT 1.24 mm ± 0.03 mm; p=0.0003) (Fig. 4-17C). In contrast, quantitative analysis of NeuN+ cells in the knockout animal revealed an increased neuron density (11%) as compared to the WT (Cyclin $D1^{-/-}$: 2845 cells/$mm^2$ ± 104 cells/$mm^2$; WT 2572 cells/$mm^2$ ± 59 cells/$mm^2$; p=0.003) (Fig. 4-17D). Thus, the absolute numbers of neurons within the measured was the same in WT and knockout animals.

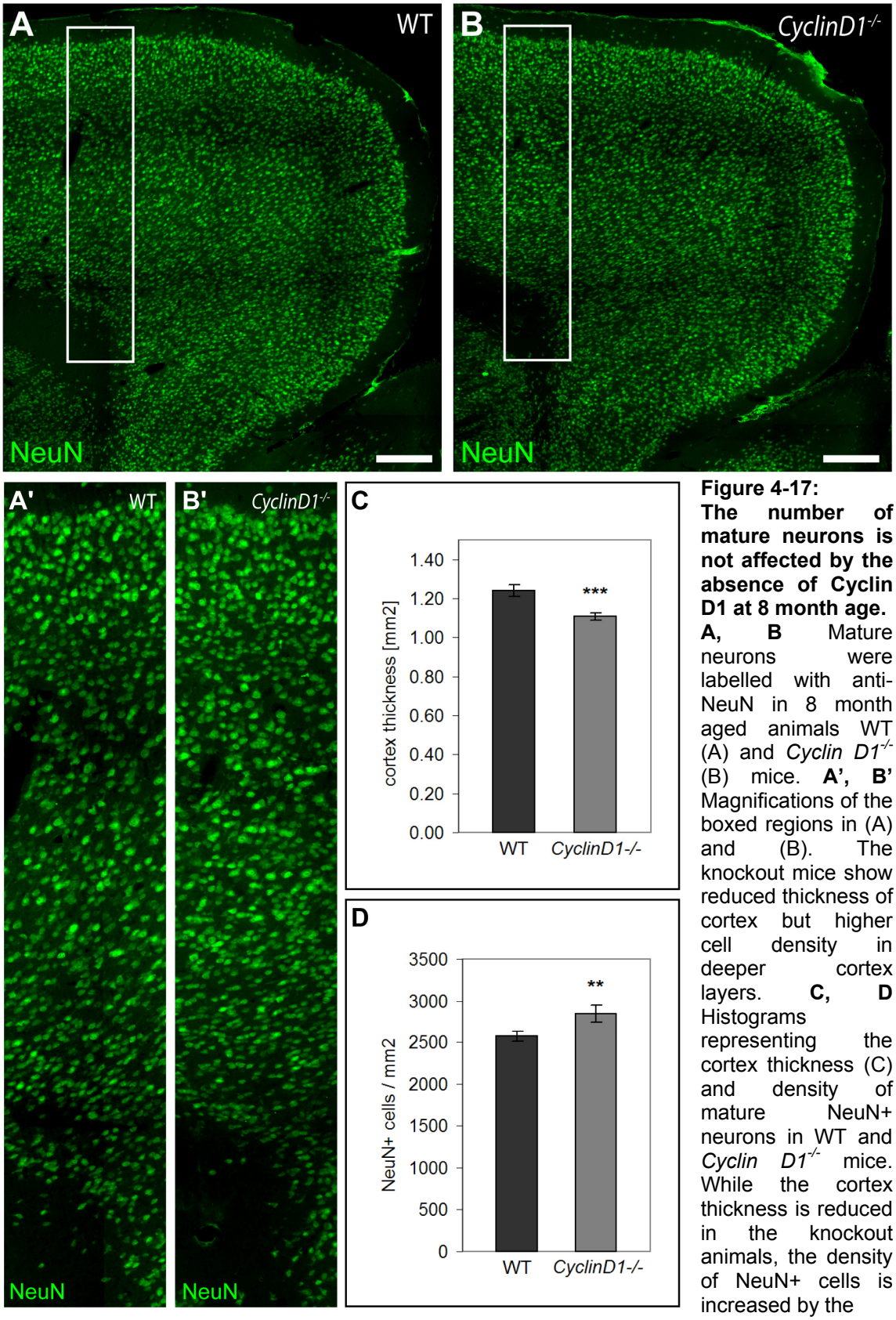
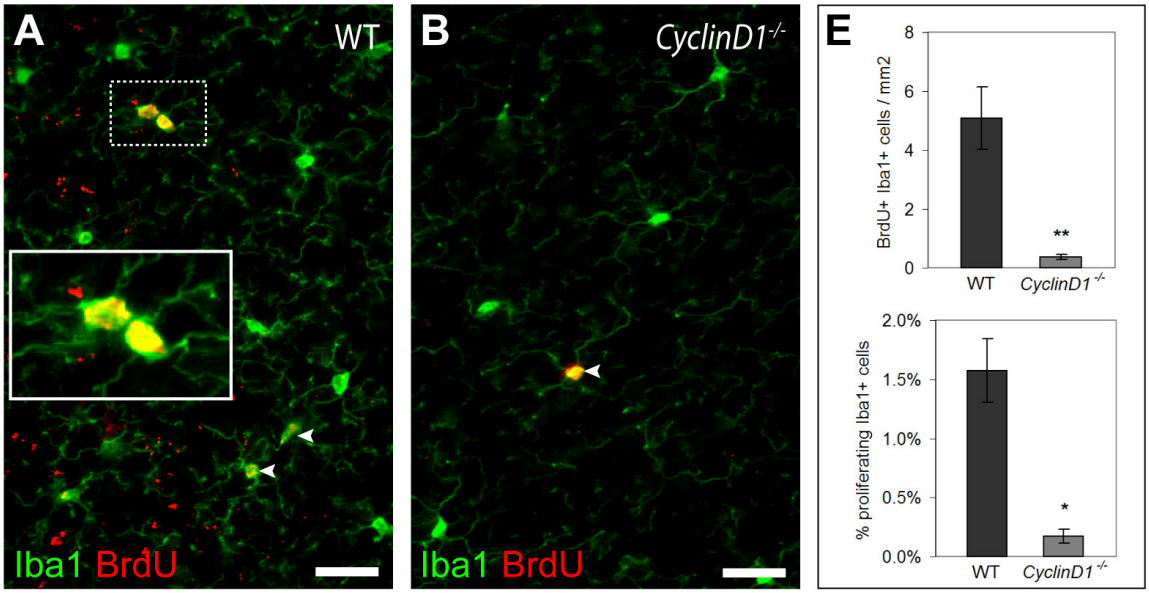



**Figure 4-17: The number of mature neurons is not affected by the absence of Cyclin D1 at 8 month age.** **A, B** Mature neurons were labelled with anti-NeuN in 8 month aged animals WT (A) and *Cyclin D1*$^{-/-}$ (B) mice. **A', B'** Magnifications of the boxed regions in (A) and (B). The knockout mice show reduced thickness of cortex but higher cell density in deeper cortex layers. **C, D** Histograms representing the cortex thickness (C) and density of mature NeuN+ neurons in WT and *Cyclin D1*$^{-/-}$ mice. While the cortex thickness is reduced in the knockout animals, the density of NeuN+ cells is increased by the same percentage. Results are expressed as means ± SEM (error bars; ***, P < 0.001) and were analyzed by a *t* test.

## 4.17 Reduced proliferation of microglia in adult *Cyclin D1*$^{-/-}$ mice

Unlike the Olig2+ cells, the number of Iba1+ microglia was already diminished at P3, implicating a defect during embryonic development. I performed experiments to analyse slow and fast proliferating microglia in the adult cortex.

In the intact cortex of WT mice slow proliferating BrdU-labelled microglia often appeared as doublets (Fig. 4-18A), while in the *Cyclin D1*$^{-/-}$ mice they mostly appeared as single cells (Fig. 4-18B). Quantitation showed that the total number of Iba1+ cells was reduced by 36% in the knockout animals (Cyclin D1$^{-/-}$ : 220 cells/mm$^2$ ± 3 cells/mm$^2$; WT 343 cells/mm$^2$ ± 9 cells/mm$^2$; p=0.002), while the proliferative fraction was even reduced by 89% (Cyclin D1$^{-/-}$: 0.4 cells/mm$^2$ ± 0.08 cells/mm$^2$; WT 5 cells/mm$^2$ ± 1 cells/mm$^2$; p=0.002) (Fig. 4-18E). Within the lesion of the injured cortex the effect seemed to be less pronounced. While the number of hypertrophic Iba1+ cells was decreased 27% by (Cyclin D1$^{-/-}$: 702 cells/mm$^2$ ± 34 cells/mm$^2$; WT 968 cells/mm$^2$ ± 45 cells/mm$^2$; p=0.041) (Fig. 4-18E) the proliferative fraction was reduced by 57% (p=0.046) (Fig. 4-18F).



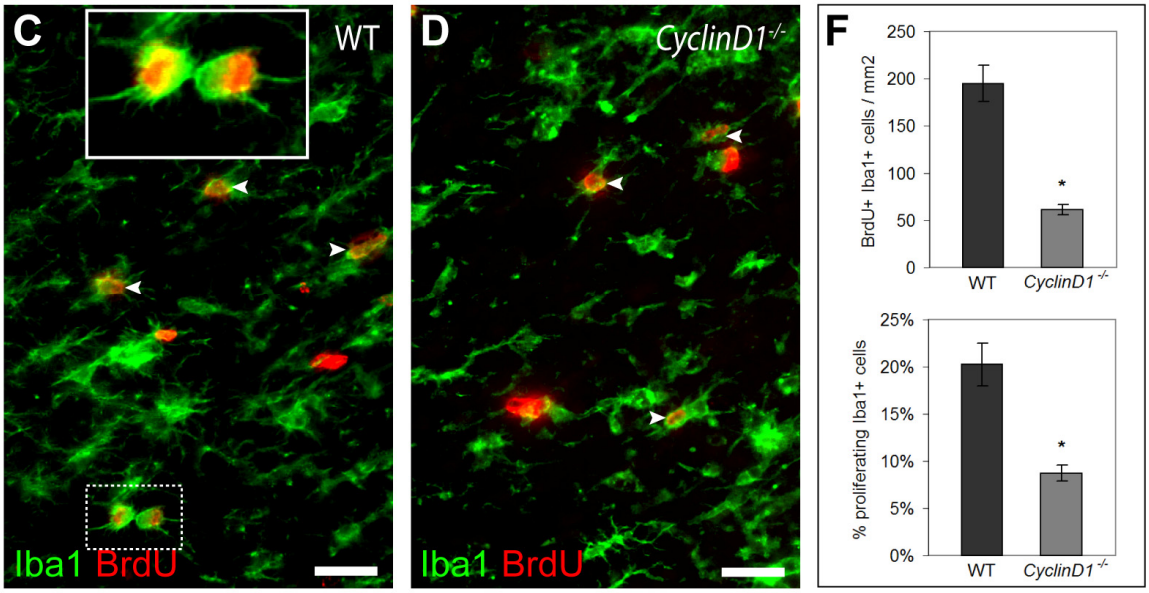



**Figure 4-18: Deficiency for Cyclin D1 strongly reduceds slow-proliferating microglia of the intact and fast-proliferating microglia of the injured cortex.**
**A, B,** BrdU staining (red) highlighting slow-proliferating Iba1+ cells (green) in adult intact cortex of WT (A) and Cyclin D1-knockout mice. **C, D** BrdU highlights fast-proliferating Iba1+ cells within the lesion of WT (C) and *Cyclin D1*$^{-/-}$ mice (D). Arrowheads indicated double-positive cells. Proliferating cells often appeared as doublets (insets). Scale bars 25 µm. **E, F** Histograms representing the number of slow- (E) and fast-proliferating (F) Iba1+ cells per analyzed area and the corresponding proliferative fraction of Iba1+ cells. Results are expressed as means ± SEM (error bars; *, $P < 0.05$; **, $P < 0.01$) and were analyzed by a *t* test.

## 4.18 Cyclin D1 regulates the proliferation of oligodendroglial cells during postnatal development and following injury

The previous results showed that the number of Olig2+ cells decreased during adulthood and following injury in mice deficient for Cyclin D1. Therefore, I assumed that the proliferation of the oligodendroglial cells might be diminished after injury. To test this hypothesis I assessed the impact of Cyclin D1 on the proliferation of Olig2+ cells in three different paradigms. First, I analysed fast proliferating Olig2+ cells at early postnatal stages, (P3). Therefore, I injected BrdU into P3-pups 2h prior to perfusion and processing for immunohistochemical analysis. In a second step I examined the slow proliferating Olig2+ cells in the non-injured adult. For this purpose mice were

fed with BrdU in their drinking water during 15 days. Finally, in a third step I analysed the fast proliferating Olig2+ cells following injury to the adult cortex. As for P3 mice, BrdU was intraperitoneally injected 2h prior to perfusion. Comparison of the obtained results for the three paradigms, would not only answer the question whether Cyclin D1 is generally required for oligodendrogenesis but also if it is specifically affecting proliferation in one of the three paradigms and therefore being differentially required within proliferating oligodendroglial cells.

Taking into account that a reduction of the total number of Olig2+ cells itself could be the reason for a reduced number of proliferating Olig2-cells, I calculated the percentage of BrdU+ Olig2+ cells of the total Olig2-cell population (proliferative fraction). At P3, knockout mice showed no difference in proliferation of cortical Olig2+ cells (Fig. 4-19A, B, G, H), whereas in the intact adult cortex slow-proliferating oligodendroglial cells were significantly reduced (Cyclin $D1^{-/-}$: 7 cells/$mm^2$ ± 1 cells/$mm^2$; WT 56 cells/$mm^2$ ± 6 cells/$mm^2$; p=0.00005) (Fig 4-19C, D, G). This decrease was also reflected by the reduced proliferative fraction which was reduced by 77% (p=0.0001) (Fig. 4-19H). In the injured cortex of adult knockout mice I could observe a considerable decline in fast-proliferating Olig2+ cells, though not as severe as observed for the slow-proliferating cells (Cyclin $D1^{-/-}$: 42 cells/$mm^2$ ± 3 cells/$mm^2$; WT 93 cells/$mm^2$ ± 8 cells/$mm^2$; p=0.0005) (Fig. 4-19E, F, G). In this case, the proliferative fraction was reduced by 33% (p=0.015) (Fig. 4-19H). Taken together, the proliferation at perinatal stage was not impaired by the deficiency for Cyclin D1, while during adulthood endogenous proliferation and injury-induced proliferation were severely diminished.

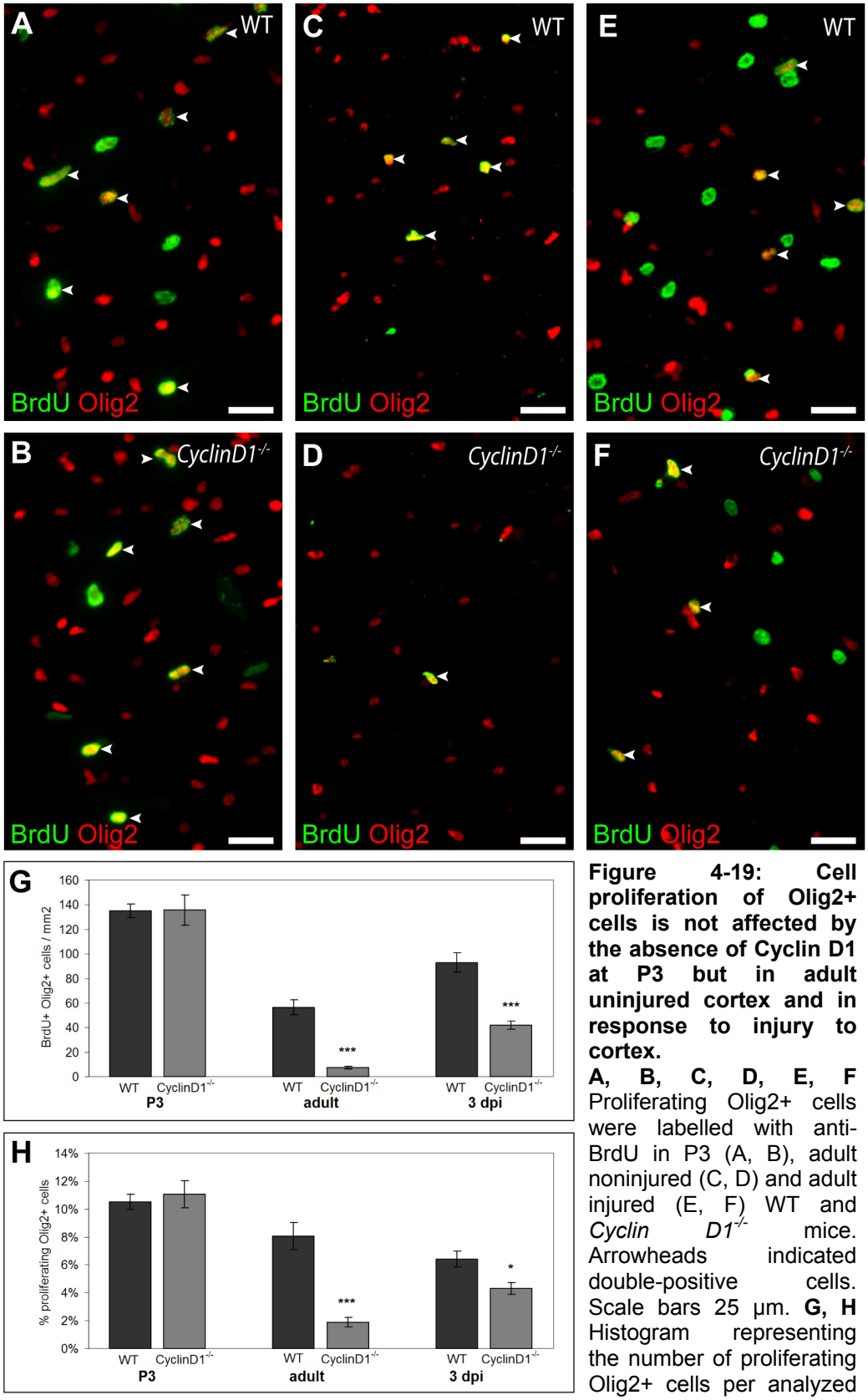



**Figure 4-19: Cell proliferation of Olig2+ cells is not affected by the absence of Cyclin D1 at P3 but in adult uninjured cortex and in response to injury to cortex.**

**A, B, C, D, E, F** Proliferating Olig2+ cells were labelled with anti-BrdU in P3 (A, B), adult noninjured (C, D) and adult injured (E, F) WT and *Cyclin D1*$^{-/-}$ mice. Arrowheads indicated double-positive cells. Scale bars 25 µm. **G, H** Histogram representing the number of proliferating Olig2+ cells per analyzed area (G) and the proliferative fraction of proliferating Olig2+ cells (H) in P3, adult and adult injured (3 dpi) mice. Results are expressed as means ± SEM (error bars; *, P < 0.05; ***, P < 0.001) and were analyzed by a *t* test.

The marker Olig2 is expressed throughout the entire oligodendroglial lineage and it is suggested that the proliferating fraction of the Olig2-population are oligodendrocyte progenitors (OPCs) (Zhou et al., 2000; Ligon et al., 2004; Ligon et al., 2006). To further support the data obtained with Olig2 I performed stainings with the NG2-antibody, which labels putative OPCs.

Comparison of the total number of NG2-cells within the cortex of WT and Cyclin D1 mice showed only a decline of 17% in the knockout (Cyclin $D1^{-/-}$: 122 cells/$mm^2$ ± 3 cells/$mm^2$; WT 146 cells/$mm^2$ ± 5 cells/$mm^2$; p=0.003) (Fig. 4-20A, B). As the decline of oligodendroglial cells labelled for Olig2 in the knockout was at 39%, the decrease in NG2-population would only account for a part of the total effect. Next I examined the proliferation of NG2-cells.

From previous studies it is known that within the cortex of adult mice, the fraction of actively dividing NG2-cells is about half of the NG2-population while the other half of the population being long-term quiescent (Psachoulia et al., 2009). This has been shown by cumulative BrdU labelling experiments. Moreover, it has been shown that with increasing time of administered BrdU the fraction of NG2-cells labelled for BrdU is increasing linearly, reaching its maximum at less than 20 days of BrdU administration.

Thus, I attempted to reproduce the results of Psachoulia et al. in the WT mice and analysed the fraction of proliferating NG2+ cells within the intact cortex of WT mice. As I was feeding mice for 15 days with BrdU I expected the fraction of proliferating NG2-cells to be between 40% and 50%. Indeed, proliferating NG2-cells made up 43% of the total NG2-cell population (43.3% ± 1.4%) (Fig. 4-20G). Proliferating NG2-cells could often be observed as doublets (Fig. 4-20C). In the knockout animals, the reduction of proliferating NG2-cells was obvious (Fig. 4-20D). Subsequent analysis in the Cyclin D1 knockout revealed that the number of proliferating NG2-cells (Fig. 4-20F) and moreover the proliferative fraction of NG2-cells was both strongly reduced by 89 % (Cyclin

D1-/-: 6 cells/mm2 ± 1 cells/mm2; WT 57 cells/mm2 ± 5 cells/mm2; p=0.000007) (Fig. 4-20G).

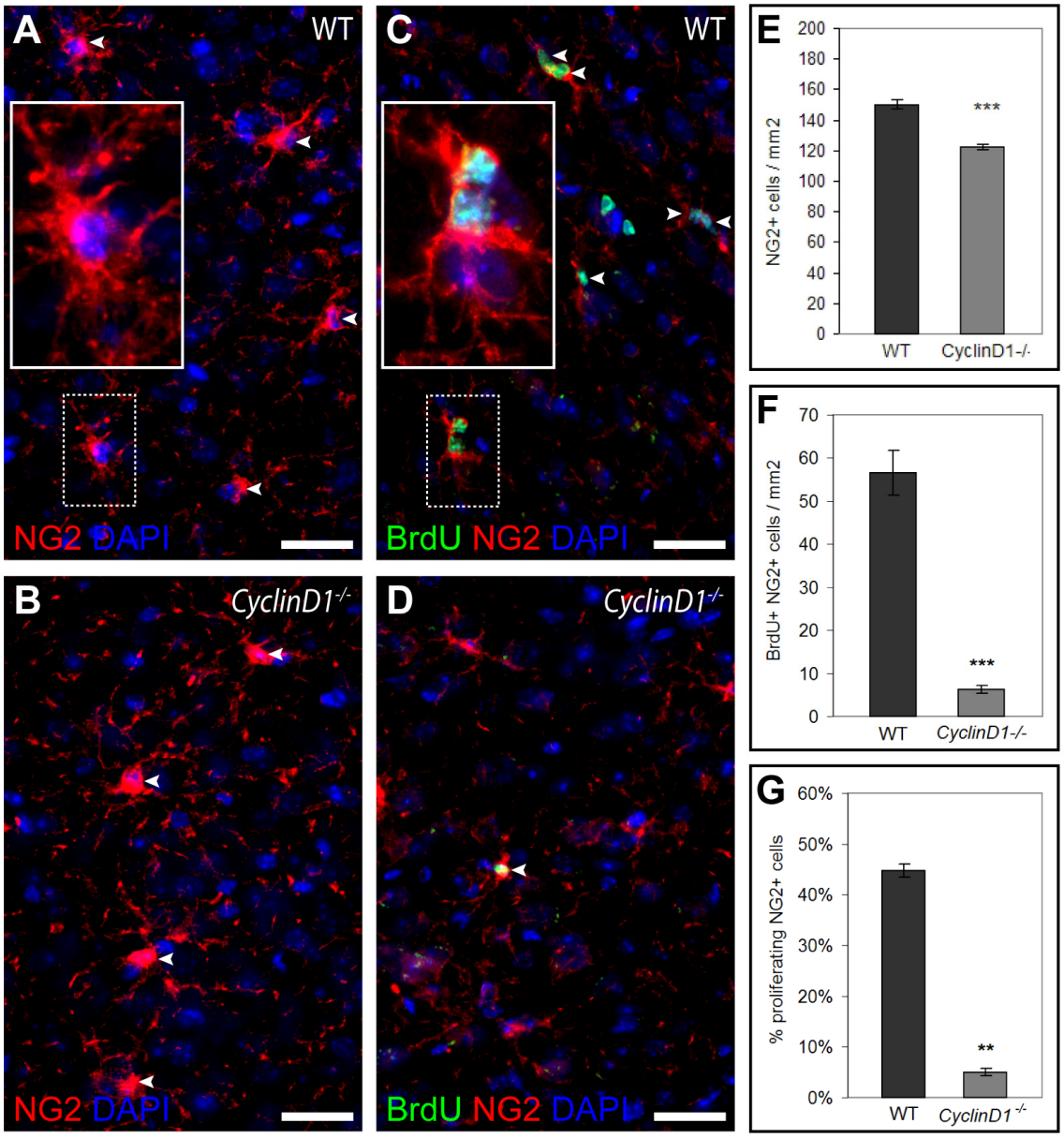



**Figure 4-20: The popu-lation and proliferation rate of NG2-cells is significantly reduced by the absence of Cyclin D1 in the adult uninjured cortex.**
**A, B,** NG2+ cells within the intact adult cortex of WT (A) and *Cyclin D1*$^{-/-}$ (B) mice. **C, D,** Proliferating NG2+ cells are labelled with anti-BrdU in adult intact cortex of WT (D) and *Cyclin D1*$^{-/-}$ (E) mice. Arrowheads indicated double-positive cells. Scale bars 25 µm. **E, F** Histograms representing the total number (E) and the proliferating number (F) of NG2+ cells per analyzed area **G** Histogram representing the proliferative fraction of NG2+ cells. Results are expressed as means ± SEM (error bars; **, P < 0.01; ***, P < 0.001) and were analyzed by a *t* test.

## 4.19 Absence of Cdk4 impairs the injury-induced proliferation of OPCs

As Cyclin D1 is the regulatory subunit of the holoenzyme formed with the catalytic subunits Cdk4 and Cdk6, one could assume that knockout of one of the catalytic subunits might have similar effects as observed in the Cyclin D1-knockout mice. It was previously shown in the PNS, that mice lacking Cdk4 show a drastic decrease in number of proliferating Schwann cells – the myelinating cells of the PNS – during development. Moreover, injury-induced proliferation of adult Schwann cells was abolished in Cdk4-knockout mice. Interestingly, ablation of Cdk2 and Cdk6 had no influence on developmental or injury-induced proliferation of Schwann cells, indicating the need for specific Cdks (Atanasoski et al., 2008). Thus, the question came up whether the same might be true for the CNS.

To answer this question I investigated on the number and injury-induced proliferation of oligodendroglial cells in Cdk2-, Cdk4- and Cdk6-knockout mice and compared it to the results obtained in the Cyclin D1-knockout mice. Therefore, the same type of injuries and immunohistochemical analysis for BrdU and Olig2 were performed in the Cdk-knockout mice as for the *Cyclin D1*$^{-/-}$ mice.

In the *Cdk2*$^{-/-}$ mice the number of Olig2+ cells within the lesion area was comparable to the one observed in WT and insignificantly lower in the *Cdk6*$^{-/-}$ mice (Cdk2$^{-/-}$: 1525 cells/mm$^2$ ± 81 cells/mm$^2$; p=0.9; Cdk6$^{-/-}$: 1346 cells/mm$^2$ ± 58 cells/mm$^2$; p=0.15; WT 1496 cells/mm$^2$ ± 118 cells/mm$^2$) (Fig. 4-21A). In contrast, *Cdk4*$^{-/-}$ mice showed a reduction of 44% (Cdk4$^{-/-}$: 834 cells/mm$^2$ ± 81 cells/mm2; p=0.0007), which was even more extensive than the one observed in *Cyclin D1*$^{-/-}$ mice (25%, Fig. 4-21A). For the absolute number of proliferating Olig2 cells (Fig. 4-21B) and the proliferative fraction of the Olig2-population I could observe a similar effect. While Cyclin D1$^{-/-}$ and Cdk4$^{-/-}$ animals showed a

significant decrease in the proliferative fraction of Olig2+ cells (Cyclin D1-/-: 4.3% ± 0.4%; p=0.015; Cdk4-/-: 2.3% ± 0.6%, p=0.005; WT: 6.4% ± 0.6%), the difference was non-significant in Cdk2- and Cdk6-knockout mice (Fig. 4-21C).

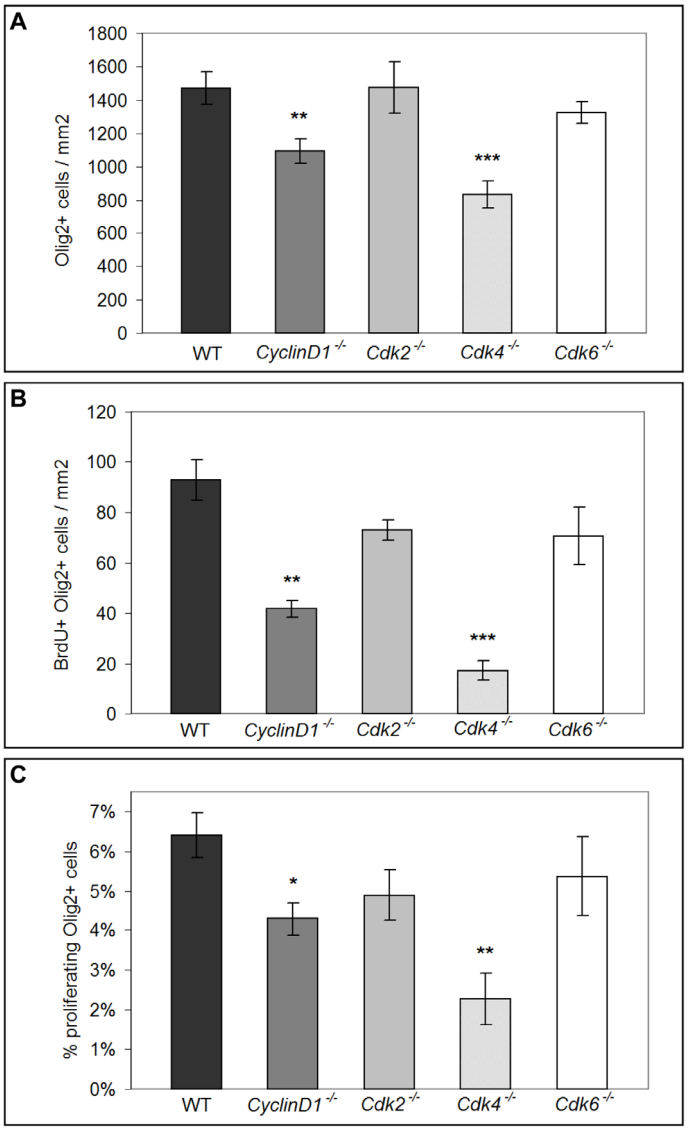



**Figure 4-21: Following cortical injury to the adult brain number and proliferation of Olig2+ cells is affected by the absence of Cyclin D1 and Cdk4 but not by the absence of Cdk2 and Cdk6**
**A, B C** Histograms repre-senting the total number (A) and the proliferating number (B) and the proliferative fraction (C) of Olig2+ within the lesion of WT, Cyclin D1-, Cdk2-, Cdk4- and Cdk6-knockout animals at 3 dpi. The three measured data showed to be significantly reduced in Cyclin D1- and Cdk4-knockout animals but not in animals deficient for Cdk2 or Cdk6. Results are expressed as means ± SEM (error bars; *, P < 0.05; **, P < 0.01; ***, P < 0.001) and were analyzed by a *t* test.

In summary, I have shown that Cyclin D1 has a direct impact on the lesion size following neurotoxic injury to the brain cortex. Furthermore, analysis of the requirement for Cyclin D1 within glial lineages revealed that it is indispensable for the development of microglia, while OPCs are only Cyclin-dependent during adult stages. In addition I propose Cdk4 to be the interaction partner of Cyclin D1, regulating injury-induced proliferation of OPCs.

# 5. DISCUSSION

I have studied the impact of Cyclin D1 in the intact and injured postnatal prefrontal cortex and observed that: (1) three days following neurotoxic injury to the cortex Cyclin D1 is highly upregulated within the neuron-depleted area; (2) absence of Cyclin D1 reduces the neurodegenerative area following injury; (3) Cyclin D1 is differentially required depending on the glial cell type; (4) absence of Cyclin D1 impairs proper generation of microglia, (5) Cyclin D1 is required for the oligodendrogenesis during adulthood but not during early development and (6) Cyclin D1 is not important for the generation of the proper number of neurons. In addition, I show that absence of Cdk4 but not Cdk2 and Cdk6 reduce injury-induced proliferation of oligodendrocyte progenitor cells.

From previous studies (Kaya et al., 1999; Di Giovanni et al., 2003; Di Giovanni et al., 2005) it was known that cell cycle proteins are significantly upregulated following injury to the CNS. In addition, it has been shown that following traumatic brain injury (TBI) there is a marked upregulation of cyclin D1 and cdk4 (Di Giovanni et al., 2005). However, after global brain ischemia Cyclin D1 and Cdk4 are not increased above normal values (Small et al., 2001). Thus, whether there is increased protein expression of cell cycle protein, may be dependent on the injury model and species.

It was shown that the upregulation of cell cycle proteins following injury to the CNS can result either in the proliferation and activation of glial cells such as astroglia and microglia, or in the apoptosis of post-mitotic cells such as neurons and mature oligodendrocytes (Byrnes et al., 2007). For example resident microglia strongly increase their proliferation rate in response to injury (Byrnes and Faden, 2007) and proliferating astroglia have been shown to play a significant role in the formation of the glial scar (Byrnes and Faden, 2007). Treatment with the cell cycle-inhibitor flavopiridol decreases injury-induced lesion volume and improves behavioural outcomes (Cernak et al., 2005),

suggesting that modulation of the cell cycle after injury might provide a mechanism for neuroprotection. However, flavopiridol is a relatively nonspecific cell cycle inhibitor that also inhibits transcription, which complicates interpretation of its mechanism of action. Treatment with roscovitine, a more specific Cdk-inhibitor decreased microglial activation after TBI, and attenuated astrogliosis (Cernak et al., 2005; Hilton et al., 2008). Taken together, these data support a multifactorial neuroprotective effect of cell cycle inhibition after TBI and suggest that multiple Cyclins and Cdks are potentially involved in this process. The critical limitation of the approach using pharmacological compounds is that one cannot connect their effect to a specific cell cycle molecule as the activity of multiple CDKs may be inhibited (Bain et al., 2007). Thus, genetic approach using Cyclin D1-deficient mice specifically targets the cell cycle molecule in order to determine its effects.

To date, most brain injury models affect not only neurons but result in various pathophysiological alterations which makes it difficult to dissect injury-induced mechanisms. An example of this is controlled cortical impact, a model for traumatic brain injury (TBI). Although it produces a focal injury it is also leading to vascular disruption, cerebral edema and elevated intracerebral pressure (Dixon et al., 1991; Cernak et al., 2005). To investigate the processes specifically occurring following neurodegeneration and to minimize side effects as mentioned before, I chose to perform local injections of ibotenic acid.

## 5.1 Upregulation of Cyclin D1 following injury

Microarray analysis in previous studies showed a marked upregulation of Cyclin D1 and Cdk4 mRNA expression by 24h- 48h after spinal cord injury (Byrnes and Faden, 2007). This up-regulation persists for at least 7 days post-injury, and disappears by 28 days. Confirmation of these microarray findings with immunohistochemistry demonstrates upregulation of the cell cycle proteins (Kaya et al., 1999; Di Giovanni et al., 2003; Di Giovanni et al., 2005).

My own analysis of the expression of Cyclin D1 three days following neurotoxic insult agree with the previous findings in other injury models, revealing a significant increase in the number of cells expressing Cyclin D1 within the lesion site. In contrast, at 1 dpi I could not observe any change in protein expression. Apparently, the upregulation of Cyclin D1 or rather the activation of the cell cycle machinery in cells residing within neurodegenerative area requires more than 24h. This delay could be partly due to the indirect mechanism of cell cycle activation within cells which react upon degeneration of neurons. At 30 days postinjury the expression level in cells residing within the neurodegenerative area had returned to basic level and the density of Cyclin D1+ cells had even dropped below values observed in the uninjured cortex. Thus, the cells had lowered their Cyclin D1 expression to the one comparable in resting state. The decrease in number is probably due to cell death induced by mechanic damage of the tissue.

## 5.2 Glial cell types within the intact and lesioned cortex

I could show that three days following neurotoxic insult to the cortex neurons completely disappeared within the neurodegenerative area (Fig. 6-1).

Simultaneously, the number of oligodendroglial cells and activated microglia markedly increased, while the number of mature astrocytes remained unchanged. (Fig. 6-1). In addition, reactive astrocytes could be observed outside at the border of the lesion (Fig. 6-1).

Concerning Cyclin D1 expression, I could show that within the intact prefrontal cortex minor fractions of the resting microglia and mature astrocytes weakly expressed Cyclin D1 in the nucleus. In contrast, a major fraction of the oligodendroglial cells expressed nuclear Cyclin D1. Although the number of microglial and oligodendroglial cells increased within the injured cortex, the percentage of microglial, astrocytic and oligodendroglial cells double positive for Cyclin D1 did not change significantly when compared to the intact situation.

Apparently, the sum of the percentages of Cyclin D1 cells positive for one of the glial markers exceeds 100% in the intact as well as in the injured condition. From previous studies it is known, that astrocytes also express Olig2 (Cassiani-Ingoni et al., 2006; Cai et al., 2007; Chen et al., 2008; Zhao et al., 2009). Thus, the expression of the markers S100β+ and Olig2+ might overlap within the Cyclin D1-cell population.

The observed expression of Cyclin D1 within microglia and oligodendroglial cells is in line with studies which examined Cyclin D1 expression before and after traumatic brain injury (Kaya et al., 1999) or focal ischemia (Li et al., 1997).

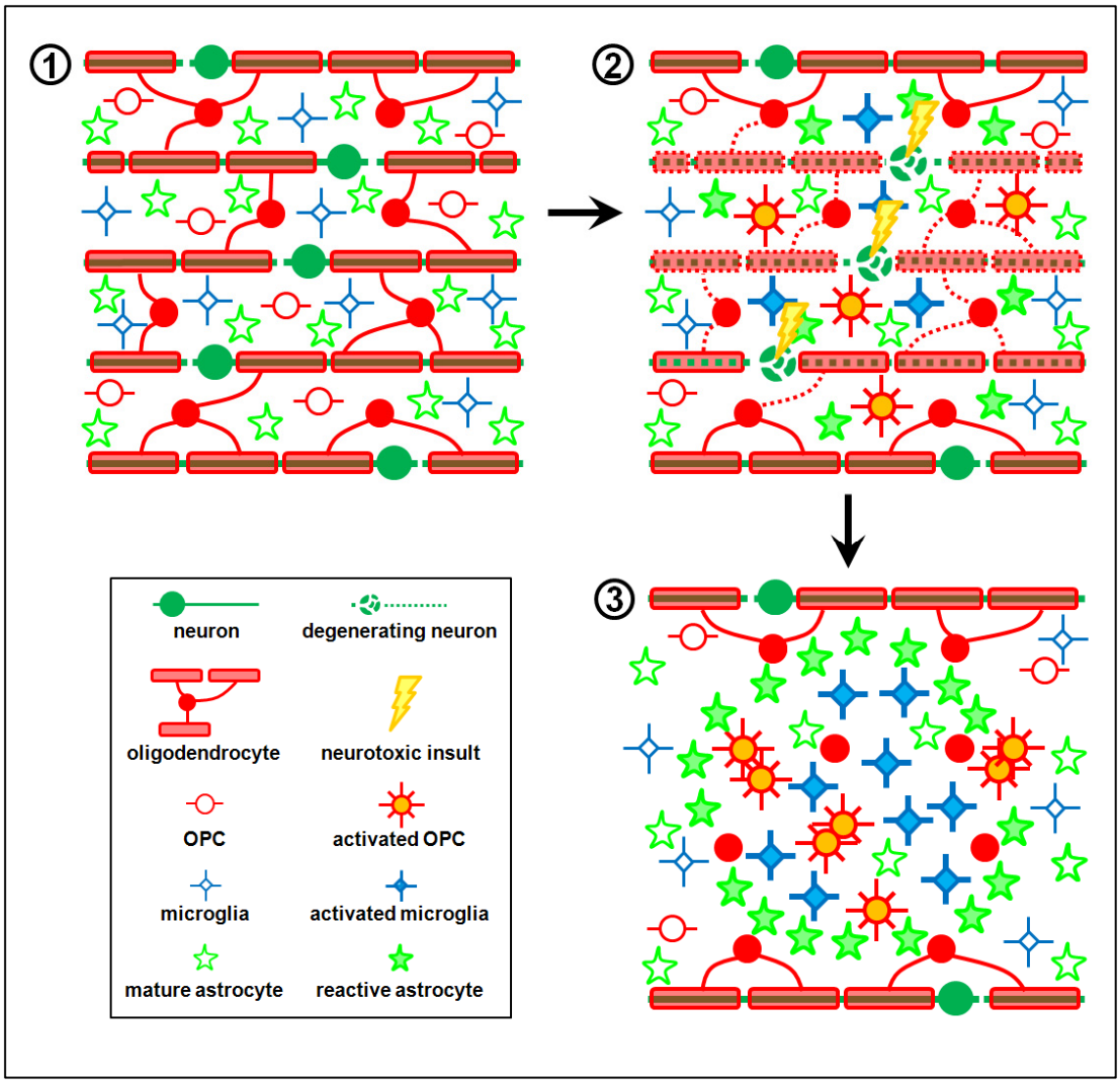



**Figure 6-1: Proposed model of injury-induced activation and proliferation of glial cells following local neurotoxic insult to the cortex**

**1** Normal adult white matter contains astrocytes, microglia, myelinating oligodendrocytes, and in addition oligodendrocyte precursor cells (OPCs). **2** Following neurotoxic insult to the cortex causing neurodegeneration astrocytes, microglia and OPCs in the proximity are activated. **3** Three days following neurotoxic insult the neurons completely disappeared within the lesion site. Microglia and OPCs proliferate while reactive astrocytes form a glial scar around the lesion.

## 5.3 Knockout of Cyclin D1 reduces the lesion area

The size of a cortical lesion provides a marker for secondary injury-mediated tissue damage and neurodegeneration. I could show that 3 days following neurotoxic injury to the cortex the neurodegenerative area was significantly smaller within *Cyclin D1*$^{-/-}$ mice. These results were in line with previous results obtained in other injury models such as focal cerebral ischemia (Zhu et al., 2007), spinal cord injury (Di Giovanni et al., 2005; Byrnes and Faden, 2007) or traumatic brain injury (Kabadi et al., 2012). The latter revealed that following traumatic brain injury in Cyclin D1-deficient mice, expression of markers of cell cycle activation, microglial inflammation, as well as neurodegeneration are strongly reduced (Kabadi et al., 2012). The authors suggest that the neuroprotective effect is due to direct inhibitory effects on cell cycle activated-dependent neuronal cell death and microglial-mediated inflammation. As I could observe a reduced number of oligodendroglial cells within the injury of *Cyclin D1-/-* mice I would not only account neurons and microglia to the smaller injury size but also oligodendroglial cells.

To definitely answer the question whether the reduced size of the lesions in the *Cyclin D1-/-* mice relies specifically upon one cell type, one would need to use mice in which *cyclin D1* is knocked out in neurons, microglia or oligodendroglial cells.

## 5.4 Deficiency for Cyclin D1 does not affect the number of reactive astrocytes

In contrast to previous studies which used pharmacological inhibitors to block the Cyclin D1 pathway (Di Giovanni et al., 2005), the *Cyclin D1-/-* mice used in my study did not display a reduction in GFAP+ cells leading to reduced glial scar

formation at 3dpi. This might be due to the fact that at 3 dpi most reactive astrocytes derive from local astrocytes whose number in the intact cortex of Cyclin D1-deficent mice does not differ from WT mice. This could be further supported by the fact that at 1 dpi I could not detect GFAP+ cells surrounding the neurotoxic area (data not shown), suggesting that activation and thereby proliferation of reactive astrocytes occurs only after some temporal delay. Furthermore, preliminary results indicated a reduced astrogliosis around the lesion site (data not shown). Thus, it is well probable that the proliferation of reactive astrocytes is reduced in *Cyclin D1-/-* mice, leading in long-term to a reduced scar formation. To assess this, one would need to examine the density of reactive astrocytes within the glial scar at a point in time beyond 3 dpi so that the potential effect can become observable.

## 5.5 Cyclin D1 is essential for the generation of microglia

Studying the postnatal brains of *Cyclin D1*$^{-/-}$ mice I could show that the number of microglia within the prefrontal cortex was already prominently reduced at early postnatal stage. As the microglial cells observed at this stage just have entered into brain parenchyma and may not be proliferating, one can assume that the absence of Cyclin D1 has an impact on microglia during embryonic development. Either it is reducing the cellular traffic through the blood brain barrier into the brain which is unlikely as the blood brain barrier is not fully developed at birth, or, more probably, by hampering the proper formation of microglia precursors before they invade the brain. At adult stage, the reduction of the pool of cortical microglia in the Cyclin D1 knockout was even more prominent. As the turnover of microglia is very low in the intact brain (Lawson et al., 1992), I performed long-term proliferation studies.

In addition to the investigation in the intact cortex I also assessed the impact of the genetic loss of Cyclin D1 on the response of microglia following injury to the cortex. At 3 dpi, microglia within the neurodegenerative area of the knockout mice were reduced in number as compared to the WT. On one hand this could be explained by a reduced number of resident microglia before injury. But determination of the proliferative fraction showed that also the proliferation of injury-induced microglia is prominently reduced. Thus, in *Cyclin D1*$^{-/-}$ mice both the number of resident microglia as well as the proliferation of activated microglia are reduced within the neurotoxic lesion.

Taken together, Cyclin D1 is important for the overall proliferation of microglia (Fig. 6-2A, B). As it was shown, that microglial activation is associated with the release of proinflammatory molecules that can cause neurotoxicity (Di Giovanni et al., 2005; Byrnes and Faden, 2007), one can assume that a reduction in the number of microglia leads to a reduced inflammatory response and thus to a reduction in secondary injury.

This matches with a recent publication which showed that genetic ablation of Cyclin D1 significantly reduced numbers of activated microglia following traumatic brain injury (Kabadi et al., 2012). Furthermore, they observed that the protein level for the reactive microglial marker Iba-1 was reduced in the *Cyclin D1*$^{-/-}$ mice, emphasizing a role of Cyclin D1 in microglial activation.

## 5.6 Absence of Cyclin D1 results in a reduced number of mature oligodendrocytes

Analysis of Olig2+ oligodendroglial cells at adult stage in *Cyclin D1*$^{-/-}$ mice revealed a reduction by almost 40% as compared to wild-type animals. As Olig2 is expressed during the complete differentiation process of oligodendroglial cells

I further examined its subtypes. Investigation on the number of adult OPCs which specifically express NG2 revealed a reduction of 17% in *Cyclin D1*$^{-/-}$ mice.

To figure out whether the observed reduction of OPCs was due to the effect of the absence of Cyclin D1 during adulthood or during development, I examined the pool of oligodendroglial cells in newborn mice. Oligodendrocytes are generated in three waves during development with the third wave starting just after birth (Kessaris et al., 2006). Thus, impact of Cyclin D1 on the production of oligodendrocytes could be best monitored at perinatal stages. Analysis of the total number of oligodendroglial cells in the prefrontal cortex of newborn WT and *Cyclin D1*$^{-/-}$ mice at P3 showed that in contrast to microglia, the pool of oligodendroglial cells was not affected by the absence of Cyclin D1. Thus, I concluded that the proliferation of oligodendroglial cells is not affected during early development but during postnatal stages. Investigation on the proliferation of oligodendroglial cells at P3 revealed that the proliferation of these fast-proliferating cells was not affected by the absence of Cyclin D1. Thus I further analysed the proliferation of oligodendroglial cells at adult stage and I could show that the population of slow-proliferating Olig2-cells is markedly reduced in *Cyclin D1*$^{-/-}$ mice. To further support this result I assessed the proliferation of adult NG2-cells. I could show that in the wild-type animals about half of the NG2-cell population was proliferating (which is in line with Psachoulia et al., 2009) and that this proliferative fraction declined to 11% in *Cyclin D1*$^{-/-}$ mice. Thus, the absence of Cyclin D1 strongly impaired the proliferation of OPCs at adult stage in contrast to early postnatal stage (P3). This in turn, implies the existence of molecular mechanisms which are able to discriminate between the proliferation of OPCs during development and adulthood.

As the OPCs observed at early postnatal stage are fast-proliferating cells and the OPCs at adult stage slow-proliferating cells, one would tend to claim that only slow-proliferating cells are affected by the absence of Cyclin D1. Thus, I

used my injury model to induce fast-proliferating OPCs in the cortex of adult mice.

Different models inducing demyelination in the adult mouse brain provide indirect evidence that OPCs are the major source of remyelinating oligodendrocytes. First of all, BrdU tracing indicates that dividing cells in adult white matter give rise to remyelinating oligodendrocytes (Gensert and Goldman, 1997). Secondly, remyelination can be achieved by transplantation of OPCs (Zhang et al., 1999). Thirdly, demyelinating lesions in which both oligodendrocytes and OPCs die are repopulated by OPCs before new remyelinating oligodendrocytes appear (Sim et al., 2002). Fourth, at the onset of remyelination cells can be identified which transitionally express molecular markers of both OPCs and oligodendrocytes, namely Nkx2.2, Olig1 and Olig2 (Fancy et al., 2004).

By performing neurotoxic injuries in the cortex of adult mice I was able to induce proliferation in oligodendroglial cells. Analysis of the fast-proliferating pool revealed that the proliferative fraction in the knockout was reduced by a third as compared to the wild-type. Thus, Cyclin D1-dependent proliferation is not restricted to slow-proliferating cells.

Taken together, the endogenous and injury-induced proliferation of adult OPCs are downregulated in *Cyclin D1*$^{-/-}$ mice (Fig. 6-2A, B). The mechanisms which drive oligodendrogenesis during adulthood and thus regulate endogenous or injury-induced remyelination are Cyclin D1-dependent. In contrast, the mechanisms driving proliferation of oligodendrocytes during development and thus regulating normal myelination are independent of Cyclin D1. Thus, one can conclude that there is a switch in the requirement for distinct cell cycle proteins driving proliferation of OPCs during development and in the adult.

This theory would match with previous investigations on the role of Cyclin D1 in the peripheral nerve system (PNS) which indicated a differential requirement

for Cyclin D1 (Atanasoski et al., 2001): while developmentally regulated proliferation was not affected in *Cyclin D1*$^{-/-}$ mice, injury-induced proliferation was impaired in immature and mature Schwann cells.

Most NG2 cells in vivo exhibit a complex stellate morphology with many fine processes that contact neurons at synapses and nodes of Ranvier – not the simple morphology one might expect of immature progenitor cells (Butt et al., 1999; Ong and Levine, 1999). Bergles and collaborators were the first to demonstrate that OPCs receive functional glutamatergic synapses from neurons in adult mice (Bergles et al., 2000). It was presumed that NG2 cells are 'listening in' to electrical activity, which at some threshold might trigger their myelination programme and thus ensure that only active circuits are myelinated (Fields, 2008). An answer to this was the identification of two different classes of OPCs: one class capable to generate action potentials and to sense its environment by receiving excitatory/inhibitory synaptic input from axons and a second class that lacks action potentials and synaptic input (Karadottir et al., 2008). Interestingly, the two electrophysiological subtypes of NG2 cells were found in almost equal proportions as the dividing and non-dividing subtypes (Psachoulia et al., 2009). The authors suggest that newly generated NG2 cells attach to unmyelinated axons, some of which fire action potentials and deliver a mitogenic signal. These NG2 cells consequently divide, renewing themselves and producing myelinating oligodendrocytes. The other NG2 cells are associated with axons that never fire, or do not fire above a sufficient threshold, so these cells are destined to remain mitotically inactive (Psachoulia et al., 2009). It is also known, that when oligodendroglial cells cease to express the OPC-marker NG2 and become postmitotic they simultaneously start with the expression of APC which is maintained during oligodendrocyte maturation (Bhat et al., 1996; Kitada and Rowitch, 2006). Thus APC can be used as a marker for mature oligodendrocytes.

I could show that in the prefrontal cortex of Cyclin D1 knockout animals the total number of APC+ cells was reduced by 39%. Thus I could conclude that the knockout of Cyclin D1 has an impact on the number of mature oligodendrocytes (Fig. 6-2A). To see whether this reduction results in decreased myelination, I examined qualitatively the MBP staining in adult *Cyclin D1*$^{-/-}$ mice but I could not detect any obvious alteration. This could be explained by the fact that the loss of Cyclin D1 does not affect the number of oligodendroglial cells at early postnatal stage. Hence, myelination in the young, intact animal is not affected. In contrast, the reduced pool of OPCs observed at adult stage means that there are less premyelinating cells capable to replace dying oligodendrocytes.

Although the number of oligodendrocytes is reduced in adult Cyclin D1 knockout animals, the mutant mice showed – except fot the leg-clasping reflex – no obvious signs of neurological deficits comparable to the ones that can be observed in mice with hypomyelination (Readhead and Hood, 1990). Thus, I concluded that although the absence of Cyclin D1 results in a severe reduction of oligodendrogenesis during adulthood, there are apparently more than enough premyelinating oligodendrocytes which were generated during early postnatal stages and could contribute to remyelination if needed. Thus, incomplete remyelination in Cyclin D1 knockout animals could only occur if the pool of premyelinating oligodendrocytes would be depleted. To answer this question, one would need to analyse ageing *Cyclin D1*$^{-/-}$ mice after a very long period which would probably exceed the normal lifespan of mice. It is known, that remyelination typically produces myelin sheaths thinner and shorter than those produced during original development (Franklin and Ffrench-Constant, 2008). Thus, if Cyclin D1 has a direct impact on remyelination, this could be revealed by analysis of the myelin at the ultrastructural level using electron microscopy.

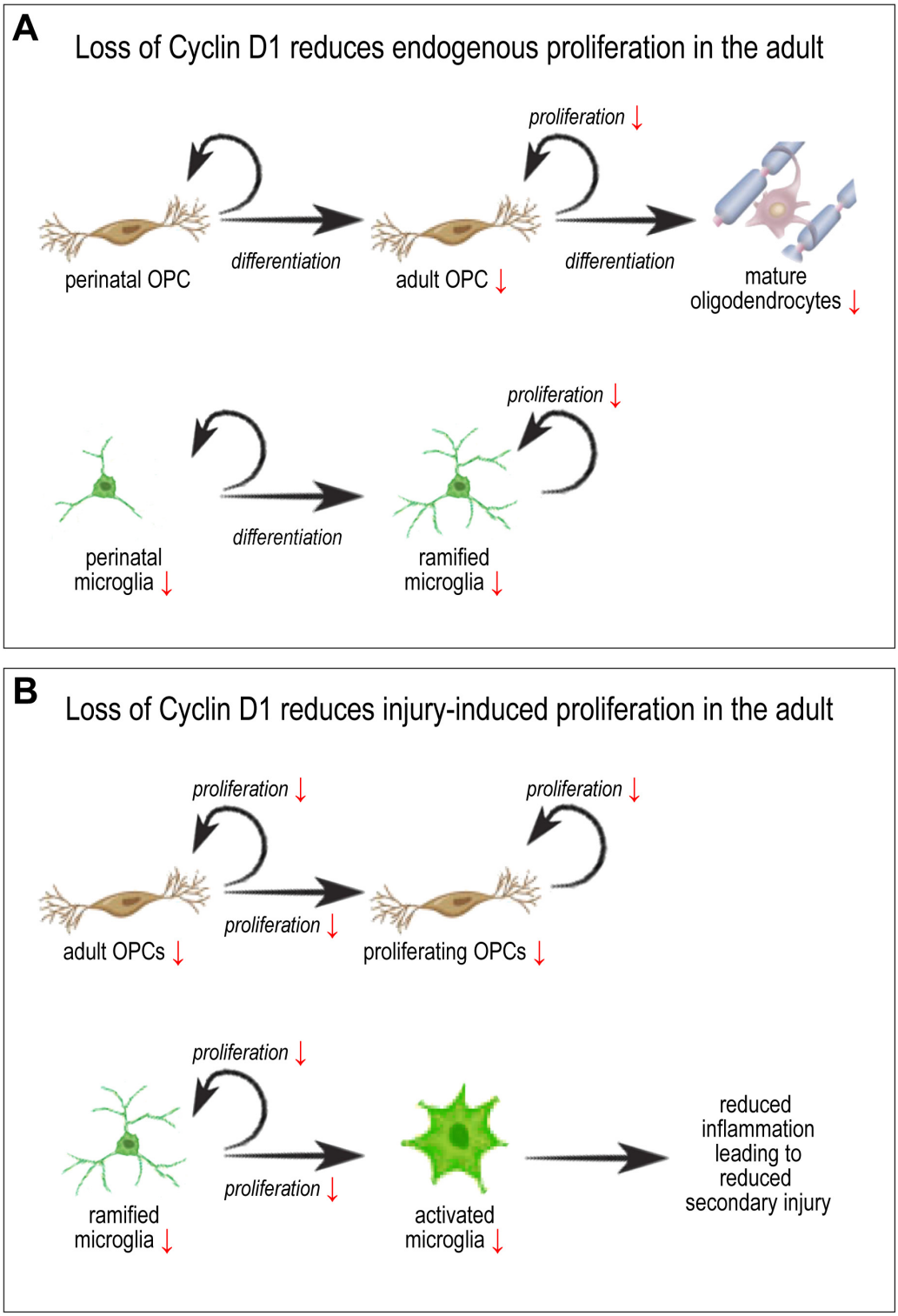



**Figure 6-2: Loss of Cyclin D1 within the intact cortex and following cortical injury**
**A** Loss of Cyclin D1 reduces proliferation of OPC and microglia leading to reduced number of premyelinating oligodendrocytes and ramified microglia. In addition, it affects early microglial development such that the number is already reduced at perinatal stage. **B** Loss of Cyclin D1 impairs injury-induced proliferation of OPCs and microglia, which leads to a lower number of activated microglia and furthermore to a reduced inflammation which is reflected in a reduced secondary injury. ↓ indicates the reduction in either cell number or proliferation rate due to Cyclin D1 knockout.

## 5.7 Knockout of Cdk4 but not Cdk2 or Cdk6 impairs the proliferation of OPCs

As Cyclin D1 is the regulatory subunit of the holoenzyme formed with the catalytic subunits Cdk4 and Cdk6, one could assume that knockout of the catalytic subunit might have similar effects as long as the absence of Cyclin D1 is not compensated by one of its other isoforms.

My analysis following cortical injury in *Cdk2*$^{-/-}$, *Cdk4*$^{-/-}$ *and Cdk6*$^{-/-}$ mice provide the evidence that the fast-proliferating oligodendroglial cells are dependent on Cdk4 but not on Cdk2 or Cdk6. Moreover, I could observe a stronger impact on the proliferation of OPCs in the Cdk4-knockout as compared to the Cyclin D1-knockout. Although the pattern of expression of D-cyclins is distinct in different tissues, functional redundancy exists among them (Ciemerych et al., 2002; Ciemerych and Sicinski, 2005). It has been shown, that during development animals retaining only one D-type cyclin lose the tissue-specific expression characteristic of that gene concomitantly upregulating the remaining D cyclin (Ciemerych et al., 2002). Thereby absence of the regulatory protein Cyclin D1 could be partially compensated by another. Redundancy or developmental compensation could mask the functions that a deleted gene may ordinarily control. In contrast, absence of the catalytic binding partner Cdk4 completely shuts down the Cdk4-pathway.

Several publications examined the Cyclin D1 binding partner Cdk4 in injury paradigms. Following controlled cortical impact injury in rat brain, Kaya et al. observed selective expression of Cyclin D1 and Cdk4 in morphologically intact or injured neurons throughout the rat brain (Kaya et al., 1999). In addition, apoptotic cells were neither immunoreactive to Cyclin D1 nor to Cdk4. Analysis of the cell types revealed that reactive astrocytes did not express Cyclin D1, whereas some astrocytes at the boundary of the lesion showed nuclear Cdk4

immunoreactivity. In contrast, microglia expressed Cyclin D1, while Cdk4 could not be detected. Another analysis in a focal stroke model showed increased Cdk4 and Cyclin D1 levels in neurons and that these signals are required for the death of neurons (Osuga et al., 2000). Taken together, it is most likely that Cyclin D1 and Cdk4 interact following injury to the adult cortex.

Concerning Cdk2, not much is known about its function in the CNS. A previous study of Caillava et al. revealed that in response to demyelination, loss of Cdk2 alters adult OPC renewal, cell cycle exit, and differentiation thereby accelerating remyelination. The authors concluded that Cdk2 is important for adult OPC renewal, and could be one of the underlying mechanisms that drive adult progenitors to differentiate and thus regenerate myelin (Caillava et al., 2011).

In the PNS, Atanasoski et al. (2008) have shown that Cdk4, but not Cdk2 or Cdk6, is essential for postnatal proliferation of Schwann cells. The Cdk4 null mice displayed a striking decrease in the number of mitotically active Schwann cells whereas no such difference was observed in mice lacking Cdk2 or Cdk6 (Atanasoski et al., 2008). Interestingly, loss of Cdk4 had no effect on the myelination of sciatic nerves. Thus, it could be concluded that postnatal proliferation is not required for the establishment of appropriate Schwann cell numbers, suggesting that the number of Schwann cells produced before birth is sufficient to ensure subsequent myelination of axons. Atanasoski et al. (2008) also studied whether Cdk4 expression was required for proliferation following nerve injury. They showed that Schwann cells lacking Cdk4 were unable to re-enter the cell cycle which completely abolished Schwann cell proliferation, while Schwann cells lacking Cdk2 or Cdk6 displayed proliferation rates comparable to controls (Atanasoski et al., 2008).

Taken together, I propose Cdk4 to be the interaction partner of Cyclin D1, regulating injury-induced proliferation of OPCs within the lesioned cortex.

## 5.8 Cyclin D1 is not essential for the adequate generation of mature neurons

From previous studies, it is known that the size of the brain is reduced in *Cyclin D1*$^{-/-}$ mice (Fantl et al., 1995; Sicinski et al., 1995). Analysis of the Cyclin D1 expression revealed that it is expressed in post-mitotic neurons in the forebrain of adult mice (Tamaru et al., 1994; Glickstein et al., 2007a). In addition, cortical thinning of cortical lamina has been observed in adult knockout brain, while the densities of parvalbumin- and somastatin-expressing interneurons remained preserved (Glickstein et al., 2007b). But it remained an open question whether the absence of Cyclin D1 affects the number of neurons in general.

Thus, I investigated whether the number of neurons is changed in the Cyclin D1 knockout mice. As previously described, I could detect a decrease in cortical thickness in the Cyclin D1 knockout. Surprisingly, I could measure an increased neuronal density in the knockout. But as this increase of density was elevated by the same ratio as the decrease in cortical thickness, the absolute number of neurons was comparable in *Cyclin D1*$^{-/-}$ and wild-type mice. As a result, one can conclude that Cyclin D1 is dispensable for the generation of the proper number of neurons. The thinning of the cortex must be due to reduced numbers of glial cells and / or reduced numbers of synapses.

## 5.9 Concluding remarks

In conclusion, the present sudy shows that absence of Cyclin D1 reduces the pool and the endogenous proliferation of OPCs in the adult brain but with no obvious effect on myelination. Furthermore, it could be shown that the proliferation of OPCs following neurotoxic injury to the cortex is dependent on Cyclin D1 and its catalytic interaction partner Cdk4. Last but not least, it could be shown that

Cyclin D1 is a prerequisite for migration and proliferation of microglia which in turn results in reduced inflammation in the case of brain injury in *Cyclin D1*$^{-/-}$ animals.

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# CURRICULUM VITAE

## PERSONAL PROFILE


| | |
|---|---|
| **Name:** | **Lionel Nobs** |
| Date of Birth: | 21st February 1981 |
| Home Town: | Münchenstein (BL), Switzerland |
| Address | Gustav Wenk-Strasse 13<br>4056 Basel |
| Phone | +41 79 289 15 72 |
| e-mail | lionel.nobs@stud.unibas.ch |


## EDUCATION AND RESEARCH

| | |
|---|---|
| 2007 - 2012 | PhD thesis in neurobiology<br>"The impact of Cyclin D1 on proliferating cells in the intact and injured mouse cortex"<br>Supervision: Prof. Dr. Suzana Atanasoski<br>Laboratory of Cellular Neurobiology, Institute of Physiology, Department of Biomedicine, University of Basel |
| 2006 | Internship, Molecular Zoology, University of Basel |
| 2005 | Master of Science in Molecular Biology<br>"Tracing single neuroblast lineages from the embryonic neurectoderm to the third instar larval brain of *Drosophila Melanogaster*"<br>Supervision: Prof. Dr. Heinrich Reichert<br>Molecular Zoology, Biozentrum, University of Basel |
| 2000 - 2004 | Bachelor of Science in Biology,<br>Major in Molecular Biology<br>Minor in Computer Science<br>University of Basel |
| 1997 - 2000 | Matura Typus C (mathematics and natural sciences)<br>Gymnasium Münchenstein (BL)<br><br>Matura thesis<br>"Chaotic Nuclear Migration (Cnm67p)"<br>Supervision: Florian Schaerer<br>Laboratory of applied microbiology, Biozentrum, University of Basel |

## FURTHER TRAINING

| | |
|---|---|
| 2008 | Lab Animal Experiments Licence: LTK Module 1, University of Zürich |

## PUBLICATIONS

**Lionel Nobs**, Sigrun Nestel, Akos Kulik, Cordula Nitsch, Suzana Atanasoski. 2012. Differential requirement for cyclin D1 in glial cells of the intact and injured cerebral cortex. *submitted.*

Robert Lichtneckert, **Lionel Nobs**, Heinrich Reichert. 2008.
Empty spiracles is required for the development of olfactory projection neuron circuitry in Drosophila. Development. 2008 Aug;135(14):2415-24.

## POSTER PRESENTATION

FENS Forum 2010 - Amsterdam
**Nobs L**, Nitsch C, Atanasoski S. Poster presentation: The role of cyclin D1 in adult mouse brain and following cortical injury. FENS Abstr., vol.5, 132.29, 2010.

## GRANT

2011 Freiwillige Akademische Gesellschaft (FAG), Basel

## COMPUTER SKILLS

2001 - 2003 Degree in Informatics (Minor), Institute for Informatics, Univ. of Basel

Confident user of all Microsoft Office applications, Good knowledge of image processing software (ImageJ, Adobe Photoshop) and microscopy software (Leica Confocal Software, Zeiss AxioVision)

## LANGUAGES

German: native language

French: native language

English: fluent written and spoken (Cambridge Certificate in Advanced English)

## REFERENCE

**Suzana Atanasoski**
Assistant Professor
Department of Biomedicine
University of Basel
Pestalozzistrasse 20
CH-4056 Basel
Switzerland
e-mail: suzana.atanasoski@stud.unibas.ch
phone: +41 61 267 35 44