Redox-dependent decrease of neurogenesis in the aging brain - Contribution of Sirt1

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„They said I couldn’t. That’s why I did!“

Für alle „unsichtbar Kranken“ – Diese Arbeit beweist:

Alles ich möglich, wenn ihr eure Erkrankung als Teil von euch akzeptiert und dafür kämpft, dass auch andere dies tun.
Lasst euch niemals unterkriegen!!!

Abstract

Aim Nowadays improvement of medical treatments results in higher life expectance of the human population, increasing the importance of fighting aging related cognitive deficits. Those deficits are mainly caused by replicative senescence, resulting in decreased neurogenesis in the adult brain, especially in the hippocampus, the structure essential for learning and memory. During the process of aging, oxidative stress increases in various tissues as well, including the brain. Along with other harmful impacts for the cells, oxidative stress leads to increased DNA damage. The NAD^{+}-dependent histone deacetylase Sirt1, mitochondrial Sirt3 and the transcription factor Nrf2 are part of the machinery challenging these oxidative conditions. Sirt1 has been shown to be neuroprotective before, while it is not clear which role Sirt1, Nrf2 and Sirt3 play regarding adult neurogenesis and if they possibly are capable of keeping neurogenesis high, even in the aged brain. Therefore this study was designed to elucidate the functions of Sirt1, Nrf2 and Sirt3 on adult neurogenesis.

Methods Immunohisto-/ -cytochemical analyses of brain sections / NPC cultures as well as gene and protein expression analyses of brain tissue / NPCs (PCR, Western Blot) were used to investigate effects of Sirt1 / Sirt3 / Nrf2 deficiency on neurogenesis and cell cycling. Results were compared to those obtained from wildtype littermate control animals / NPCs. Neurogenesis can be separated into proliferation, differentiation and maintenance of stemness of neuronal precursor cells. To assess proliferation, BrdU assays (*in vitro* and *in vivo*), immunohistochemical staining for proliferation markers like H_3Ser_{10} as well as measurement of neurophere forming capacity (*in vitro*) was performed. To analyse differentiation of neuronal precursor cells, the number of $Sox2^{+}$ stem cells, $Olig2^{+}$ or $NG2^{+}$ oligodendrocyte precursors and DCX^{+} neuronal precursors was quantified *in vitro* as well as *in vivo*. Maintenance of stemness / induction of stem cell exhaustion was assessed by measuring gene induction of genes relevant for cell cycling /cell cycle inhibitors as well as immunocytochemical staining for markers of DNA repair mechanisms.

Results In wildtype animals, hippocampal neurogenesis is decreased upon aging, accompanied by upregulated gene expression of sirtuins, antioxidant enzymes and cell cycle inhibitors. In adult mice, Sirt1 is expressed in stem cells of the hippocampal subgranular zone (SGZ) and is upregulated upon aging. In aged mice, systemic as well as brain specific loss of Sirt1 function significantly increased the subgranular zone stem cell pool. Migratory capacity of maturating stem cells is not affected by loss of Sirt1 function in animals which underwent Sirt1 silencing *in utero* by targeted electroporation. *In vitro,* wt neuronal precursor cells exposed to in prolonged culturing conditions show decreased proliferative capacities, increased Sirt1 expression and hallmarks of aging or replicative senescence. Neuronal precursor cells expressing unfunctional Sirt1 showed higher proliferation rates than wt neuronal precursor cells, even in prolonged culturing conditions. This effect was positively correlated with the concentration of unfunctional Sirt1. In contrast to wt cells, neuronal precursor cells expressing unfunctional Sirt1 do not stop proliferating upon oxidative stress induced by BSO or metabolic stress induced by glucose deprivation.

Conclusion Aging-associated oxidative stress affects HC neurogenesis. Upregulation of Sirt1 upon aging might be caused by increased oxidative stress in the aged brain. In general Sirt1 may play a role in limiting stem cell proliferation in the subgranular zone. The same holds true *in vitro* especially under stressful culturing conditions and prolonged culturing. Sirt1 might be an important factor in control of mitotic activity of neuronal precursor cells in response to metabolic stress.

Zusammenfassung

Hintergrund Die immer besser werdende medizinische Versorgung des Menschen bedingt eine höhere Lebenserwartung, wodurch die Bekämpfung altersbedingter kognitiver Defizite immer wichtiger wird. Grund dieser Defizite ist zu einem Großteil die replikative Seneszenz, die eine verringerte Neurogenese im adulten Gehirn hervorruft. Der Hippocampus als essenzielle Struktur für Lernen und Erinnerungsvermögen ist davon besonders betroffen. Während des Alterns steigt der oxidative Stress in verschiedensten Geweben, unter anderem auch im Gehirn, an. Zusammen mit anderen zellschädigenden Einflüssen schädigt er die DNA. Die NAD^{+}-abhängige Histondeacetylase Sirt1, mitochondrielles Sirt3 und der Transkriptionsfaktor Nrf2 sind Bestandteile der Maschinerie zur Bekampfung des oxidativen Stresses. Eine neuroprotektive Funktion von Sirt1 ist bereits bekannt, unklar ist jedoch, welche Rolle Sirt1, Nrf2 und Sirt3 bei der adulten Neuro-genese spielen und ob sie die Neurogenese im adulten Gehirn sogar erhöhen könnten. Die vorliegende Arbeit soll also die Funktionen von Sirt1, Nrf2 und Sirt3 im Prozess der adulten Neurogenese aufklären.

Methoden Um die Effekte von Sirt1 / Sirt3 / Nrf2 auf die Neurogenese und den Zellzyklus zu untersuchen, wurden Immunohisto-/ -zytochemische Analysen von Hirnschnitten / NPC Kultuen sowie Gen- und Proteinexpressionsanalysen (PCR, Western Blot) durchgefüht, Die Ergebnisse wurde mit denen von Wildtyp (wt)-Tieren / Zellkulturen verglichen. Der Prozess der Neurogenese gliedert sich in Proliferation, Differenzierung und Erhalt der Stammzelleigenschaften der neuronalen Vorläuferzellen (NPCs). Zur Untersuchung der Proliferation, wurden sowohl BrdU-Analysen (*in vitro* und *in vivo*) und immunohistochemische Färbungen von Proliferationsmarkern wie H_3Ser_{10}, als auch Messungen der Kapazität von NPCs, Neurospären zu bilden (*in vitro*), herangezogen. Zur Analyse der Differenzierung von NPCs wurden $Sox2^{+}$ Stammzellen, $Olig2^{+}$ bzw. $NG2^{+}$ Oligodendroztenvorläuferzellen und DCX^{+} neuronalen Vorläuferzellen *in vitro* sowie *in vivo* quantifiziert. Der Erhalt der Stammzelleigenschaften bzw. beginnender Verlust selbiger durch zelluläre Seneszenz wurde mit Hilfe von Messungen der Induktion von Genen untersucht, die relevant für den Zellzyklus bzw die Inhibierung des Zellzyklus sind. Zusätzlich wurden immunozytochemische Färbungen von Markern für DNA Reparaturmechanismen ausgewertet.

Ergebnisse In wt-Tieren nimmt die Neurogenese während des Alterns ab, was einhergeht mit einer erhöhten Genexpresionen von Sirtuinen, antioxidativen Enzymen und Zellzyklusinhibitoren. In adulten Mäusen wird Sirt1 in Stammzellen der hippokampalen subgranulären Zone (SGZ) exprimiert. Die Expression wird während des Alterns hochreguliert. In gealterten Mäusen führt systemischer sowie hirnspezifischer Verlust von funktionellem Sirt1 zu einer signifikanten Vergrößerung des subgranulären Stammzellpools. Bei Tieren, in denen Sirt1 durch gezielte *in utero* electroporation ausgeschaltet wurde, bleibt die migratorische Kapazität der reifenden Stammzellen unverändert. *In vitro* weisen NPCs in Langzeitkultur verminderte Porilferation, erhöhte Sirt1 Expression und Anzeichen von Alterung und replikativer Seneszenz auf. NPCs, die unfunktionelles Sirt1 exprimieren, zeigen im Vergleich dazu eine höhere Proliferationsrate, selbst in Langzeitkultur. Dieser Effekt korreliert positiv mit steigender Konzentration von unfunktionellem Sirt1. Im Gegensatz zu wt-NPCs stoppt die Proliferation von NPCs, die unfunktionelles Sirt1 exprimieren, bei Induktion von oxidativem Stress mit Hilfe von BSO oder metabolischem Stress durch Glucoseentzug nicht.

Fazit Oxidativer Stress, der während des Alterns auftritt, beeinflusst die hippokampale Neurogenese. Im adulten Gehirn könnte die Hochregulation von Sirt1 während des Alterns eine Reaktion auf erhöhten oxidativen Stress sein. Generell könnte Sirt1 eine Rolle bei der Limitierung der Stammzellproliferation in der SGZ spielen. Dies zeigt sich auch *in vitro*, speziell in stressinduzierenden Kulturbedingungen und Langzeitkulturen. Sirt1 könnte ein wichtiger Faktor für die Kontrolle der mitotischen Aktivität von NPCs als Reaktion der Zelle auf metabolischen Stress sein.

Content

1. Introduction

Prevention of aging related diseases and ensuring healthy aging becomes increasingly important, as people are getting older today due to better medical treatments and food supply. Severe aging-related diseases like Alzheimer's or Parkinson's Disease occur at a higher incidence, due to this trend. Even without these diseases, aging is accompanied by memory loss, loss of learning abilities and dementia. Since 1965 it is known that neurogenesis occurs during the whole life of a mammal, but declines severely upon aging (Altman & Das 1965; Couillard-Després 2013). Neurogenesis is of high importance to maintain memory and learning abilities. In the hippocampus (HC) decreased neurogenesis in aging is maily caused by the loss of proliferative capacity of SGZ stem and progenitor cells (Bondolfi et al., 2004). Differentiation capacity is not decreased upon aging, as morphology, dendritic spine density and electrophysiological properties do not differ in newborn granular neurons in HC of young compared to old mice (van Praag et al., 2005; Couillard-Despres et al., 2006).

HC neurogenesis has been proven to be essential for spatial relational memory in adult mice. A mouse model for induced, specific death of HC NPCs showed severely decreased spatial relational learning abilities in behavioural tests like object recognition test and Morris water maze (Dupret et al., 2007). Supporting this, interventions enhancing HC neurogenesis improved cognitive function of mice (van Praag et al., 2005). These results lead to the assumption that age-related decrease in HC neurogenesis may contribute to cognitive deficit in elderly subjects.

Another important hallmark of the aging brain is the increase of oxidative stress (Dorszewska 2013), which is suspected to decrease neurogenesis. Molecules like silent mating type information regulator 1 (Sirt1), Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and Sirt3 have been shown to fight oxidative stress in various tissues, including the brain (Prozorovski et al., 2008; Sandberg et al., 2014; Wu et al., 2014). Therefore this study was designed to elucidate the still unknown function of these molecules regarding maintenance of adult neurogenesis.

1.1 Adult neurogenesis

Neurogenesis is an ongoing process in the adult brain. The new-born neurons contribute to memory and learning function as well as olfaction. Neurogenesis in the adult mouse brain has been observed in two areas: The hippocampal (HC) subgranular zone (SGZ) and the subventricular zone (SVZ) of lateral ventricle. SVZ neurogenesis can be divided into four stages (fig. 1): During the first stage, neuronal stem cells (NSCs) proliferate (B cells). During the second stage, called fate specification, the cells start to differentiate into immature neurons (neuroblast / A cells). During the third stage neurons migrate to the olfactory bulb (OB) via the rostral migratory stream (RMS). In the fourth and last stage the immature neurons differentiate into mature neurons and develop synaptic contacts.

SGZ neurogenesis can be divided into five stages (fig.1). During the first stage, stem cells proliferate, with their cell bodies located within the subgranular zone (SGZ) of the dentate gyrus (DG). In the second stage they start to differentiate into immature

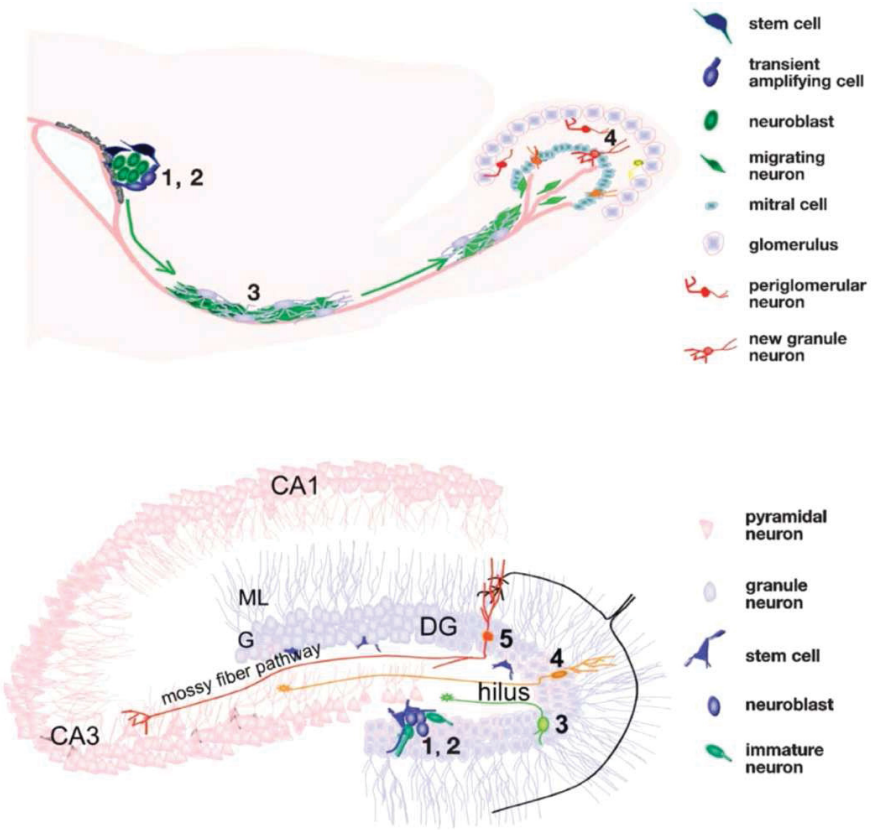

Figure 1: Adult neurogenesis. Adult neurogenesis occurs in the SVZ (top) and SGZ (bottom). Top: NSCs proliferate in the SVZ (1,2) migrate via the RMS (3) towards the OB (4) where they differentiate into mature neurons and integrate into the granular layer. Bottom: In the HC, NSCs proliferate in the SGZ (1,2). During differentiation they migrate into the granular layer of the DG (3,4). After full maturation they form mossy fibre connections to the CA3 region and the molecular layer (ML) (5). (figure from Ming & Song, 2005)

neurons. Migration into the granule cell layer takes place during the third stage. Stage four is known as the axon / dendrite targeting stage: The immature neurons extend their axonal projections along mossy fibre pathways to the CA3 pyramidal cell layer. The dendrites grow the opposite way, towards the molecular layer. In the fifth and last stage synaptic integration of the now fully differentiated new granule neurons takes place. During those stages different stem cell populations are active, which can be distinguished by different immunohistological markers (fig. 2): Type-1 progenitor cells ($GFAP^+/Nestin^+/Sox2^+$) are considered to be the resident stem cell population in the adult HC and can be subdivided into two populations, the actively proliferating, though slow cycling Type-1a cells and the quiescent Type-1b cells (Kempermann et al., 2004). Type-1b cells are proliferating exclusively under special stimuli like seizures (Indulekha et al., 2010). Upon differentiation stimuli, Type-1 progenitors maturate and become fast cycling, $GFAP^-$ Type-2 progenitors ($Nestin^+/Sox2^+$) (Kempermann et al., 2004). Those can be subdivided into two populations, too: Type-2a ($Nestin^+/Sox2^+$) and Type-2b ($Nestin^+/Sox2^+/DCX^+$,$NeuroD1^+$) cells (Steiner et al., 2006). Proliferation of Type-2a cells can be enhanced by physical activity, whereas Type-2b cells show increased proliferation upon seizures or in mice living in enriched environments (Steiner et al., 2008). Upon differentiation stimuli, Type-2 cells further develop into Type-3 neuroblasts ($DCX^+/NeuroD1^+$). The latter finally give rise to mature granule cell neurons (Ming & Song, 2005).

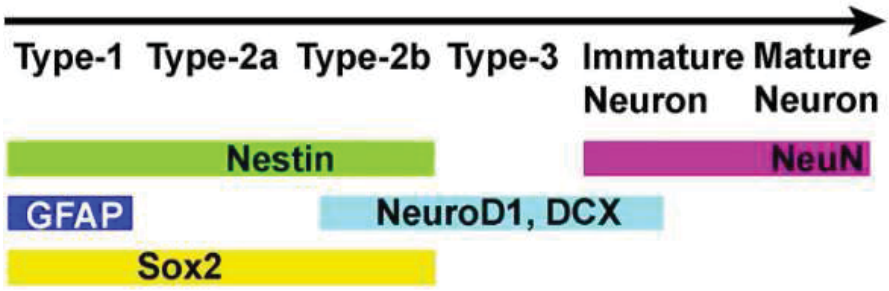

Figure 2: Stages of HC neurogenesis. During neurogenesis, stem cells can be subdivided into different cell types regarding their status of maturation. Those types can be distinguished by immunocyto/histochemical staining of the markers Nestin, neuronal nuclear antigen (NeuN), glial fibrillary acidic protein (GFAP), Neurogenic differentiation 1 (NeuroD1), doublecortin (DCX) and sex determining region Y-box 2 (Sox2) (modified after Kim et al., 2011b).

Most progeny of HC NPCs develop into dentate granule neurons, but some become glia (Cameron et al., 1993). Newborn dentate granule cells (DGCs) exhibit much differrent characteristics compared to mature DGCs. A long process of physiological and morphological changes is necessary for their maturation (Zhauo et al., 2006).

1.1.1 Regulation of adult neurogenesis

Adult neurogenesis is regulated by many intrinsic and extrinsic factors at different stages, including proliferation, fate specification, migration, integration and survival. Many proliferating, gliogenic and neurogenic signals can be found in the neurogenic niches. Following, the most important processes regulating adult neurogenesis are described:

It has been shown that active Notch signalling persists in the HC and Notch is highly expressed in neurons of the adult HC (Berezovska et al., 1998). Notch signalling is essential for NSC maintenance, proliferation, and survival during development and controls stem cell maintenance and dendritic morphology of newborn granule neurons in the adult brain (Ehm et al., 2010; Wang et al., 2004; Imayoshi et al., 2010; Breuning et al., 2007; Lugert et al., 2010).

Bone morphogenic protein (BMP) ligands like Noggin, as well as BMP receptors have been shown to be expressed by Nestin$^+$ NSCs in the adult HC (Mira et al., 2010), limiting BMP signalling to proliferative cells of the SGZ. BMP maintains cells in quiescent (mitotically not active) state, blocks differentiation and thus, is involved in long-term activity of NSCs in the adult HC (Mira et al., 2010; Bonaguidi et al., 2008; Colak et al., 2008). Additionally, the regulation of NSC proliferation and differentiation by p53 is BMP-dependent (Liu et al., 2013).

Another signalling pathway involved in regulation of adult neurogenesis is Wnt-signalling. Wnt ligands are mostly secreted by astrocytes. Activated Wnt signalling, has been shown to increase proliferation in the adult SVZ and SGZ (Adachi et al., 2007; Ikeda et al., 2010; Cui et al., 2010). In contrast to this, canonical Wnt signalling leads to the transition of a subset of proliferating progenitor cells to a quiescent state (Lugert et al., 2010). Wnt3 seems to be the most important molecule of the Wnt family for regulation of adult HC neurogenesis (Lie et al., 2005).

Neuronal nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signalling has been shown to affect adult neurogenesis by maintenance of neuronal survival, proliferation, migration, differentiation, synaptogenesis, neural plasticity, learning and memory (Fridmacher et al., 2003; Mattson & Meffert 2006; Camandola & Mattson 2007; Boersma et al., 2011; Rolls et al., 2007; Widera et al., 2007; Zhang et al., 2012).

Sonic hedgehog (Shh) is essential for neural development and regulates adult HC stem cell proliferation (Lai et al., 2003; Ahn & Joyner, 2005; Favaro et al., 2009).

Additionally it is required for progenitor cell maintenance in the adult HC (Machold et al., 2003).

Foxo transcription factors, especially Foxo3, have been shown to preserves adult NPC function (Paik et al., 2009). Additionally, Foxos seem to play some role in differentiation of NPCs (Renault et al., 2009).

Several factors of the INK4 family are also known to regulate neurogenesis: Cyclin-dependent kinase 4 inhibitors C ($p18^{INK4c}$) and D ($p19^{INK4d}$) are expressed during neurogenesis, whereas p15INK4b and p16INK4a are absent during development. p19 is crucial for keeping cells in a non-cycling state. It permanently blocks cycle of postmitotic cells at the G1 phase, hindering already differentiated cells to enter the cell cycle again (Coskun & Luskin 2001; Zindy et al.,1997).

p21, a cyclin dependent kinase inhibitor, controls adult neurogenesis by regulating Sox2 gene expression. Loss of p21 leads to loss of Sox2, replicative stress and induces DNA damage responses. Those lead to cell growth arrest and premature exhausttion of the stem cell pools (Marqués-Torrejón et al., 2013). Additionally, p21 deficient NPCs show higher proliferation rates *in vivo* and *in vitro*. Those cells mainly give rise so DCX^+ neuroblasts. p21 is especially induced in inflammatory conditions, where it might lead to increased astrogliogenesis (Zonis et al., 2013). The cyclin-dependent kinase inhibitor p16 inhibits proliferation and differentiation of NPCs, keeping them in a non-cycling, quiescent senescent state. It specifically leads to G1 cell-cycle arrest, by acting in two ways: On the one hand by activation of the Rb pathway, on the other hand by increasing intracellular ROS levels (Takahashi et al., 2006). p16 is also used as a biomarker for detection of cellular senescence.

HC-dependent learning is one of the major regulators of HC neurogenesis (Gould et al., 1999). The number of adult-born granular neurons is increased by learning of HC-dependent, but not independent tasks (Gould et al., 1999; Epp et al., 2007; Leuner et al., 2004 and 2006). As an example, neurogenesis is regulated by HC dependent spatial learning in the Morris Water Maze. This type of learning leads to enhanced survival of newborn granular neurons in the DG (Dupret et al., 2007).

Aged animals show decreased abilities to solve behavioural test like those explained above (van Praag et al., 2005). This might be caused by the aging systemic environment, leading to cellular senescence, which results in impaired neurogenesis (Villeda et al., 2011).

1.1.2 Adult neurogenesis and aging induced cellular / replicative senescence

Cellular senescence has been described as a stable arrest of the cell cycle coupled to stereotyped phenotypic changes (Campisi & d'Adda di Fagagna 2007; Collado et al., 2007; Kuilman et al., 2010). Nowadays, cellular senescence is thought to be the major reason for mammalian aging. Additionally it is known as a mechanism for tumour suppression. Declining regenerative potential due to cellular senescence of stem cells has also been found in the aged mouse forebrain (Molofsky et al., 2006). Neurogenesis in SGZ (Kuhn et al., 1996; Kempermann et al., 2002b) as well as SVZ (Jin et al., 2003). Bromodeoxyuridine^{+} (BrdU^{+}) dividing cells as well as DCX^{+} neuroblasts are severely reduced in the brains of aged mice, already starting by middle age (Luo et al., 2006).

In vivo cellular senescence is crucial to avoid the development of cancer. Additionally it is supposed to induce inflammation associated with aging and cancer. Hallmarks for cellular senescence like permanent, non reversible cell cycle arrest, β-galactosidase expression and morphological changes are induced by several stimuli. The best known stimulus is telomere shortening. Telomeres are regarded as molecular clock, reflecting the replicative history of a primary cell (Harley et al., 1990). With each cell division, a bit of telomere length is lost. If they reach a critical minimal length, their DNA protecting function is lost, making the DNA more sensitive towards damages. If this damage occurs, are activated. This can be visualized via immunostaining by an increased amount of phosphorylated histone H2AX inside the cells (D'Adda di Fagagna et al., 2003). DNA-damage responses lead to the phosphorylation and thereby activation of cell cycle proteins like tumour suppressor p53 (Fujita et al., 2009). Transient cell cycle arrest is induced, until DNA damage is repaired. If repair is not accomplished, cells either become senescent / stop to proliferate or apoptotic responses are induced. Telomerase deficient mice show accelerated aging. After restoring telomerase activity in these mice, the CNS rejuvenates: Telomeres are rapidly elongated, leading to restored endogenous CNS stem cell function, increased neurogenesis and oligodendrocyte numbers and reversion of age-related myelin loss (Jaskelioff et al., 2011).

Another factor contributing to cellular senescence is the RB tumour suppressor pathway, including for example the cyclin-dependent kinase inhibitors p16^{INK4a} and alternate reading frame tumour suppressor (p19^{ARF}). p16^{INK4a} is induced by culture

stress and upon telomere or DNA damage (Le et al., 2010) and upon aging in mice as well as in humans (Zindy et al., 1997; Liu et al., 2009). Activation of $p16^{INK4a}$ leads to a reduction of progenitor cell numbers in multiple tissues including the brain. $p16^{INK4a}$ deficient mice show smaller age-related reductions of neurogenesis, at least in the SVZ, but increased cancer incidents, supporting the role of $p16^{INK4a}$ and cellular senescence in general for avoiding cancer (Molofsky et al., 2006).

$p19^{ARF}$ is the main senescent marker in mice. Arf acts as a tumour suppressor in rodents (Kamijo et al., 1997).

Together with activation of p53 and its downstream effecttors (e.g. cyclin-dependent kinase inhibitor ($p21^{CIP}$)), the aforementioned pathways are essential for induction of cellular senescence and are seen in mouse as well as human cell lines (Ben-Porath & Weinberg 2005; Chen et al., 2005b). Additionally to the pathways described above, neurogenesis also decreases due to lower levels of growth factors like FGF2 and EGF in the aged brain (Bernal & Peterson, 2011).

Senescence of cultured cells by prolonged passaging has been proven as a model for mammalian aging. Stem cell cultures can undergo only a distinct amount of divisions, before cell cycle arrest is reached (Campisi 2000; Hayflick & Moorhead 1961). Cells stay viable, but lose their ability to proliferate. Cellular senescence can be induced prematurely by stressors like inadequate culturing conditions (Sherr & DePinho 2000) or reactive oxygen species (ROS) (Lu & Finkel 2008). *In vitro* Sirt1 levels are decreased in senescent compared to young cells (i.e. 2BS cells (fetal lung fibroblasts) (Huang et al., 2008)).

1.2 Impact of Redox state and oxidative stress on neurogenesis

Intracellular Redox state has been shown to regulate the balance between self-renewal and differentiation in glial precursor cells: Upon self-renewal stimuli, progenitors are characterized by a higher reduced state compared to differentiating cells. During differentiation they become more oxidized (Smith et al., 2000). However, Le Belle and colleagues found that in SVZ NSCs and NPCs show higher levels of ROS as compared to differentiated neurons. These ROS were essential for proliferation and differentiation of those cells (Le Belle et al., 2011). Additionally it has been

shown, that low ROS levels stimulate differentiation of neuronal precursor cells (NPCs) and NSC (Kennedy et al., 2012).

An important molecule for amelioration of oxidative stress in the aging brain is the redox-sensitive transcription factor Nrf2. Upon oxidation, it induces the expression of antioxidant, anti-inflammatory and cytoprotective genes. It has been shown that an imbalance of Nrf2-driven antioxidant / anti-inflammatory genes in the SVZ already starts to occur by middle age. Those effects severely decrease neurogenesis in the SVZ (L'Episcopo et al., 2013).

Using embryonic cultures of NPCs and early postnatal brains, our group previously showed that oxidative conditions suppress proliferation of NPCs and induce their differentiation towards astrocytes at the expense of neurons. In line with this, reducing conditions support specification of NPCs towards the neuronal lineage (Prozorovski et al., 2008). This effect depends on Sirt1, but if Sirt1 regulates neurogenic processes in adult brain remains largely unknown.

1.3 Sirtuins

Sirtuins are highly conserved proteins occurring in all species from prokaryotes to mammals. They possess either mono-ADP-ribosyltransferase, or deacylase activity, including deacetylase, desuccinylase, demalonylase, demyristoylase and depalmitoylase activity. They all share a common core domain of approximately 200–275 amino acids. Sirtuins regulate many cellular processes like aging, transcription, apoptosis, inflammation, stress resistance, mitochondrial biogenesis and energy efficiency (Abdellatif, 2012; Chalkiadaki & Guarente, 2012; Yu & Auwerx, 2009; Duan, 2013). The NAD^+ dependent histone deacetylase Sirt1 as well as mitochondrial Sirt3 recently attracted attention regarding a possible neuroprotective function (Pfister et al., 2008; Kim et al., 2011a). In mitochondria, the generation of reactive oxygen species (ROS) takes place. Sirt3 has been shown to deacetylate the critical antioxidant enzyme manganese superoxide dismutase (SOD2), which is located in the mitochondrial matrix. This leads to an increased specific activity of SOD2 towards fighting ROS (Qiu et al., 2010). Sirt3 has also been shown to have anti-aging effects in mammals. For example, it reduces the calorie restriction mediated reduction of oxidative damage in various tissues via regulation of the

glutathione antioxidant system and mediates the prevention of age-related hearing loss (Someya et al., 2010). Deficiency of Sirt3 leads to dramatic hyperacetylation of mitochondrial proteins, suggesting that Sirt3 is the major mitochondrial deacetylase. Due to its regulation of mitochondrial electron transport chain activity It is considered essential for maintainance of energy homeostasis (Dali-Youcef et al., 2007).

1.3.1 Sirt1

Mouse Sirt1 consists of 737 amino acids that can be divided into an enzymatic core domain and two flanking non-catalytic N- and C-terminal domains. The enzymatic core, which is encoded on exon 4, spans the amino acids from position 236 to 490 (www.ensembl.org). The important regulatory part for functionality of Sirt1 is the ESA (essential for Sirt1 activity) motif consisting of a small region from amino acids position 641–665. It is essential for interaction with the catalytic domain and increases the affinity towards Sirt1 substrates (Kang et al., 2011). The N-terminal extension of Sirt1 contains two functional nuclear localization sequences (NLS) and two nuclear export sequences (NES). The nuclear-cytoplasmic distribution of Sirt1 is regulated by external signals. Upon differentiation stimuli, Sirt1 shuttles from the nucleus to the cytosol (Tanno et al., 2007). Sirt1 regulates the activity of broad spectrum of proteins due to its ability to shuttle between the nuclear and cytosolic compartments (Tanno et al., 2007).

Sirt1 is a member of the Sirtuin family of nicotinamide adenine dinucleotide (NAD^+/NADH)-dependent class III histone deacetylases. It hydrolyses one NAD^+ for each acetyl group removed from a substrate, with release of the nicotinamide moiety (Landry et al., 2000). As NAD^+-dependent deacetylase, its activity is regulated by its ligand NAD^+ or NADH.

1.3.1.1 Regulation of Sirt1

Sirt1 is regulated by different intrinsic and extrinsic factors (fig. 4). It can be activated by resveratol and other chemical Sirt1 activators (fig. 4, top left). The first known direct regulator of Sirt1, active regulator of Sirt1 (AROS) regulates Sirt1 by direct protein-protein interaction. It enhances the activity of Sirt1 to deacetylate p53. This

leads to an inhibition of p53, thereby increasing cell-survival under conditions of DNA-damage (Kim et al., 2007b).

Sirt1 expression, its deacetylase activity and intracellular NAD^+ levels are also upregulated upon calorie restriction *in vivo* in a cAMP response element-binding protein (CREB) dependent manner (Cohen et al., 2004; Qin et al., 2006; Fusco et al., 2012).

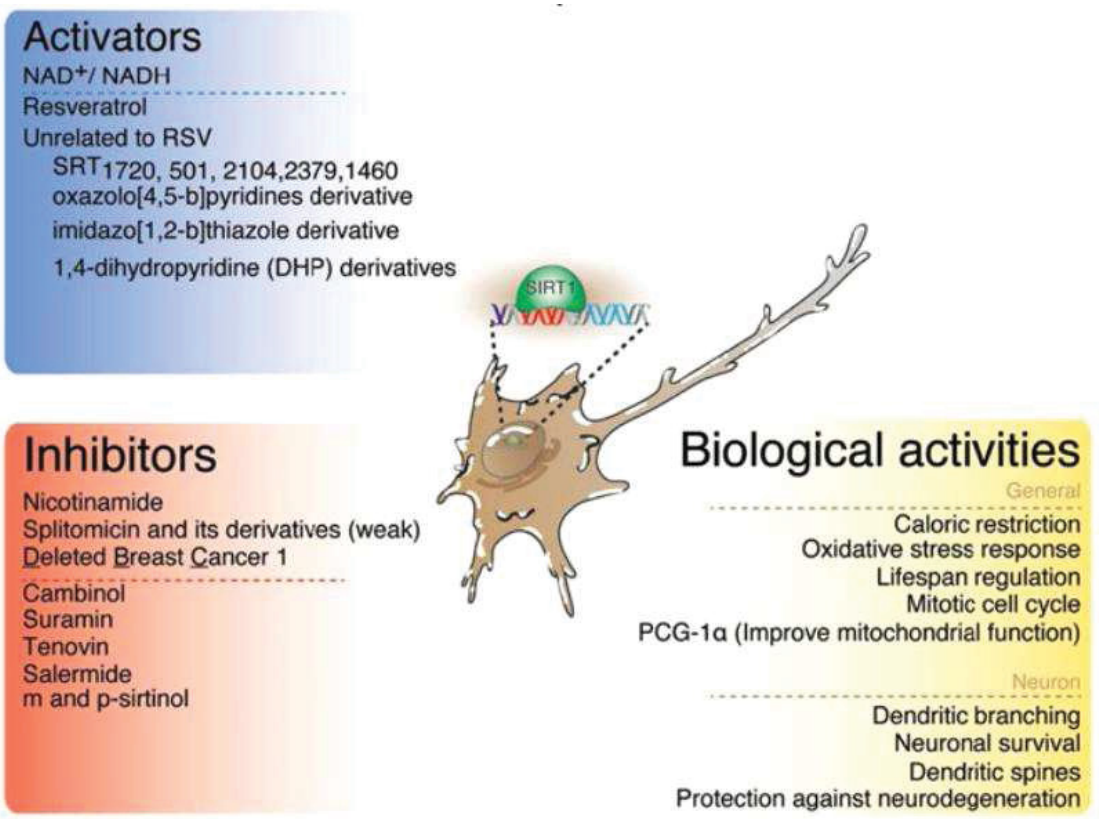

Figure 3: Activators, inhibitors and biological activity of Sirt1. (figure from Godoy et al., 2014)

Sirt1 is inhibited by compounds like Nicotinamide, Deleted in breast cancer-1 (DBC-1) and others (fig. 4, bottom left). DBC-1 regulates Sirt1 by protein-protein interaction and inhibits the activity of Sirt1 upon oxidative stress, thereby leading to enhanced cell apoptosis (Kim et al., 2008). Sirt1 has also been shown to be post-transcriptionally regulated by miRNAs. In mouse embryonic stem cells, they downregulate Sirt1 during differentiation and maintain low levels of Sirt1 expression in differentiated tissues (Saunders et al., 2010).

The RNA-binding protein human antigen R (HUR) binds the 3′ untranslated region of Sirt1 mRNA, thereby stabilizing it. This effect is abolished under oxidative stress (Abdelmohsen et al., 2007).

Sirt1 is also regulated by posttranslational modifications. It contains 13 phosphorylation sites. Seven of these are found in the N-terminal region, including

Ser27 and Ser47, and six in the C-terminal region, including Thr530. Thr530 and Ser540 are supposed to be substrates of cyclin B/cyclin-dependent kinase 1 (CDK) complexes. These two phosphorylation sites seem to be required for normal cell cycle progression. This had been proven by experiments, in which wildtype (wt) Sirt1 has been shown to rescue the growth defect of cells without endogenous Sirt1. In contrast to this, a mutant form of Sirt1, in which Thr530 and Ser540 were substituted by alanines, could not rescue the KO cells (Sasaki et al., 2008). Additionally upon oxidative stress Sirt1 is phosphorylated at residues Ser27, Ser47, and Thr530 by c-Jun N-terminal kinase (JNK). This leads to an enhanced nuclear localization of Sirt1 as well as an increased substrate-specific enzymatic activity towards histone 3 (H3) (Nasrin et al., 2009). In contrast to this, phosphorylation of Ser47 alone leads to an inhibition of Sirt1 deacetylase activity (Back et al., 2011). CK2 can also phosphorylate Sirt1, thereby stimulating its catalytic activity and enhancing its interaction with p53 (Kang et al., 2009). Phosphorylation and sumoylation increase the activity of Sirt1, leading to an anti-apoptotic response or cell cycle progression, respectively (Yang et al., 2007; Sasaki et al., 2008). Small Ubiquitin-like Modifier (SUMO) attaches close to the C-terminal end of Sirt1 at Lys734 leading to an increased catalytic activity measured by deacetylation of p53. Upon stress Sirt1 is desumoylated, which reduces its catalytic activity leading to an activation of p53 (Yang et al., 2007).

S-glutathiolation reversibly affects the function of Sirt1 as a redox regulator, as it makes Sirt1 less sensitive to activation by activating compounds like resveratol (Zee et al., 2010). Sirt1 can also be methylated. Upon interaction with the methyltransferase Set7/9 the binding of Sirt1 to p53 is disrupted, leading to an enhanced activation of p53 (Liu et al., 2011b).

Transnitrosylation of Sirt1 by nitrosylated glyceraldehyde 3-phosphate dehydrogenase (GAPDH) leads to a decrease of its deacetylation activity. Nitrosylation is even thought to result in Sirt1 protein misfolding (Kornberg et al., 2010).

Also the subcellular localization of Sirt1 regulates its function: Upon oxidative stress, Sirt1 shuttles from the nucleus to the cytoplasm. By this it is not longer co-located with its nuclear, mainly anti-apoptotic substrates, making the cell more prone to apoptosis (Tanno et al., 2007). Additionally, oxidative stress, which also can lead to

DNA damage, has been shown to decrease Sirt1 expression in the rat HC and cortex (Wu et al., 2006).

1.3.1.2 Sirt1 expression in the central nervous system

In the CNS Sirt1 is predominantly expressed in neurons (Hisahara et al., 2008) with high expression levels in cortex, HC, cerebellum and hypothalamus. Lower levels are reached in the white matter (Singh et al., 2004). In the HC Sirt1 can be found in actively proliferating $Nestin^+$ stem cells, neurons of the granular layer, DG and the pyramidal neurons in CA3, CA2 and CA1 areas (Michan et al., 2010; Ramadori et al., 2008). In neurons Sirt1 functions as regulator of energy metabolism (Ramadori et al., 2008), consistent with the role of Sirt1 in linking the status of energy availability with cellular functions in various peripheral tissues.

Upon aging, Sirt1 protein level is decreased in different tissues as well as in cells after prolonged culturing, but not in immortalized cells or post-mitotic organs (Panossian et al., 2011; Sasaki et al., 2006). Loss of Sirt1 is further accelerated in brains of mice exhibiting premature senescence (P44 mitogen-activated protein kinase (p44) transgenic mice, in which a truncated form of p53 is expressed). In contrast to that, loss of Sirt1 cannot be observed in long-lived growth hormone-receptor knockout (KO) mice (Sasaki et al., 2006). On the cellular level, during aging cytoplasmic Sirt1 levels are increased at the expense of nucleic levels, suggesting an age-related translocation of Sirt1 to the cytoplasm. *In vivo*, loss of Sirt1 led to a senescence-like phenotype with impairments in wakefulness, loss of dendrites, orexinergic boutons and neurotransmitter synthesis as well as accelerated lipofuscin accumulation (Panossian et al., 2011). Lipofuscin consists of highly oxidized and heavily cross-linked proteins and lipids and cannot be degraded. Therefore it accumulates over time, impairing proteasomal degradation and autophagy and subsequentially leading to increased oxidative stress (Jung et al., 2007). This effect is ameliorated by normal levels of Sirt1 under physiological conditions.

1.3.1.3 Functions of Sirt1 in the adult central nervous system

The first known function of Sirt1 is its function as histone deacetylase (HDAC). Upon inflammatory stimuli it leads to repression of inflammatory genes. Additionally Sirt1 remains bound to tumour necrosis factor α (TNFα) and interleukin 1β (IL-1β) promoter regions to recruit additional components of repressor complexes, such as histone 1 (H1), Relin B (RelB), methyltransferases and non-histone proteins like p65 / RelA (Liu et al., 2011). Sirt1 also deacetylates histone 4 (H4K16) to terminate NF-κB-dependent transcription. Sirt1 ameliorates inflammation by enhancing the activities of histone methyltransferases. For example, it deacetylates histone methyltransferase SUV39H1. This leads to activation of SUV39H1 resulting in increased levels of the trimethylated histone 3 (H3K9) (Vaquero et al., 2007), resulting in suppressed expression of inducible inflammatory genes (Saccani & Natoli, 2002).
Additionally to this well known function, Sirt1 has been shown to be a key regulator of cell defences and survival in response in diverse type of cellular stress (Brunet et al., 2004; Vaziri et al., 2001; Luo et al., 2001; Langley et al., 2002; Motta et al., 2004). Sirt1 deacetylates several regulatory proteins like tumour protein p53 (p53), PGC-1alpha, forkhead-box-protein O (Foxo), Heat shock factor protein (HSF) and hypoxia-inducible factor-2α (HIF-2α), activating pathways leading to resistance against stress-sors like oxidation, starvation, heat or hypoxia and to inhibition of apoptosis (Guarente, 2009). These defence mechanisms may also be the reason of Sirt1's implication to longevity: Sirt1 overexpression has been shown to increase longevity in yeast (Kaeberlein et al., 1999), worms and flies (Rogina & Helfand, 2004; Tissenbaum & Guarente, 2001). There are first data indicating, that brain specific overexpression of Sirt1 leads to increased longevity in mammals, too (Satoh et al, 2013).

1.3.1.4 Sirt1 and DNA homeostasis / damage

Defect DNA repair mechanisms result in cognitive decline in aging and neuro-degenerative diseases (Borgesius et al., 2011; Canugovi et al., 2013). Stability and integrity of DNA is continuously endangered by factors like exo-genous, biological or physical agents. Additionally DNA is challenged by endogenous threats like DNA replication errors, spontaneous hydrolysis or ROS (Hoeijmakers, 2009).

In the HC, the number of pyramidal and granule cells is not decreased upon aging, but DNA damage accumulates (Rutten et al., 2007). Sirt is important for reparation of these damages: In dopaminergic neurons, oxidative DNA damage and apoptosis is decreased upon pharmacological activation of Sirt1 (Okawara et al., 2007; Bureau et al., 2008). However, after the cells detect DNA-damage like double strand breaks DSB (occurring for example under conditions of oxidative stress), Sirt1 dissociates from these loci. It relocalizes to DSB, where it promotes repair. Thereby it triggers transcriptional changes paralleling those in the aging mouse brain (Oberdoerffer et al., 2008). Importantly, the authors note that “two thirds of Sirt1-bound genes that were derepressed by oxidative stress in vitro were also derepressed during aging. Moreover, Sirt1-target genes were significantly overexpressed among age-upregulated genes”. These effects can be ameliorated by increased Sirt1 activity. Pharmacological activation of Sirt1 has been shown to promote survival in an irradiation-induced genomic instability mouse model. Additionally it has been shown to suppress age dependent transcriptional changes (Oberdoerffer et al., 2008).

Sirt1 has also been shown maintain genomic stability in neurons in collaboration with ataxia telangiectasia mutated (ATM) and the neuroprotective HDAC1. Upon DNA DSB Sirt1 was rapidly recruited to the damaged region in an ATP dependent manner. It showed a synergistic relationship with ATM, stimulating its autophosphorylation and activity and stabilizing it at DSB sites. Additionally, upon DNA damage Sirt1 binds to HDAC1, deacetylates it and stimulates its enzymatic activity. This process is important for DSB repair. Pharmacological activation of Sirt1 promoted HDAC1 deacetylation and reduced DNA damage even in mouse models of neurodegeneration (Dobbin et al., 2013).

Together with the cell-cycle and apoptosis regulator E2F1 Sirt1 regulates DNA-damage induced apoptosis (Wang et al., 2006).

Another mechanism occurring upon DNA damage is the acetylation-dependent activation of p53, leading to either growth arrest or apoptosis of cells. Sirt1 deacetylates p53 in mice and human, thereby inactivating its sequence-specific transcriptional activity and repressing p53-mediated cell growth arrest and apoptosis in response to DNA damage and oxidative stress (Vaziri et al., 2001; Luo et al., 2001). Additionally Sirt1 inhibits p53 function to express its target genes in stem cells upon differentiation (Lhee et al., 2012).

1.3.1.5 Sirt1, replicative senescence and cell cycle control

Recently the role of Sirt1 in cellular replicative senescence attracted attention. Sirt1 rescues primary mouse embryonic fibroblasts (MEFs) from PML-mediated premature cellular senescence via inhibition of p53 (Langley et al., 2002). In human diploid fibroblasts Sirt1 activation / overexpression increases proliferation and antagonizes cellular senescence. Senescence associated biomarkers like β-galactosidase and senescence-associated heterochromatin foci (SAHF) were reduced (Huang et al., 2008). Similar results were found by Michishita and colleagues: Overexpression of Sirt1 in human fibroblasts extended their lifespan (Michishita et al., 2005).

Sirt1 has been shown to keep human embryonic lung fibroblasts (2BS) in a proliferative state, even during long term culture. Transfection with Sirt1 led to postponed gap-phase 1 (G1-phase) arrest but had less effect on synthesis (S) and G2 phase. The authors conclude that Sirt1 leads to higher proliferation due to suppression of G1 arrest and initiation of G1/S transition. Additionally Sirt1 promotes phosphorylation of kinases associated with cell proliferation, like ERK and ribosomal protein S6 kinase beta-1 (S6K1) (Huang et al., 2008).

In human lung fibroblasts and MEFs Sirt1 has been shown to decrease with serial passaging, as cells stop dividing. This effect was enhanced in cell lines exhibiting rapid premature senescence and delayed in those exhibiting delayed senescence. Additionally Sirt1 was inversely correlated with senescence-activated β-galactosidase activity and positively correlated with proliferating cell nuclear antigen (PCNA), a DNA processing factor which is expressed during the S-phase. Sirt1 has been shown to be regulated on protein, not on RNA level, concluding that Sirt1 levels are changed post-transcriptionally in serially passaged cells (Sasaki et al., 2006).

Similar results to those described above have been shown for primary neuroblasts (Horio et al. in 2003) as well as cells from mice with Sirt1-deficiencies (Lemieux et al., 2005): Low levels of Sirt1 could be linked to reduced cellular proliferation.

In contrast to the results described above, some groups also showed negative effects of Sirt1 on cell survival and senescence: Chua et al. describe enhanced senescence in Sirt1 deficient MEFs. Additionally those showed higher proliferation in chronic but sublethal stress conditions (Chua et al., 2005).

1.3.1.6 Sirt1 and protection against cellular stressors

One of the main reasons for decreased neurogenesis in the adult brain is oxidative stress. With aging, the brain becomes more and more vulnerable to oxidative stress, as self-defence responses are reduced (Mariani et al., 2005; Venkataraman et al., 2013). One of these reduced defence mechanism is the downregulation of Sirt1 upon aging. Oxidative stress, induced by feeding rats a diet high in saturated fat, severely reduced Sirt1 levels in HC as well as cortex. The effect could be abolished by additional feeding of vitamin E (Wu et al., 2006).

Upon oxidative stress, Sirt1 has been shown to regulate mitochondrial dependent apoptosis in mouse embryonic stem cells by controlling p53 subcellular localization (Han et al., 2008). Compromised mitochondrial function is a critical factor for enhanced ROS. Mitochondrial biogenesis has been shown to be regulated by PGC-1α, which is a deacetylation target of Sirt1. Therefore Sirt1 together with PGC-1α regulates the adaptation to factors like nutrient availability (Nemoto et al., 2005).

Sirt1 also has been described to challenge oxidative stress by deacetylation and activation of PPARγ co-activator 1α (PGC-1α), a molecule known to orchestrate enhanced anti-oxidant defenses, improved fatty acid oxidation and also mitochondriogenesis (Rodgers et al., 2005; Fernandez-Marcos & Auwerx 2011). After activation of Sirt1 via resveratol or SRT1720, several authors found protection from metabolic damage and improvement in mitochondrial respiration, due to activation of the PGC-1α pathway (Baur et al., 2006; Feige et al., 2008; Lagouge et al., 2006; Minor et al., 2011).

Another mechanism by which Sirt1 promotes cell survival and stress resistance is the deacetylation of three of the four known Forkhead Box Class O (Foxo) transcription factors: Foxo1, Foxo3a and Foxo 4. During the interaction Sirt1 binds to the LXXLL motif of Foxo1. Especially Foxo3, which is also expressed in neurons, is regulated by Sirt1 leading to reduced apoptosis in response to stress conditions. Additionally it increases the expression of genes regulating DNA repair and cell cycle checkpoints (Motta et al., 2004; Brunet et al., 2004).

Apparently it is of high importance to keep Sirt1 levels up to fight oxidative stress in the aging brain, as Sirt1 contributes to cellular adaptation to oxidative stress conditions (Prozorovski et al., 2008). Activation of Sirt1 has been shown to reduce oxidative stress, enhance mitochondrial function and promote cell survival in neuronal cells (Khan et al., 2012). Supporting these results, Lin et al. found that in

yeast reduced calorie intake enhances Sirt1 expression (Lin et al., 2004). This may be of particular interest for maintenance of NSCs in the aged brain, where oxidative stress is supposed to have a major negative effect on neuronal function and neurogenesis (see chapter 1.3.6).

Upon oxidative stress, neuroprotection is also performed by astrocytic Sirt1. It suppresses expression of pro-inflammatory cytokines and increases expression of superoxide dismutase 2 and catalase, thereby decreasing the production of ROS (Cheng et al., 2014).

1.3.1.7 Sirt1 as neuroprotector

Additionally to challenging oxidative stress, Sirt1 is neuroprotective in several further ways:

It is important for the maintenance of the amount of neuronal mitochondria by regulating PGC1-α, a master regulator of mitochondrial number and function. PGC1-α-KO mice suffer from high neuronal loss, whereas increased PGC1-α levels have been shown to be neuroprotective under oxidative stress conditions (St-Pierre et al., 2006).

In brains of patients suffering from Multiple Sclerosis, Sirt1 is decreased during relapses (Tegla et al., 2014), whereas it is increased in $GFAP^+$ cells near lesions in experimental autoimmune encephalomyelitis (EAE) mice, a mouse model of Multiple Sclerosis, as shown by our group (Prozorovski et al., 2008). Pharmacological activation of Sirt1 prevented neuronal loss and suppressed ROS in this mouse model (Khan et al., 2014). Clinical symptoms were improved, axonal injury decreased as well as the amount of apoptotic cells. Additionally brains of Sirt1 overexpressing mice showed higher levels of brain derived neurotrophic factor (BDNF) and NAD (Nimmagadda et al., 2013). In line with this, pharmacological inhibition of Sirt1 attenuated the aforementioned neuroprotective effects (Shindler et al., 2007; 2010).

Sirt1 has been found to play a role in nearly all widespread neurodegenerative diseases, like Alzheimer's, Huntington's and Parkinson's Disease as well as in Amyotrophic Lateral Sclerosis and Multiple Sclerosis (fig. 4). It is upregulated in mouse models for Alzheimer's Disease, Amyotrohic Lateral Sclerosis and in primary neurons challenged with neurotoxic insults (Kim et al., 2007a). It can protect neurons from β-amyloid-induced toxicity via inhibition of NF-κB signalling in microglia or

downregulation of the serine / threonine ROCK1 (Rho-kinase) expression in neurons, leading to the induction of α-secretase (Chen et al., 2005a; Qin et al., 2006). Sirt1 also shows neuroprotective effects in neuronal cultures incubated with huntingtin. This effect seems to be Sirt1-dependent, as it is abolished by blocking Sirt1 activity with inhibitors like Sirtinol or nicotinamide (Borrell- Pagès et al., 2006).

In HC slice cultures, Sirt1 has been shown to be upregulated upon ischemic preconditioning (IPC). This effect was abolished by Sirtinol, which blocks Sirt1. Interestingly, resveratol-induced activation of Sirt1 alone can mimic the neuroprotective effect of IPC (Raval et al., 2006).

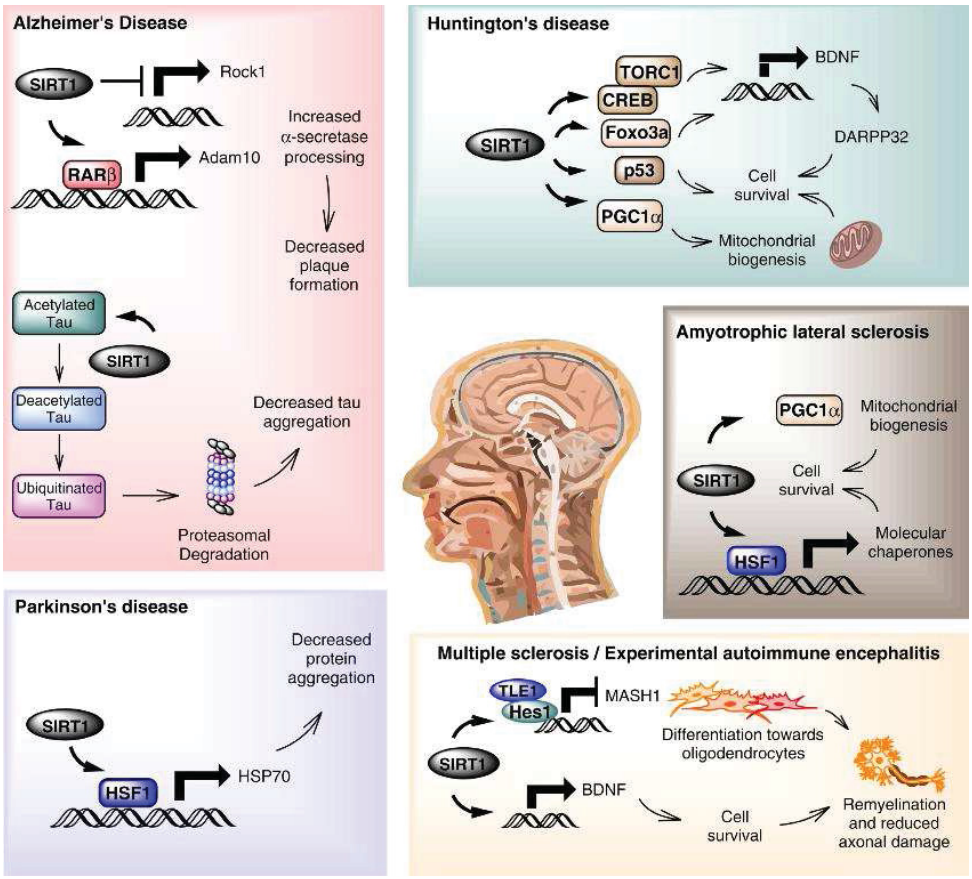

Figure 4: Sirt1 contributes to several neurodegenerative diseases (figure from Herskovits & Guarente 2014)

In a Wallerian Degeneration $(Wld)^s$ mice, an mouse model for delayed Wld, Sirt1 is involved in the beneficial effects of the protein Wld^s leading to delayed axonal degeneration. This neuroprotective effect could be abolished by inhibition or silencing of Sirt1 (Araki et al., 2004). Additionally Sirt1 is involved in autophagy processes leading to removal of damaged mitochondria, toxic misfolded proteins which accumulate upon nearly all neurodegenerative disease (Lee et al., 2008) and other

neurotoxic substances like α-synucleins (Wu et al., 2011). Sirt1 also has been shown to be an important neuroprotector for wake neurons in young animals (Panossian et al., 2011) and protects neurons against cytotoxicity induced by mutant polyglutamines (Parker et al., 2005). Sirt1 has been shown to affect psychiatric disorders like depression, anxiety disorders or bipolar disorders, in which neurogenesis is decreased (Abe et al., 2011; Kishi et al., 2010; 2011; Libert et al., 2011).

1.3.1.8 Sirt1 – A regulator of adult neurogenesis?

Several working groups have shown contradictory results regarding Sirt1 as a regulator of adult neurogenesis.

In mice, neuron-specific overexpression of Sirt1 has been shown to promote neurite outgrowth and improve cell viability, an effect lost upon blocking of Sirt1 activity (Guo et al., 2011). Regarding differentiation of HC neurons, Sirt1 has been shown to be essential for dendritic development and increases dendritic arborisation (Braidy et al., 2012). In brains of Sirt1 KO mice, neurons show reduced numbers of dendritic branches, decreased branch length and complexity (Michan et al., 2010).

Sirt1 has been shown to modulate Wnt signalling: The levels of all Dishevelled proteins are downregulated upon lost Sirt1 function. Additionally Sirt1 leads to changes in gene expression of Wnt target genes and Wnt-stimulated cell migration is inhibited upon Sirt1 inhibition. These mechanisms are also important for HC neural progenitor proliferation and neurogenesis (Holloway et al, 2010).

Sirt1 is involved in stem cell function by controlling cell fate decision. It is down-regulated during human embryonic stem cell differentiation at both, messenger RNA (mRNA) and protein level. This leads to reactivation of key developmental genes such as the neuroretinal morphogenesis effectors delta like ligand (DLL4), T-box transcription factor 3 (TBX3), and paired box protein 6 (PAX6), which are epigenetically repressed in pluripotent human embryonic stem cells. (Calvanese et al., 2010).

Our group has shown that activation of Sirt1 in embryonic NPCs inhibits their proliferation and directs their differentiation toward the astroglial lineage at the expense of the neuronal lineage. This can be induced by direct activation of Sirt1 as well as mild oxidative stress. Mild oxidative stress can be induced by low, non-toxic

concentrations of buthionine sulfoximine (BSO), an inhibitor of glutathione synthase or diethyldithiocarbamate (DETC), which suppresses superoxide dismutase, thereby enhancing the amount of superoxide anions in the cells. In reducing conditions, induced by application of reducing agents like lipoic acid on N-acetylcysteine (NAC), the opposite effect could be seen: Proliferation increased, as well as the amount of NPCs giving birth to neurons. Our group also showed that under oxidative conditions Sirt1 is upregulated *in vitro* as well in vivo. Under these conditions it binds to the transcription factor hairy and enhancer of split 1 (Hes1), stabilizing transducin like enhancer of split 1 (TLE-1) containing repressor complex which inhibits pro-neuronal Mash1. This pathway has been shown to be disrupted after knockdown of Sirt1 (Prozorovski et al., 2008).

Supporting our findings, Saharan and colleagues showed that Sirt1 expression in SGZ as well as SVZ are markedly reduced during NPC differentiation. Lentiviral Sirt1 knockdown did not alter the amount and proliferation of NPCs in these areas, but led to increased production of neurons. In contrast to this, enhancement of Sirt1 signalling (by overexpression or direct activation of Sirt1) led to a decreased number of new-born neurons, in vitro as well as in vivo (Saharan et al., 2013).

Similar data, though focused more on the development of oligodendrocytes, were shown by Rafalski and colleagues: Inactivation of Sirt1 in the adult mouse brain led to increased production of oligodendrocyte precursor cells (OPCs), which developed normally to fully functional oligodendrocytes. This increase was not at the expense of other cell types, as the proliferation of NPCs was also enhanced. The authors showed that effects of Sirt1 on the development of oligodendrocytes are mediated via p38 mitogen-activated protein kinases ($p38^{MAPK}$) and AKT signalling. Additionally, in Sirt1 deficient cells they found an upregulation of genes important for cell metabolism and growth factor signalling (e.g. alpha-type platelet-derived growth factor receptor (PDGFRα)) and a downregulation of genes important for neurogenesis and neuronal function (Rafalski et al., 2013).

Similar data have been shown in human embryonic stem cells. Sirt1 is downregulated during human embryonic stem cell differentiation at both, mRNA and protein levels, leading to reactivation of key developmental genes such as the neuroretinal morphogenesis effectors DLL4, TBX3, and PAX6, which are epigenetically repressed by this HDAC in pluripotent human embryonic stem cells (Calvanese et al., 2010). Additionally, several groups proved that Sirt1 is essential for

neurite outgrowth, spine architecture and dendritic arborisation of HC neurons (Michan et al., 2010; Codocedo et al., 2012).

Some authors show contrasting results to those described above. Hisahara et al. found a decrease of neuronal differentiation in embryonic and adult NPCs after inhibition of Sirt1. On the other hand, Sirt1 overexpression led to an increase of neuronal differentiation. The authors conclude that Sirt1 represses Notch1-Hes1 signalling to regulate differentiation of NPCs. Additionally they found that Sirt1 is located in the cytoplasm, whereas it is transiently localized in the nucleus during differentiation stimuli (Hisahara et al., 2008). Also Zhang and colleagues showed that in human embryonic stem cells inhibition of Sirt1 promotes neural progenitors to differentiate into motor neurons (Zhang et al., 2011).

These results are supported by Tiberi and colleagues, who showed that Sirt1 is essential for BCL6-dependent neurogenesis in vitro and in vivo. Sirt1 and BCL6 build a complex, in which BCL6 recruits Sirt1 to repress Hes5. Thereby Hes5 is silenced, which ensures maturation of neurons, even with ongoing Notch signalling. Inhibition of Sirt1 led to a breakdown of these pathways, leading to decreased neurogenesis (Tiberi et al., 2012).

2. Aim of this work

As data regarding Sirt1 as a regulator of adult neurogenesis are still contradictory (see above), this work was designed to elucidate the function of Sirt1 on adult HC neurogenesis *in vitro* and *in vivo*. Additionally it addressed the question whether HC Sirt1 expression is affected by aging of mice. As the characteristics of brain stem cells are proliferation, migration and differentiation, this work shall elucidate possible roles of Sirt1 in this processes. Oxidative stress is thought to decrease neurogenesis. Therefore this work will also further investigate the function of Sirt1 on neurogenesis under oxidative conditions during aging.

3. Methods

3.1 Laboratory equipment

Automatic Cell Counter	BioRAD, Munich, Germany
Centrifuge 5417R	Eppendorf, Hamburg, Germany
Centrifuge Rotanta 460R	Hettich, Tuttlingen, Germany
CO_2 cell culture incubator	Thermo Scientific, Massachusetts, USA
Dako Pen	Dako, Hamburg, Germany
F-View fluorescence camera	Soft Imaging System, Munich, Germany
Freezer / fridge	Liebherr, Biberach an der Riss, Germany
Hera Safe horizontal laminar flow	Heraeus, Frankfurt, Germany
Kodak X-Omat 1000 processor	Kodak, Stuttgart, Germany
Leica CM1900 UV (cryostat)	Leica Microsystem, Wetzlar, Germany
Microplate reader Genios Pro	Tecan, Männedorf, Switzerland
Microwave MW-1035M	Eurotec Riga International, Lana, Italy
Mini Protean Tetra Cell for WB	BioRAD, Munich, Germany
Nanodrop 2000 spectrophotometer	Thermo Scientific, Massachusetts, USA
Odyssey infrared imaging system	Li-Cor, Nebraska, USA
Olympus BX51	Olympus, Hamburg, Germany
Olympus U-RFL-T Burner	Olympus, Hamburg, Germany
Pipettes	Eppendorf, Hamburg, Germany
Pipetus	Hirschmann Laborgeräte,Eberstadt,Germany
Precision balance	Kern, Balingen, Germany
Rocking Platform WT15/WT16	Biometra, Göttingen, Germany
Sonicator, UW 2070	Bandelin electronic, Berlin, Germany
Thermocycler T gradient TC10	Biometra, Göttingen, Germany
Trans Blot Turbo	BioRAD, Munich, Germany
Transfer pack for Trans Blot Turbo	BioRAD, Munich, Germany
7500 Pro real-time PCR systems	Applied Biosystems, Darmstadt, Germany
Vacuboy	Integra Biosciences, Fernwald, Germany
Vortex Genie	Scientific Industries, New York, USA
Water bath	GFL, Burgwedel, Germany
Wilovert, inverted microscope	Hund, Wetzlar, Germany

3.2 Chemicals

2-Deoxyglucose	Sigma-Aldrich, Munich, Germany
Agarose	Biozym Scientific, H. Oldendorf, Germany
B27 (with and without retinoic acid)	Invitrogen (Gibco), Karlsruhe, Germany
BrdU	Sigma-Aldrich, Munich, Germany
BSA	Merck, Darmstadt, Germany
BSO	Sigma-Aldrich, Munich, Germany
DNA ladder	PeqLab Biotechnologie, Erlangen, Germany
DNA loading buffer 6x	PeqLab Biotechnologie, Erlangen, Germany
DNAse/RNAse free H2O (destilled)	Invitrogen (Gibco), Karlsruhe, Germany
DMSO	Sigma, Munich, Germany
Dulbecco's PBS	PAA, Pasching, Austria
EDTA	Sigma-Aldrich, Munich, Germany
EGF	Invitrogen (Gibco), Karlsruhe, Germany
Ethanol	Invitrogen, Karlsruhe, Germany
Ethidium bromide	Sigma-Aldrich, Munich, Germany
bFGF	Immuno Tools, Friesoythe, Germany
Glutamax	Invitrogen (Gibco), Karlsruhe, Germany
HBSS ($^{+/+}$ and $^{-/-}$)	Invitrogen (Gibco), Karlsruhe, Germany
HCl	Merck, Darmstadt, Germany
Hoechst 33258	Invitrogen, Karlsruhe, Germany
Immunomount	Thermo Scientific, Pittsburgh, USA
Isofluran	Actavis, Dublin, Ireland
Milk powder	Sigma-Aldrich, Munich, Germany
Mini-Protean TGX Gels for WB	BioRAD, Munich, Germany
NaCl	Merck, Darmstadt, Germany
NBM	Invitrogen (Gibco), Karlsruhe, Germany
NGS	Invitrogen (Gibco), Karlsruhe, Germany
Nuclease free water	Invitrogen (Gibco), Karlsruhe, Germany
PFA	Roth, Lauterbourg, France
PBS	Invitrogen (Gibco), Karlsruhe, Germany
Penicillin / streptomycin	Invitrogen (Gibco), Karlsruhe, Germany
peqGOLD TriFast	Peqlab, Erlangen, Gemany
Poly-L-ornithine 0.01% solution	Sigma-Aldrich, Munich, Germany

Power SYBRGreen	Applied Biosystems, California, USA
Protease inhibitor cocktail tablets	Roche, Mannheim, Germany
Protein Loading buffer 4x	Li-Cor, Nebraska, USA
Prestained Protein Marker V peqGold	PeqLab Biotechnologie, Erlangen, Germany
Protease inhibitor cocktail tablets	Roche, Mannheim, Germany
Proteinase K	Sigma-Aldrich, Munich, Germany
RIPA buffer	Sigma-Aldrich, Munich, Germany
SDS	Sigma-Aldrich, Munich, Germany
Sodium-deoxycholate	Sigma-Aldrich, Munich, Germany
Sucrose	Roth, Lauterbourg, France
TaqMan Rev. Transcription Reagents	Applied Biosystems, California, USA
Tetraborate	Sigma-Aldrich, Munich, Germany
Tissue Tek	Sakura Fintek, Alphen aan den Rijn Netherlands
Tris / Glycerine / SDS Buffer for WB	BioRAD, Munich, Germany
Tris-HCl	Merck, Darmstadt, Germany
Triton-X-100	Merck, Darmstadt, Germany
Trypsine	Invitrogen, Karlsruhe, Germany
Tween 80	Sigma-Aldrich, Munich, Germany
2-Propanol	MERCK, Darmstadt, Germany

3.3 Kits

BC Assay Protein Quantification Kit	Interchim, Montluçon, France
High Capacity cDNA Reverse Transcription Kit	Life Technologies, Carlsbad, USA
Nucleo Spin RNA/Protein extraction Kit	Macherey-Nagel, Düren, Germany
TaqMan Reverse Transkriptase Kit	Applied Biosystems, California, USA
Sybr Green / Taq Man Kit	Applied Biosystems, California,USA

3.4 Plastics and consumables

If not stated otherwise, reactions tubes, pipettes and similar were from:
Applied Biosystems, B.Braun, Eppendorf, Falcon, Costar, GE Healthcare, Roth, Beckton Dickinson and Sarstedt.

3.5 Software

Following software programs or packages were used for his work:

Adobe Acrobat Reader 8.0	Image J 1.46r
Adobe Photoshop CS4	Microsoft Office 2010
Adobe Illustrator CS4	Odyssey Imaging System Software
Graph Pad Prism 5	7500 Real Time PCR System

3.6 Animals

All animal experiments were performed in strict accordance with German animal protection law (TierSchG) and approved by the responsible state office Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (LANUV) under protocol numbers G197-09, G163-11 / 84-02.2011.A163. The mice were housed and handled in accordance with good animal practice and all efforts were made to minimize suffering as defined by Federation of European Laboratory Animal Science Associations (FELASA) and the national animal welfare body Gesellschaft für Versuchstierkunde - Society for Laboratory Animal Science (GV-SOLAS).

3.6.1 Sirt1$^{(+/-)}$ mice

Mice with an inactive form of Sirt1, in which exon 4 of the catalytic domain is deleted, were generated by Cheng and colleagues in 2003. These mice produce a mutant form of Sirt1 lacking the catalytic domain. Therefore this mutant form is catalytically inactive. For better understandability of this work, in the following text these mice will

be called “Sirt1 KO” or “Sirt1 deficient”, though the mutant form of Sirt1 is still expressed in the cells.

According to the initial descriptions by Michan et al. and Cheng et al., Sirt1 KO mice were smaller than wt littermates and showed developmental defects of the retina and heart and adults exhibited dramatically reduced sperm numbers. Some of the embryos exhibited exencephaly (fig. 5). Brains of Sirt1 KO mice show normal morphology and synaptic spine density, but decreased dendritic branching, branch length and complexity of neuronal dendritic arbores (Michan et al., 2010). Additionally they had a lower postnatal survival rate than wt littermates (Cheng et al., 2003). In 2005 Lemieux and colleagues described this strain further. They found that the decrease in body weight of Sirt1 KO mice is independent from growth hormone expression. Sirt1 KO mice have been shown to have particularly small white adipose tissue deposits. They also found that insulin like growth factor binding protein 1 (IGFB1) expression was three-fold higher in liver and kidney of Sirt1 KO mice, due to a disrupted Foxo signalling pathway. Under normal conditions, Sirt1 deacetylates Foxo, thereby regulating its activity. From behavioural aspects Sirt1 KO mice were described as less aggressive and lethargic in comparison to their wt littermates. Additionally they are less active but show more stereotypic behaviour. Lemieux describes a lack in bone mineralization in Sirt1 KO mice. Since homozygous mice of this strain die prior birth, for this work heterozygous mice (following called $Sirt^{(+/-)}$) were used for the analysis of functional loss of Sirt1 in the adult brain.

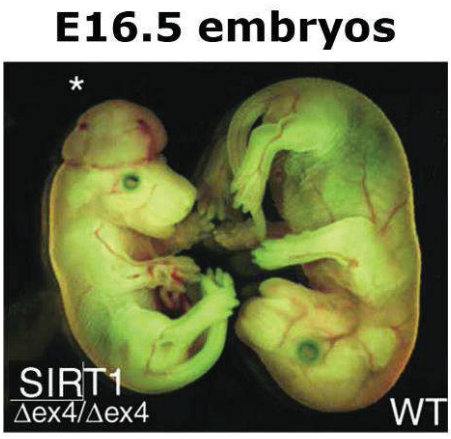

Figure 5: Sirt1 KO embryos exhibit developmental defects. Sirt1 KO embryos (left) are smaller than their wt littermates (right) and exhibit developmental defects like exencephaly.E16.5=embryonic day 16.5 (figure modified after Cheng et al. 2003).

3.6.1.1 Genotyping of Sirt1$^{(+/-)}$ mice

For genotyping, tail- or ear-cuts of mice were lysed over night in 100 µl genotyping buffer (100 mM Tris, 200mM NaCl, 5mM ethylenediaminetetra-acetic acid (EDTA), 0.2% sodium dodecyl sulphate (SDS)) plus 10 µl Proteinase K. After lysis, samples were cooked at 90°C for ~20 mins, centrifuged at 8000 rpm for 5 minutes and diluted 1:20. PCR was performed as described below, using following primers:

Primer	Sequence 5'→ 3'
Sirt1KO-F1	CTT GCA CTT CAA GGG ACC AA
Sirt1KO-R1	GTA TAC CCA CCA CAT CTG AG
Sirt1KO-R2	CTA CCA CTC CTG GCT ACC AA

expected size of wt: 500 bp; expected size of KO: 800 bp

PCR program:

1. 95°C, 5min
2. 33x: 95°C (40 sec), 54°C (50 sec), 72°C (90 sec)
3. 72°C, 10 min
4. 4°C → storage

3.6.2 NestinCre;Sirt1 LoxP mice

3.6.2.1 Breeding of NestinCre;Sirt1LoxP mice

To investigate Sirt1 function *in vivo*, the cre/loxP site-specific recombination strategy was used to generate mice with specific deletion of Sirt1 in neuronal tissues. For this, NestinCre mice (Tronche et al., 1999) and Sirt1LoxP mice (Li et al., 2007) were crossed. Constructs of both mice are shown in figure 6.

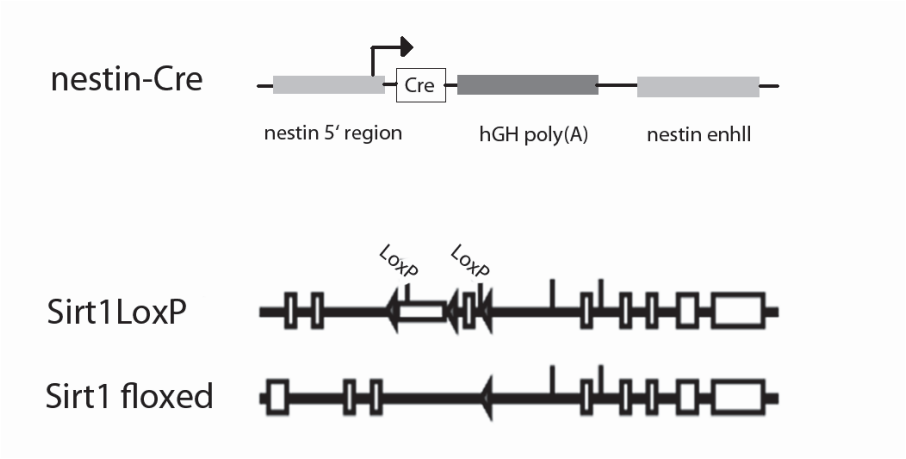

Figure 6: Constructs of NestinCre mice (top) and Sirt1LoxP mice (bottom).

3.6.2.1.1 NestinCre mice

NestinCre mice were available in the animal facility of the University Clinic Düsseldorf. In NestinCre mice, cyclization-recombinase (Cre-recombinase) is expressed under the nestin promoter, which allows expression of the enzyme in nestin expressing cells (fig. 6, top). Following breeding procedure for this work, activity of Cre-recombinase in germ cells of the male lineage was observed, consistent with previous publications (i.e. Rempe et al., 2006). Therefore a new strain of Sirt1LoxP/NestinCre mice was created by crossing female NestinCre mice with male Sirt1LoxP mice.

3.6.2.1.2 Sirt1LoxP mice

Sirt1LoxP mice were purchased from The Jackson Laboratory (stock no. 008041). These mice were generated by insertion of locus of X-over P1 (LoxP) sites down- and upstream of exon 4. In Sirt1LoxP mice, a part of the catalytic domain of Sirt1 is flanked by LoxP sites. These can be recognized by the Cre-recombinase, which cuts out the flanked part, leading to a catalytically-inactive mutant protein of Sirt1 (fig. 6, bottom).

3.6.2.2 Genotyping of NestinCre;Sirt1 LoxP mice

Primer	Sequence 5'→ 3'
Cre_F1	GCATTACCGGTCGATGCAACGAGTGATGAG
Cre_R1	GAGTGAACGAACCTGGTCGAAATCAGTGCG
Sirt1LoxP_F7	GTG TGT CTT GCC CTC TCC TTT G
Sirt1LoxP_R7	AGA CGT CCC TTG TAA TGT TTC CC
Sirt1LoxP_F9	CCA TTA AAG CAG TAT GTG GCA GAT

Sirt1LoxP_F7 + Sirt1LoxP_R7: detection of LoxP-cassette
Sirt1LoxP_F7 + Sirt1LoxP_F9: detection of "0"-allele

expected size of Cre: 400 bp

for primers Sirt1LoxP_F7 and R7:	**for primers Sirt1LoxP_F9 and R7:**
expected size of $Sirt1^{(+/+)}$: 200 bp expected size of $Sirt1^{(+/-)}$: 200 + 400 bp expected size of $Sirt1^{(lox/-)}$: 400 bp	expected size of $Sirt1^{(+/+)}$: 700 bp expected size of $Sirt1^{(+/-)}$: 700 + 300 bp expected size of $Sirt1^{(lox/-)}$: 300 bp

PCR programs:

program Sirt1 LoxP	program Cre
1. 94°C, 3min 2. 40x: 94°C (30 sec), 51,7°C (60 sec), 72°C (60 sec) 3. 72°C, 2 min 4. 4°C → storage	1. 95°C, 2min 2. 35x: 95°C (30 sec), 65°C (45 sec), 72°C (60 sec) 3. 72°C, 2 min 4. 4°C → storage

3.6.2.3 NestinCre;Sirt1 LoxP mice for experiments

Mice at generation 2 were used for analysis. The following genotypes were investigated: 1.) Control (ctrl) mice (NestinCre;$Sirt1^{(+/+)}$), 2.) mice with systemic deletion of exon 4 in one Sirt1 allele, in which Sirt1 function is lost in the whole body (systemic) (NestinCre;$Sirt1^{(+/-)}$) and 3.) mice with deletion of the catalytic domain of

Sirt1 in cells of the CNS on one allele and systemic Sirt1 deletion on the second allele (NestinCre;Sirt1$^{(lox/-)}$).

3.6.3 *In utero* electroporated mice

In utero electroporation experiments have been performed in collaboration with AG Prof. Schumacher (Institute of Molecular and Cellular Anatomy, University Ulm). Histological material for analysis of Sirt1 deficiency in the embryonic brain has been provided by Dr. Timur Prozorovskiy. *In utero* electroporation is a technique to introduce transgenes into developing brains. The *in utero* electroporation experiments were carried out as previously described (Prozorovski et al., 2008) in accordance with a protocol approved by the local ethic committee. Briefly, DNA is injected into the lateral ventricle of the embryonic brain. After putting electrodes to both sides of the embryos head, the negatively charged DNA wanders to the anode, thereby entering the cells and nuclei. Embryos then continue their development and are born like in normal conditions. We used pregnant C57BL/6 mice on the stage E15.5 (post coitum) and embryonic / pup brains were collected 4 days later at postnatal day 0.5 for cryopreservation and histological analysis.

3.6.4 Nrf2$^{-/-}$ mice

Nrf2 KO mice have a targeted deletion of part of exon 4 and 5. This includes the cap-n-collar, DNA binding and leucine zipper domains. Therefore, Nrf2 KO mice, like Sirt1 KO mice, are not lacking Nrf2, as the protein is expressed in the cells, though in a non-functional mutant form. Mice with homozygous Nrf2 KO are fertile, develop normally, reach adulthood and show normal behaviour (Chan et al., 1996).

3.6.4.1 Genotyping of Nrf2$^{-/-}$ mice

Primer	Sequence 5'→ 3'
mNrf2_F3	ATCAGGCCCAGTCCCTCAAT
mNrf2_R3	CCAGCGAGGAGATCGATGA

Primers were used for cDNA to detect Nrf2 wild type form (228 bps).

PCR program:

1. 94°C, 2min
2. 35x: 94°C (15 sec), 60 °C (40 sec), 72°C (30 sec)
3. 72°C, 2 min
4. 4°C → storage

3.6.5 Sirt3$^{-/-}$ mice

129Sv Sirt3$^{-/-}$ mice (Jackson, no. 012755) were generated by Dr. Frederick Alt (Lombard et al., 2007) via deletion of exon 2-3 of the Sirt3 gene. Therefore, like Sirt1 KO and Nrf2 KO mice, these mice are not deficient for Sirt3 but express a mutant, unfunctional Sirt3 protein. Mice were bred to 129S1/SvImJ inbred background (Stock No. 002448) for at least one generation to establish the colony. Apart from hyperacetylation of mitochondrial proteins, Sirt3$^{-/-}$ mice develop normally in basal breeding conditions. However, if Sirt3$^{-/-}$ mice are challenged with stressful stimuli, they exhibit reduced fatty acid oxidation and metabolic abnormalities like hepatic steatosis or reduced ATP levels (Hirschey et al., 2010). Additionally, Sirt3$^{-/-}$ mice do not show diminished oxidative stress upon calorie restriction, a paradigm known to inhibit oxidative stress induced damage (Qiu et al., 2010).

3.6.5.1 Genotyping of Sirt3$^{-/-}$ mice

Primer	Sequence 5'→ 3'
Sirt3_common	TGCAACAAGGCTTTATCTTCC
Sirt3_wt_Fw	CTTCTGCGGCTCTATACACAG
Sirt3_KO_Rev	TACTGAATATCAGTGGGAACG

Expected size of wt: 562 bp
Expected size of KO: 200 bp

PCR program:

1. 94°C, 2min
2. 38x: 94°C (30 sec), 60 °C (45 sec), 72°C (45 sec)
3. 72°C, 5 min
4. 4°C → storage

3.6.6 BrdU treatment

BrdU for injections was dissolved in 0.9 % NaCl in sterile conditions up to 10 mg/ml, filtered and normalized to pH 7,35. Aliquots were stored at -20°C. Animals received intraperitoneal (i.p.) injections of 50mg/kg (ca. 100 µl per animal) twice a day on 3 following days and were perfused 7 days later.

3.7 Cortical neuronal precursor cell cultures

All cell culture experiments were performed in a sterile bench HERASafe under strict aseptic conditions. Cultures were incubated in Heraeus-incubators in 37°C, 5% CO_2 and 95% humidity. NPCs were prepared and cultured according to Reynolds & Weiss 1992, Reynolds et al. 1992 and Vescovi et al. 1993. Shortly, NPCs were isolated from mice embryos at day 17 of embryogenesis or from 1-3 days old pups. The frontal part of the brain (without cerebellum and brain stem) was used for culturing. Meninges were removed and the brains were cut into small pieces under a stereozoom microscope. Brain tissue was washed, typsinized (1% Trypsin (v/v) in Hanks' Balanced Salt solution without Ca and Mg ($HBSS^{-/-}$)), and washed once more. The pellet obtained after centrifugation was resuspended in neurobasal medium (NBM) and cultured in proliferation medium (NBM with 2% (v/v) B27 supplement (without retinoic acid), 1% (v/v) glutamax, 1% (v/v) penicillin-streptomycin mix (P/S), 10ng/ml basic fibroblast growth factor (bFGF), 1ng/ml epidermal growth factor (EGF)) on non-adhesive substrates to generate suspended clusters of cells (neurospheres).
In the adhesive culture paradigm, NPCs grow as mono-layers on poly-ornithine coated plates according to Ray et al., 1993.

Every third day, cells of the free floating cultures were passaged: They were centrifuged, washed and trypsinized to split the neurospheres into single cells again. The cells were counted and seeded on plates at following densities: Petri plates 1 mio cells in 10ml medium, 6 well plates 200.000 cells in 2ml medium. To maintain self-renewal, FGF and EGF were added to the proliferation medium. After removal of these (differentiation medium consisting of NBM with 2% (v/v) B27 supplement (with retinoic acid), 1% (v/v) glutamax, 1% (v/v) P/S) cells started to differentiate. Addition of retinoic acid promoted neuronal differentiation. The cells were allowed to differentiate for 7 days, with exchange of medium or addition of growth factors every second to third day. For proliferation assay, the cells were seeded in proliferation medium on 6 well plates. They were allowed to grow for 3 days. After this period, pictures of the cultures were taken to count single cells and neurospheres. From this, the percentage of cells able to create neurospheres was calculated.

3.7.1 Cell treatments

For immunocytochemistry experiments, 20.000 NPCs in 0.5ml proliferating medium per well were seeded on 24-well plates containing poly-ornithine covered glasses. For RNA isolation, cells were plated in 6 well plates (200.000/well).

For induction of oxidative stress, cells were treated with 5µM **BSO** (dissolved in in proliferating medium) for 24 hours.
For starvation paradigm, 100mM 2-deoxyglucose (**DG**) stock was created in sterile HBSS. After filtration, 5µl was added to the 500µl cultures to reach a final concentration of 1mM. Ctrl cultures were treated with the same amount of glucose instead of DG.
To assess the amount of actively proliferating cells in culture nucleotide retention assay (**BrdU**-pulse assay) was performed. 10 µM BrdU was given to the medium of the cultures and allowed to incubate for 2h under normal culturing conditions. During this time, BrdU is incorporated into the DNA of proliferating cells and can be stained with BrdU antibodies later on.

3.8 Staining protocols

3.8.1 Immunohistochemistry

After perfusion of the mice, brains were cut sagitally. One half of the brains were used for WB or PCR experiments, the other for histology. The part used for histology was fixed in 4% paraformaldehyde (PFA) over night. After some washing steps, the brains were put into 20% sucrose solution over night to dehydrate. Following, brains were embedded, frozen in Tissue-Tek and stored at -20°C or 80°C.
Brains were cut into 20µm thin sectionss using a cryostat. Sections used for histology were dried at room temperature (RT). After that Tissue-Tek was removed using a forceps. The sections were encircled with a Dako-Pen. Samples were blocked for 1h at RT, using blocking buffer (phosphate buffered saline (PBS) with 10% (v/v) normal goat serum (NGS), 0.5% (v/v) Triton X, 1% (v/v) bovine serum albumin (BSA)).

3.8.2 Immunocytochemistry

Primary cells were seeded on poly-ornithine covered glasses in a 24-well plate, as explained above. At the end of experiments, cells were fixated with 4% PFA for 10-20min at RT and washed three times with PBS. Afterwards blocking buffer was added for 2 hours.

Following steps are the same for immunohisto- as well as -cytochemistry:

After blocking, the incubation with primary antibodies was performed on a shaker over night at 4°C or at RT in blocking buffer diluted 1:2 with PBS. After three washing steps, they samples were incubated with secondary antibodies for 1h at RT. After additional washing, samples were dried and mounted using Immuno-Mount.

primary antibody	company	host	dilution ICC	dilution IHC	target
anti-BrdU	AbD Serotec	ms	1:200	1:200	incorporated BrdU
anti-CNPase	Sigma	ms	1:500	-	oligodendrocytes
anti-DCX	GeneTex	goat	-	1:200	immature neurons
anti-GFAP	SySy	guinea pig	1:1000	1:1000	astrocytes
anti-H2A.X	Millipore	ms	1:500	-	DNA damage
anti-H3Ser10	Epitomics	rabbit	-	1:500	mitotic cells
anti-Ki67	Dako	ms	1:100	-	mitotic cells
anti-Ng2	Millipore	rabbit	-	1:500	OPCs
anti-Olig2	Millipore	rabbit	-	1:200	oligodendrocytes
anti-p19arf	Santa Cruz	rabbit	1:50	-	Senescence-associated cell cycle inhibitor
anti-Sirt1	Millipore	rabbit	1:500	1:500	Sirt1
anti-Sox2	Santa Cruz	goat	-	1:400	stem cells

secondary antibody	company	target	dilution
Cy2	Invitrogen	ms / rabbit / guinea pig / rat / chicken	1 : 500
Cy3	Millipore	ms / rabbit / guinea pig / rat / chicken / goat	1 : 500
Cy5	Millipore	ms / rabbit / guinea pig / rat / chicken	1 : 500

3.8.3 BrdU staining protocol

For BrdU staining, samples were fixed as described above. After fixation and washing with PBS, DNA hydrolysis was performed for 30min at 37°C in 200µl 2N HCl. Afterwards samples were washed quickly in PBS, following a washing step in saturated tetraborate solution. Then samples were washed with PBS twice. Blocking and staining were performed as described above.

3.9 Protein expression analysis

3.9.1 Protein isolation

Cells were grown to confluency, harvested, washed twice in ice-cold PBS and pelleted by centrifugation (1000 rpm, 5 min). Cells / brain tissue was put in 2x radioimmunoprecipitation assay (RIPA) buffer. Protease inhibitor cocktail and phenylmethanesulfonylfluoride (PMSF) was added to a final concentration of 10mM. Samples were homogenized, grinded in Eppendorf tubes using a mortar and / or ultrasonificated. Lysates were clarified by centrifugation (14000 rpm, 5 min, 4 °C).

3.9.2 Protein quantification

Proteins were measured via BCA assay. A standard curve was prepared for each measurement. Colorimetric intensity was assessed by Tecan plate reader at 562 nm wavelength.

3.9.3 SDS-Polyacrylamide gel electrophoresis

Mini-Protean TGX gels were used to separate the proteins using Mini Protean Tetra Cell electrophoresis chambers. Samples were mixed with 4x protein loading buffer, cooked for 5 min at 90°C and applied to the gel.

3.9.4 Western Blot

Blotting onto a polyvinylidene fluoride (PVDF) membrane has been performed using the Transfer Pack with the Trans Blot Turbo system for 10min at 10V. Afterwards membranes were incubated with blocking buffer (5% skimmed milk in PBS/Tween 0.05%) for 1h at RT. Membranes were incubated with primary antibodies diluted in blocking buffer on a shaker at 4°C over night. After incubation with the first antibody, the membranes were washed 3 x 5-10min at RT in washing buffer (PBS with 0.05% Tween-20). LI-Cor IRDye conjugated secondary antibodies, which allow detection with the Odyssey Infrared Imaging System, were applied to the membrane for 1h on

a shaker at RT. Washing 3x 5-10min with PBS/Tween 0.05% removed unbound antibody. The emitted light of the antibody-bound proteins on the membranes was detected at 680 or 800nm. Antibodies used are listed below.

Primary antibodies	Dilution	Secondary antibodies	Dilution
ms anti-β-Actin (Sigma-Aldrich)	1:5000	anti-ms-IgG 680 (Li-Cor)	1:20000
ms anti-NAMPT (Serotec)	1:1000	anti-ms-IgG 800 (Li-Cor)	1:20000
rabbit anti-Sirt1 (Millipore)	1:1000	anti-rabbit-IgG 680(Li-Cor)	1:20000
rabbit anti-Sirt3 (Cell Signalling)	1:1000	anti-rabbit-IgG 800 (Li-Cor)	1:20000
rabbit anti-Sirt6 (Sigma-Aldrich)	1:2000		

3.9.5 Analysis of optical density

Optical density (OD) was measures using Image J software. OD was normilized to β-actin.

3.10 Gene expression analysis

3.10.1 RNA isolation

To purify RNA from cell culture lysates or from tissue, the Nucleo Spin RNA / Protein Extraction Kit was used following the manufacturer's manual. Isolated RNA was measured with the Nanodrop 2000 spectrophotometer and directly applied for complementary DNA (cDNA) synthesis.

3.10.2 Complementary DNA synthesis

For complementary DNA (cDNA) synthesis, the following master mix (Applied Biosystems, USA) was used: 2,5µM oligo deoxythymines (dTs), 2,5mM deoxyribonucleotides (dNTPs), 5,5mM MgCl, 10U RNAse inhibitor, 25U reverse transcriptase. The mix was brought to a volume of 20 µl using the buffer supplied with the kit. 1µg of RNA was applied to this mix.

During initiation of cDNA synthesis at 25°C for 10min, oligo-dTs anneal to the RNA. Reverse transcription is done at 48°C for 30min, followed by an inactivation step at 95°C for 5min. At the end, cooling to 4°C preserves the freshly synthesized cDNA. cDNA was stored at -20°C and diluted 1:5 or 1:6 in DNAse / RNAse-free H_2O before amplification with quantitative real-time polymerase chain reaction (qRT PCR).

3.10.3 Quantitative real-time polymerase chain reaction

qRT-PCR was performed in duplicates with the 7500 Real Time PCR System. For quantification, Power SYBRGreen fluorescent dye or dual-labelled TaqMan (5'FAM, 3'TAMRA) probe were used. These reagents intercalate in newly synthesized DNA resulting in a fluorescent signal. Using the ΔΔCt-method, relative expression of the target gene was calculated. Two master mixes were prepared, one for the target gene and one for the housekeeping gene (GAPDH). Master mixes consisted of 2 x PCR master mix (Taq- polymerase, dNTPs, MgCl and ROX or SybrGreen), forward and reverse primers, fluorescent probes (if available) and water. The volume of the reaction solution was 20µl. qRT-PCR consists of 45 cycles. Each of these contains 15s of denaturation at 95°C and annealing at 60°C. The relative amount of RNA was normalized to the houskeeping gene GAPDH.

For visualization, qRT-PCR products from Power SYBRGreen samples were probed with 6x DNA loading buffer. 10µl sample and 2µl DNA ladder were loaded on a prepared 2% agarose gel and run at 100V for ~1h.

Primers were designed and ordered from Eurofins MWG Operon, Ebersberg, Germany. Stocks were diluted in DNAse-RNAse-free H_2O and used at a concentration of 10mM. Gene-specific primer pairs used for qRT-PCR are listed below.

Primer	Sequence
Catal	F1: AATCCTACACCATGTCGGACAGT R1: TCCGGTGGTCAGGACATCA
GAPDH	F3: CCAGCCTCGTCCCGTAGAC R3: TGGCAACAATCTCCACTTTGC
Lef1	F2: GGCGACTTAGCCGACATCAA R2: TCGTGGTAGGGCTCCTGAGA
Mash1	F2: CAAGAGCTGCTGGACTTTACCA R2: ACGTCGTTGGCGAGAAACAC

Primer	Sequence
NeuroD1	F1: CCACGCAGAAGGCAAGGT R1: GCTGTATGATTTGGTCATGTTTCC
Nrf1	F1: CCGCCTCTCACCATCGAT R1: CAGATAGGCTTGCAGCTTTCTTT
Nrf2	F3: ATCAGGCCCAGTCCCTCAAT R3: CCAGCGAGGAGATCGATGA
$p16^{INK4A}$	F1: AGACCGACGGGCATAGCTT R1: CGCTAGCATCGCTAGAAGTGAA
$p19^{ARF}$	F1: CGCTCTGGCTTTCGTGAAC R1: GTGAACGTTGCCCATCATCA
Sirt1	F2: GATGACGATGACAGAACGTCACA R2: GGATCGGTGCCAATCATGAG
Sirt3	F1: CCTGCAAGGTTCCTACTCCATATG R1: TTCAGACAAGCTGGCAAAAGG
SOD1	F1: TGGGTTCCACGTCCATCAGT R1: TGCCCAGGTCTCCAACATG
SOD2	F1: TCTGTGGGAGTCCAAGGTTCA R1: ATCCCCAGCAGCGGAATAA

3.10.4 Genomic DNA isolation

For isolation of genomic DNA (gDNA), samples were incubated with proteinase K (56°C, over night). Lysates were mixed with water (2:1 ratio). Ethanol was added to a final concentration of 60-65%. This mixture was incubated for at 15-30min on a shaker at RT. For higher purity, 300µl of 70% Ethanol was added before centrifugation for 10min at maximum speed. The pellet was incubated with 300µl of 70% Ethanol for 15-30min on a shaker at RT. After removal of the supernatant, pellets were dried for 5-15min at 56°C. The dried pellet was solubilized in 40µl dH_2O. Concentration of DNA was measured using a NanoDrop 200. Optimal $A_{260/280}$ ratio of the purified DNA was 1.7-1.9.

3.11 Image analysis

Pictures of living cell cultures for proliferation analysis were taken with the inverted microscope (magnification 10). Pictures for immunocyto- and –histochemistry were

taken with a fluorescence camera attached to a fluorescence microscope (magnification 20). For Olig2 and Sox2 analysis, $Sox2^+$ cells were counted in the HC DG using Photoshop. For this analysis, whole area of the DG was encircled to calculate the investigated area (fig. 7 left). Positive cells in this area were counted and normalized to the area of $1mm^2$. For DCX analysis, DCX^+ cells were counted in the SVZ (fig. 7 right). Length of the SVZ parts investigated was measured using Photoshop. Positive cells were normalized to the length of 1mm per investigated cell layers of SVZ. All image analyses were performed for at least 3 animals per experimental group. For all of these animals at least 3 glasses, containing 3-8 sections were investigated.

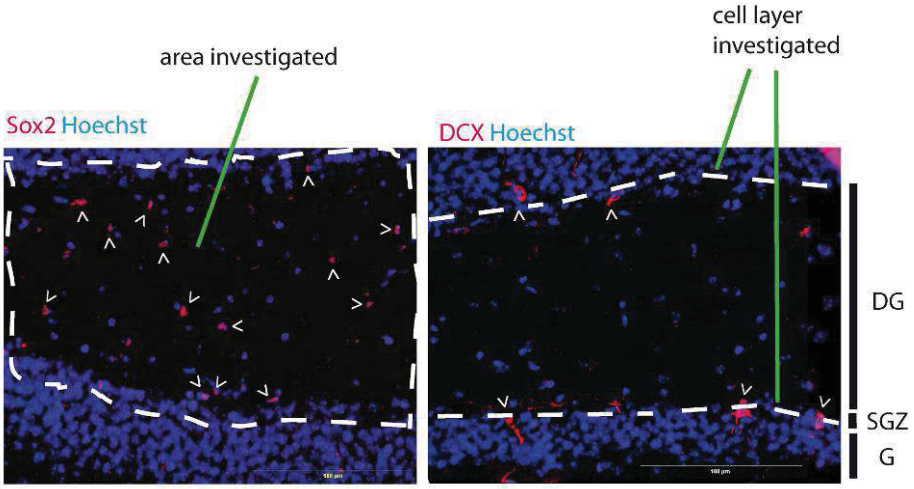

Figure 7: Image analysis of HC sections. For Sox2 (red; left) as well as Olig2 and BrdU analysis, positive cells were counted in the DG (encircled in white). Positive cells per area were calculated as $n(Sox2^+/mm^2)$. For DCX analysis (red; right), positive cells were counted in the SGZ. As this area only consists of very few cell layers, positive cells were calculated per length of SGZ as $n(DCX^+/mm)$. Nuclei were counterstained using Hoechst. Arrowheads give examples of counted cells. Scale bars display 100 µm.

3.12 Statistical analysis

If not stated otherwise, all experiments were performed in triplicate. All statistical analyses were done using One-Way ANOVA and Dunnett / Bonferri or unpaired t-test. Analysis was performed with the programme GraphPad Prism 5. All data are shown as mean plus standard error of the mean (SEM). Significances are shown as following: p :*<0.05; **<0.01; ***<0.001.

4. Results

4.1 Sirt1 and adult neurogenesis

4.1.1 Sirt1 and adult neurogenesis - *in vivo* data

4.1.1.1 Sirt1 in the adult hippocampus

Since 1965 it is known that neurogenesis occurs during the whole life of a mammal, but declines severely upon aging (Altman & Das 1965). In the present study, mouse hippocampal neurogenesis was assessed by analysis of BrdU labelled proliferating cells in the SGZ of young (3 months old) versus middle-aged (12 months old) C57/Bl5 wildtype mice (fig. 8). Those evaluations show that the number of actively proliferating $BrdU^+$ cells is severely decreased in middle-aged compared to young mice (mean of young: 4,3 $BrdU^+$ cells per mm^2, mean of middle-aged: 1,5 $BrdU^+$ cells per mm^2) (fig. 8, B+C). To assess, which cell pools are mostly affected, HC sections were stained for the general stem cell marker Sox2, and Olig2 as marker for OPCs, which can develop out of $Sox2^+$ stem cells. Decreased proliferation shown by BrdU analysis is associated with a significantly diminished number of $Sox2^+$ stem / progenitor cells in SGZ (mean of young: 1.271,7 $Sox2^+$ cells per mm^2, mean of middle-aged: 547,7 $Sox2^+$ cells per mm^2), whereas there is no significant difference in the $Olig2^+$ cell pool in young compared to middle-aged mice (mean of young: 399,0 $Olig2^+$ cells per mm^2, mean of middle-aged: 522,4 $BrdU^+$ cells per mm^2) (figure 8, D+E).

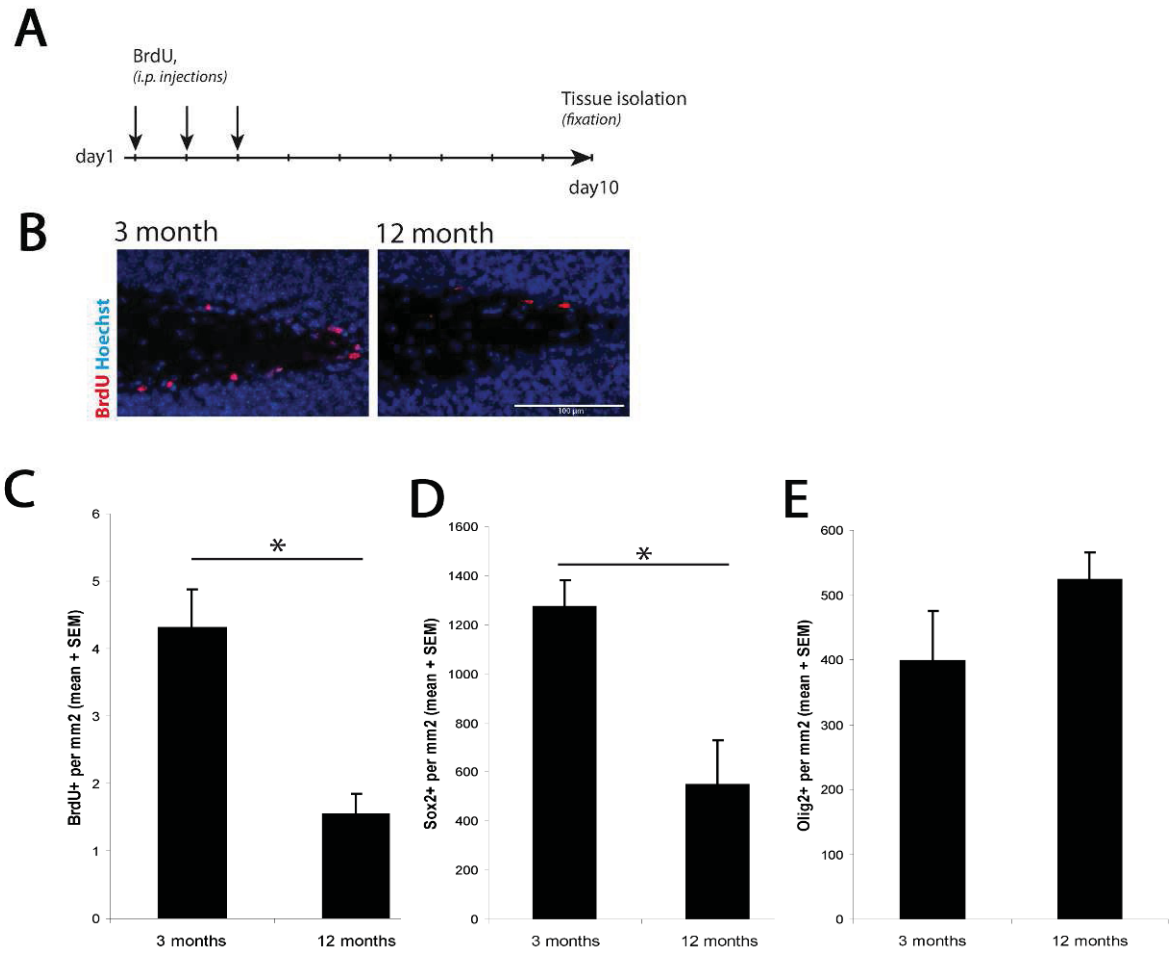

Figure 8: HC stem cells in young vs. aged mice. (A) Experimental design of BrdU pulse assay. BrdU was injected into 3 months old (n=3) and 12 months old (n=3) C57Bl6 wt mice on 3 following days, twice per day. 7 days after the last injection, on day 10, mice were sacrificed, brains were isolated, fixated, frozen and cut for further immunohistochemical analysis. (B) Reprentative image of immunohistochemical analysis of the HC DG. $BrdU^+$ cells (red) costained with nuclei (Hoechst; blue). Scale bar displays 100µm. (B, C) Evaluation of $BrdU^+$ cells per mm^2 SGZ reveals a significantly decreased number of $BrdU^+$ cells in the HC subgranular zone (SGZ) of 12 months old compared to 3 months old mice. (D) The amount of $Sox2^+$ stem cells is significantly decreased upon aging, whereas (E) the oligodendrocyte transcription factor 2 $(Olig2)^+$ oligodendrocyte cell pool is unaffected. For each mouse at least 6 brain sections were investigated. Mean per mouse of positive cells per mm^2 of DG was calculated, from which mean per genotype and significance were calculated; unpaired t-test, *$p < 0.05$.

To further assess changes of HC neurogenesis upon aging, real time PCR (rtPCR) was performed using HC tissue of young and middle-aged mice. Expression of sirtuins, antioxidant enzymes and cell cycle inhibitors was evaluated. Factors of all 3 groups are in current focus regarding neurogenesis:

Sirtuins, especially Sirt1, has been shown to be essential for neuronal differentiation and improves viability of new born cells (Guo et al., 2011; Braidy et al., 2012; Michan et al., 2010).

Earlier work of our group showed that oxidative conditions suppress proliferation of NPCs and induce their differentiation towards astrocytes at the expense of neurons (Prozorovski et al., 2008), emphasizing the importance to clarify the regulation of antioxidant enzymes in the aging brain.

Declining regenerative potential due to cellular senescence of stem cells has been found in the aged mouse forebrain (Molofsky et al., 2006). Stem cells can undergo only a distinct amount of divisions, before cell cycle arrest is reached (Campisi 2000; Hayflick & Moorhead 1961). This also has implications on adult neurogenesis.

Therefore induction of cell cycle inhibitors may give a possible explanation for reduced stem cell proliferation in this experiment.

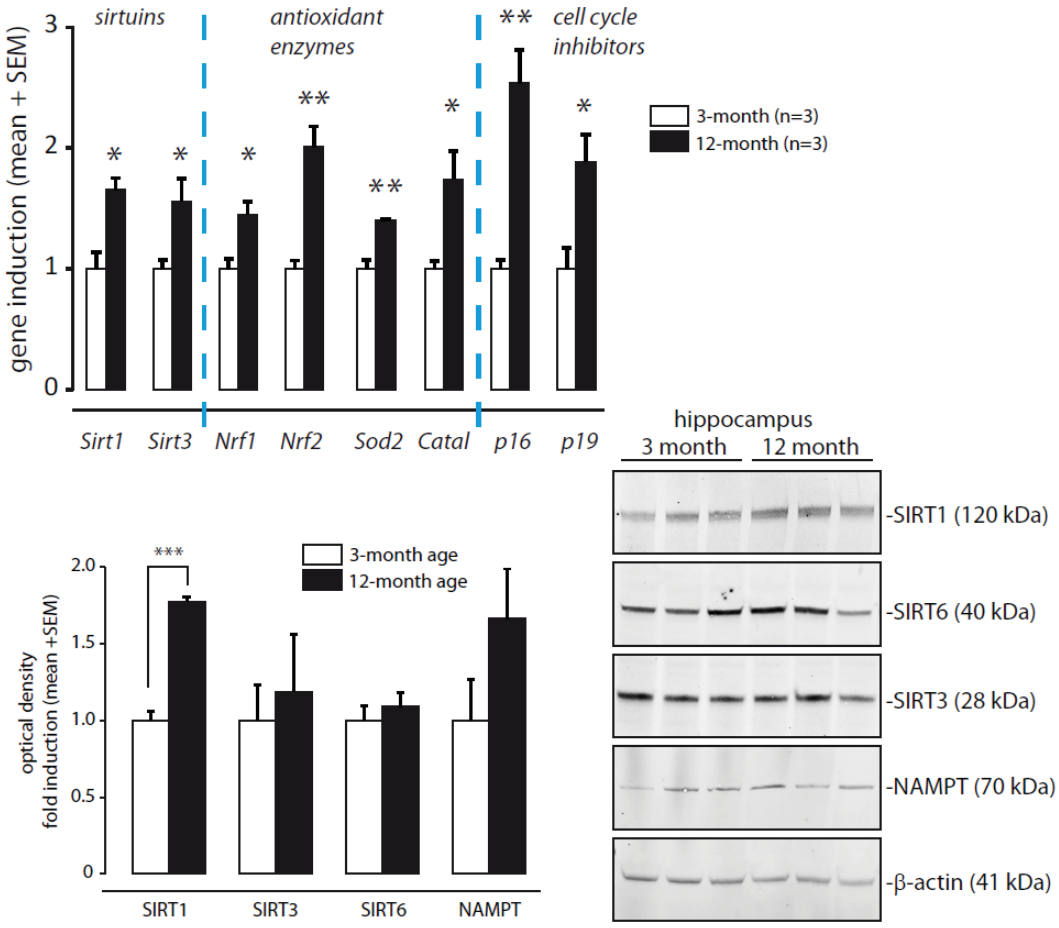

Figure 9: Gene and protein expression of sirtuins, antioxidant enzymes and cell cycle inhibitors in the HC of young (3 months old, n=3) vs. middle-aged (12 months old, n=3) mice. Top: Gene induction of Sirtuins (Sirt1, Sirt3), antioxidant enzymes (Nrf1, Nrf2, superoxide dismutase (Sod2), catalase (Catal) and cell cycle inhibitors (p16, p19) assessed by qRT-PCR is significantly upregulated in the HC upon aging. Data presented as fold gene induction normalized to induction of 3 months old mice. Bottom: WB analyses of Sirt1, Sirt3, Sirt6, and NAMPT in HC of middle-aged mice (contol: β-actin) also shows a significant upregulation of Sirt1 in middle-aged compared to young mice. Data presented as fold induction of optical density normalized to induction of 3 months old mice. Unpaired t-test, *p < 0.05, **p < 0.01, ***p < 0.001.

Comparison of gene expression in the HC of young versus aged mice showed a significant upregulation of Sirtuins (Sirt1 and Sirt3), antioxidant enzymes (Nrf1, Nrf2, Sod2 and Catalse) and cell cycle inhibitors (p16 and p19) in aged mice (fig. 9, top). $p16^{INK4a}$ is induced in several tissues upon aging in mice as well as in humans (Zindy et al., 1997; Liu et al., 2009). This work shows that those effects also occur in the aged rodent brain. Regarding Sirt1, those effects could also be revealed on protein level (fig. 9, bottom). Sirt1 has been shown to have several effects on neurogenesis (see chapter 1.3.1.8).

Next, Sirt1 was stained in the HC of young (3 months old) and middle-aged (12 months old) mice, to assess possible differences regarding localization or intensity of Sirt1 immunoreactivity. Microscopy evaluations revealed that Sirt1 is expressed in the granular layer of the HC in young as well as in middle-aged mice (fig. 10). Additionally, the intensity of Sirt1 immunoreactivity does not differ in young compared to aged mice, as Sirt1 expression is not blocked, but a mutant, unfunctional form of Sirt1 without exon 4 is still expressed and recognized by our αSirt1 antibody.

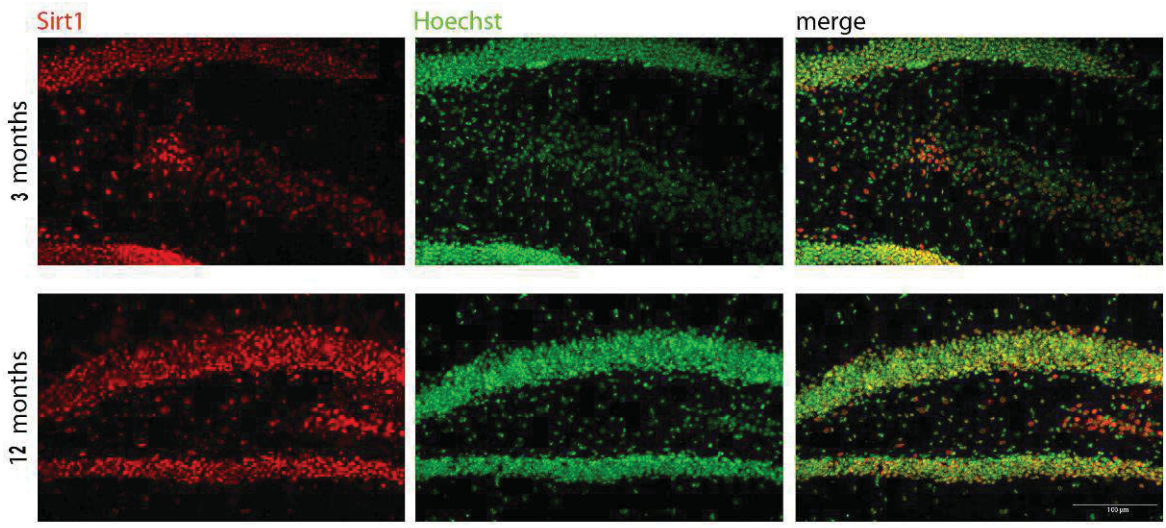

Figure 10: Expression of Sirt1 in the HC upon aging. Immunostaining of the HC DG. Sirt1 (red) is ubiquitously expressed in neurons of the HC granular layer and hilus cells in young (3 months old) and middle-aged (12 months old) mice**.** Nuclei were counterstained using Hoechst. Scale bar displays 100 µm.

To check if Sirt1 is also expressed in cells of the SGZ, the HC stem cell niche, brain sections were co-stained with antibodies against Sirt1 and GFAP, a HC stem cell marker of the DG (fig.11). Evaluation revealed that Sirt1 is also expressed in SGZ stem cells. This raised the issue which functions Sirt1 might have for adult HC neurogenesis. To answer this question further experiments were performed in aged Sirt1 deficient mice, as the before mentioned results indicated that changes in neurogenesis would rather occur in aged brains.

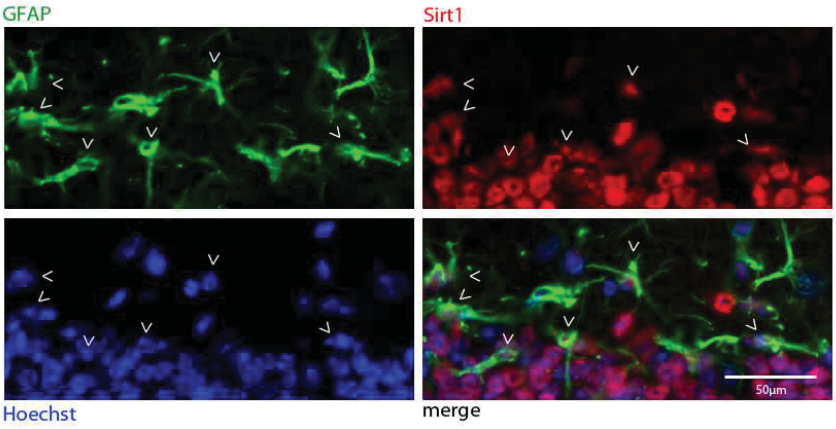

Figure 11: Expression of Sirt1 in the SGZ. Shown is an immunostaining of the HC SGZ. Sirt1 (red) is expressed in GFAP^{+} stem cells (green) of the HC SGZ. Coexpressions indicated by white arrowheads. Nuclei counterstained using Hoechst. Scale bar displays 50µm

4.1.1.2 Hippocampal neurogenesis in aged systemic Sirt1$^{(+/-)}$ mice

As systemic deletion of both Sirt1-alleles has been shown to be lethal, Sirt1$^{+/+}$ mice were compared to heterozygous Sirt1 KO mice, Sirt1$^{+/-}$.

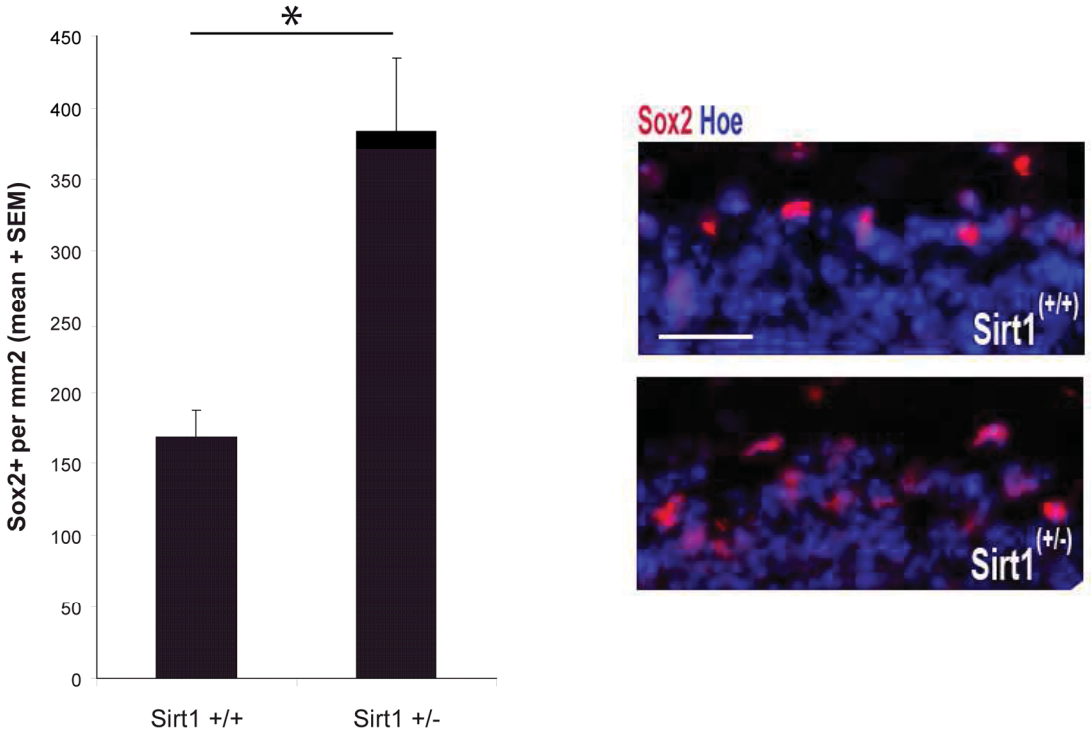

Figure 12: Quantification of Sox2^{+} cells in the HC of 12 months old Sirt1$^{+/-}$ vs. Sirt1$^{+/+}$ mice. n=3 per genotype; for each mouse at least 5 brain sections were investigated. Mean per mouse of positive cells per mm^2 of DG was calculated, from which mean per genotype and significance were calculated; unpaired t-test, *p < 0.05.; Representative images of the HC SGZ display Sox2^{+} cells in red. Nuclei were counterstained using Hoechst (blue). Scale bar displays 20 µm.

12 months old Sirt1$^{+/-}$ mice showed a significantly increased amount of Sox2^{+} SGZ stem cells (mean of Sirt1$^{+/+}$: 169,6 Sox2^{+} cells per mm^2, mean of Sirt1$^{+/-}$: 383,9 Sox2^{+} cells per mm^2) (fig. 12). A similar, though not significant effect was visible regarding the Olig2^{+} cell pool (mean of Sirt1$^{+/+}$: 130,6 Olig2^{+} cells per mm^2, mean of Sirt1$^{+/-}$: 293,5 Olig2^{+} cells per mm^2) (fig. 13).

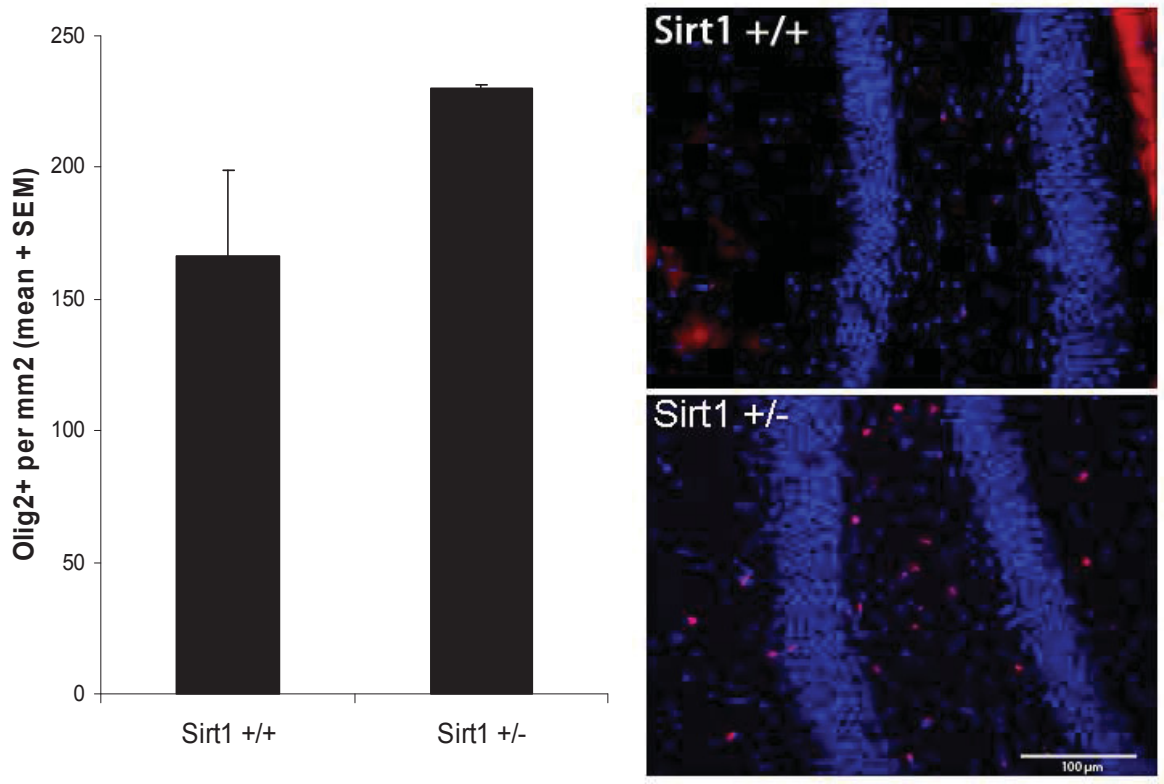

Figure 13: Quantification of Olig2^{+} cells in the HC of 12 months old Sirt1$^{+/-}$ vs. Sirt1$^{+/+}$ mice. n=3 per genotype; data presented as mean of positive cells per mm^2 of DG; for each mouse at least 8 brain sections were investigated. Mean per mouse of positive cells per mm^2 of DG was calculated, from which mean per genotype and significance were calculated; unpaired t-test did not reveal any significance. Representative images of the HC DG display Olig2^{+} cells in red. Nuclei were counterstained using Hoechst (blue). Scale bar displays 100µm.

These first evaluations hint at an increase of neurogenesis in the HC of Sirt1$^{+/-}$ mice. Though, as the mouse strain used for these analyses exhibits Sirt1 deficiency in the whole body (systemic), the next step was to rule out side effects of this systemic loss of Sirt1 function on adult HC neurogenesis. Additionally to get more solid data, it was necessary to create a mouse with homozygous Sirt1 deficiency, which was not possible for the strain with systemic loss of Sirt1 function, as those animals die prior birth. For this reasons a new mouse line was generated, in which Sirt1 functional loss is restricted to Nestin expressing cells.

4.1.1.3 Hippocampal neurogenesis in mice with brain specific expression of unfunctional Sirt1

The new mouse strain was created by breeding NestinCre mice with Sirt1LoxP mice (see chapter 3.6.2). Since in addition to CNS cells nestin is ubiquitously expressed in the male germline, this breeding resulted in offspring of 3 different genotypes: 1.) NestinCre;Sirt1$^{+/+}$, expressing the wt form of Sirt1, 2.) NestinCre;Sirt1$^{+/-}$ with systemic loss of Sirt1 function on one allele and 3.) NestinCre;Sirt1$^{lox/-}$, in which Sirt1 function is lost systemically as well as in Nestin expressing cells of the CNS.

First expression of Sirt1 in all three genotypes was assessed, using immunohistochemical stainings evaluated by fluorescence microscopy (fig. 14). The specific deletion in the Sirt1 allele did not lead to a loss of Sirt1 protein in either of the genotypes created. This was proven by immunohistochemical staining for Sirt1, as well as via agarose gel gel analysis of brain tissue (fig. 15). Those results are not surprising, as Sirt1 protein is not lost in this strain, but a mutant, inactive from of Sirt1 is expressed (floxed Sirt1), which can also be detected by the αSirt1 antibody. The band of the floxed Sirt1 protein runs at a lower size, as the floxed part is cut out (mut Sirt1).

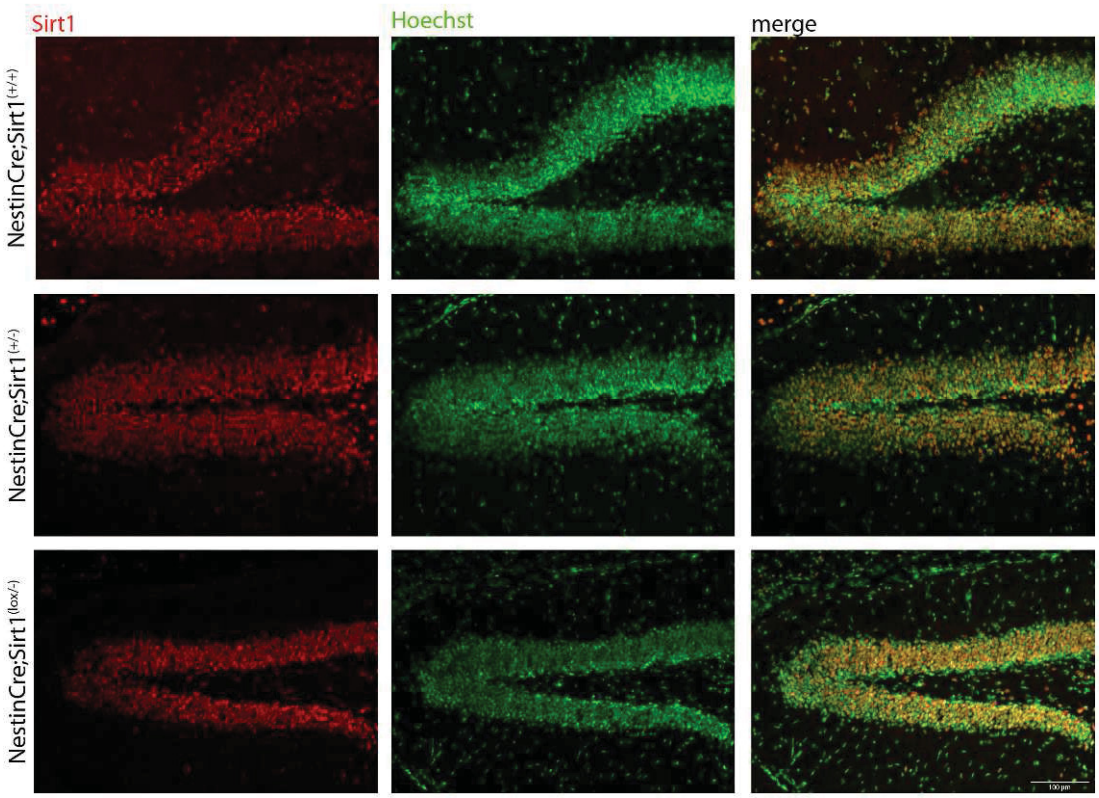

Figure 14: Expression of Sirt1 in the HC of young adult (3 months) NestinCre;Sirt1$^{+/+}$, NestinCre;Sirt1$^{+/-}$ and NestinCre;Sirt1$^{lox/-}$ mice. Shown is the immunostaining of the HC DG. Sirt1 (red) is expressed in the granular layer of all genotypes. Nuclei counterstained with Hoechst (green). Scale bar displays 100µm.

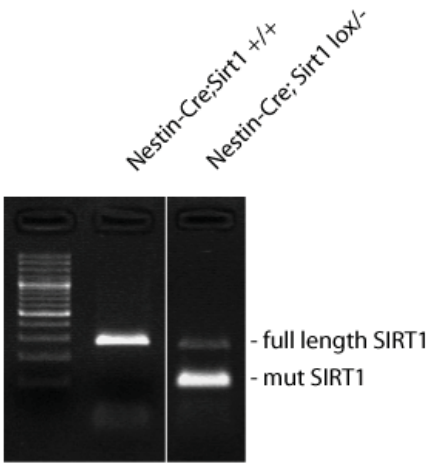

Figure 15: Expression of Sirt1 protein in the HC of young (3 months) NestinCre;Sirt1$^{+/+}$ vs NestinCre;Sirt1$^{lox/-}$ mice. Agarose gel image. In the floxed form of Sirt1 (mut Sirt1; right) the catalytic domain is cut out, indicated by the lower basepair running size on the gel. A small band for full length Sirt1 is still visible, indicating that Sirt1 is not floxed in brain cells without Nestin expression (e.g. blood vessels).

To assess if the stem cell pools are affected by loss of Sirt1 function in this mouse strain, brain sections were examined by performing immunohistochemical stainings with antibodies against the stem cell marker Sox2 and neuronal marker DCX (fig. 16). Upon brain-specific deletion of Sirt1 activity, the Sox2$^+$ stem cell pool (mean NestinCre;Sirt1$^{+/+}$: 312,5 Sox2$^+$ cells per mm^2, mean NestinCre;Sirt1$^{+/-}$: 666,3 Sox2$^+$ cells per mm^2, mean NestinCre;Sirt1$^{lox/-}$: 1.563,8 Sox2$^+$ cells per mm^2) as well as the number of DCX$^+$ neuronal progenitor (mean NestinCre;Sirt1$^{+/+}$: 18,7 DCX$^+$ cells per mm, mean NestinCre;Sirt1$^{+/-}$: 26,7 DCX$^+$ cells per mm, mean NestinCre;Sirt1$^{lox/-}$: 51,2 DCX$^+$ cells per mm) were significantly increased, in negative correlation with the amount of unfunctional Sirt1. This is in line with the data from systemic Sirt1 KO mice, suggesting that specific deletion of Sirt1 function in Nestin-expressing cells does not modulate HC stem cell pools to a higher extent, than systemic Sirt1 deficiency. The effect is exclusively due to the deletion of Sirt1 in brain cells and more pronounced in mice with brain specific deletion of Sirt1.

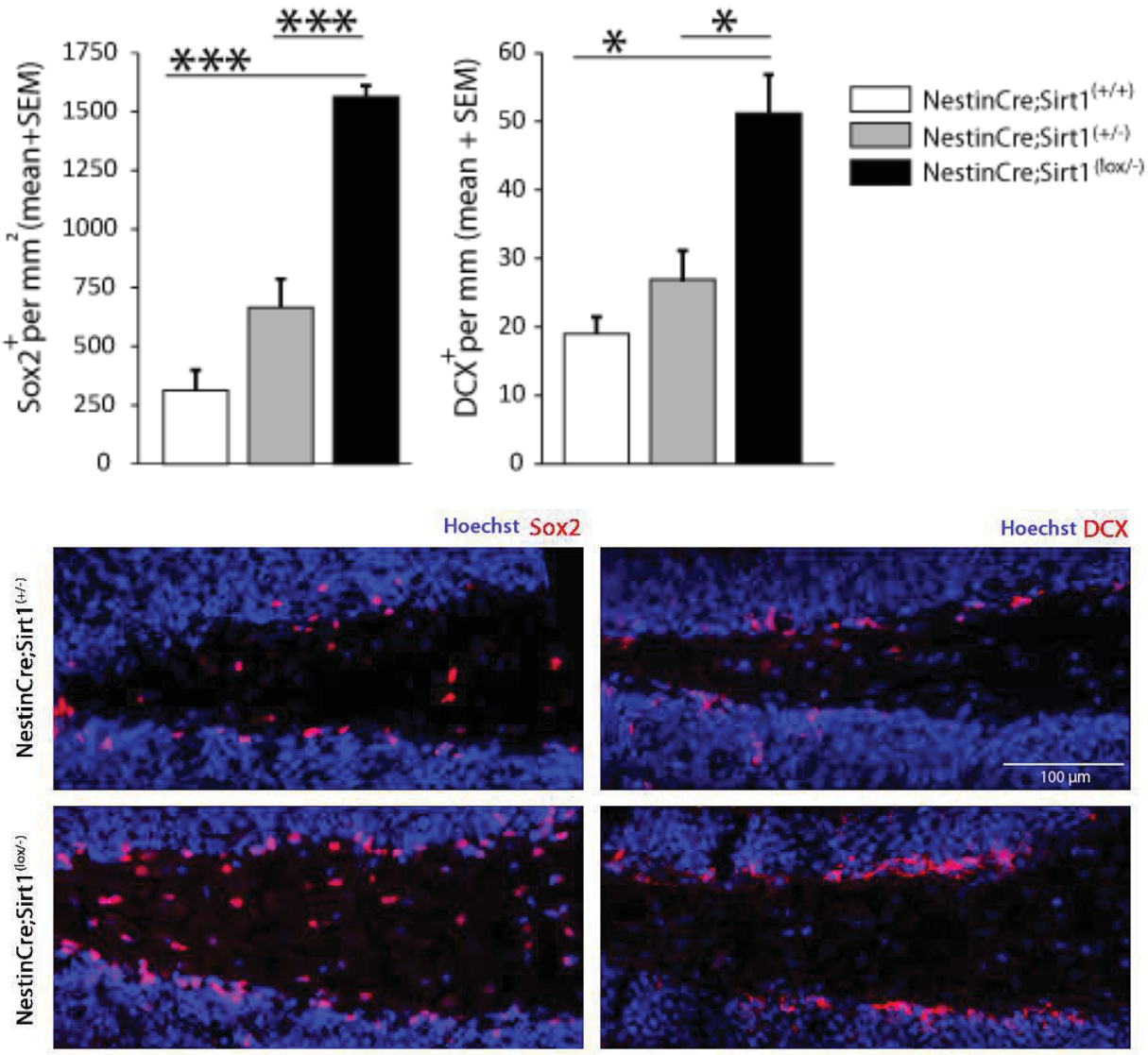

Figure 16: Expression of Sox2 and DCX in HC of young (3 months) NestinCre;Sirt1$^{+/+}$, NestinCre;Sirt1$^{+/-}$ and NestinCre;Sirt1$^{lox/-}$ mice. n=3 per genotype; Top: To assess HC neurogenesis, immunohistochemical staining of brain sections for expression of the stem cell marker Sox2^{+} (left) and DCX^{+} (neuronal precursor cells; right) in the DG (for Sox2) and SGZ (for DCX) of NestinCre;Sirt1$^{+/+}$, NestinCre;Sirt1$^{+/-}$ and NestinCre;Sirt1$^{lox/-}$ mice were analysed. Data presented as mean of positive cells per mm^2 of DG for Sox2 and per mm of SGZ for DCX. For each mouse at least 6 brain sections were investigated. Mean per mouse of positive cells per mm^2 of DG (for Sox2) or per mm of SGZ (for DCX) was calculated, from which mean per genotype and significance were calculated. Unpaired t-test, *p < 0.05; ***p < 0.001. Bottom: Representative images of immunohistochemical staining of the HC DG used for analysis. SOX2^{+}/DCX^{+} cells (red) counterstained with Hoechst, staining nuclei (blue). Scale bar displays 100µm.

As the increase in Sox2^{+} stem cells described in figure 16 could be explained by higher mitotic activity in the SGZ of Sirt1 deficient mice, mitotic activity was assessed by staining for phosphorylated histone 3, occurring in actively mitotic cells (H3Ser10 staining) (fig. 17).

This analysis showed that overall proliferation of stem cells in HC of mice with brain specific Sirt1 deficiency might be increased (mean NestinCre;Sirt1$^{+/+}$: 0,5 H3Ser10^{+} cells per mm, mean NestinCre;Sirt1$^{lox/-}$: 2,7 H3Ser10^{+} cells per mm). Though this data did not show significance, they are line with data obtained from mice with systemic Sirt1 deficiency (see chapter 4.1.2.).

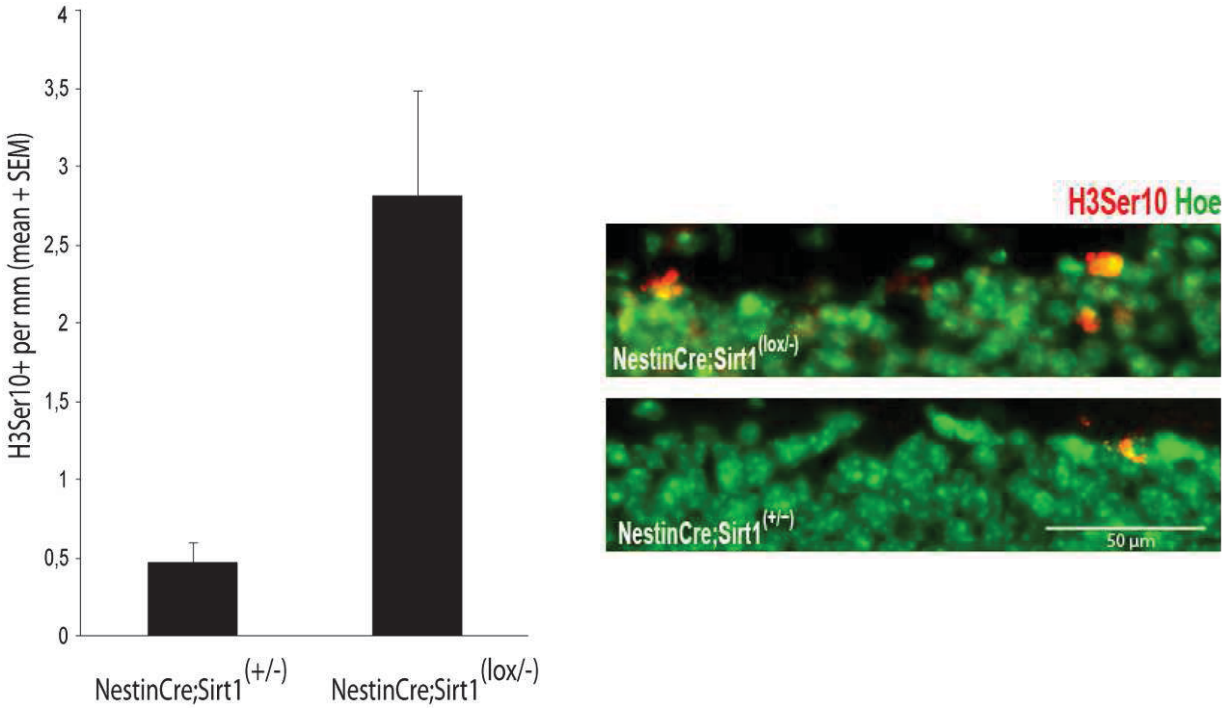

Figure 17: Analysis of proliferation in the HC SGZ of young adult (3 months) NestinCre;Sirt1$^{(+/-)}$ vs. NestinCre;Sirt1$^{(lox/-)}$ mice. Staining for H3Ser10 was used, as histone 3 becomes phosphorylated an serine 10 at the end of the prophase. Thereby this staining indicates actively proliferating cells. n=3 per genotype; Left: Analysis of H3Ser10^{+} cells in the HC SGZ of NestinCre;Sirt1$^{+/+}$ vs NestinCre;Sirt1$^{lox/-}$ mice. Data presented as positive cells per mm SGZ; for each mouse at least 3 brain sections were investigated. Mean per mouse of positive cells per mm of SGZ was calculated, from which mean per genotype and significance was calculated; Unpaired t-test did not reveal significance. Right:. Representative images of Immunohistochemical staining of the HC SGZ used for analyis. H3Ser10^{+} cells (red) counterstained with nuclei stained by Hoechst (green). Scale bar displays 50µm.

As proliferation is not the only paradigm by which stem cells can be described, next it was examined whether Sirt1 deficiency might affect migration, differentiation and functional integration of new born neurons into their target layers. To answer these questions, immunohistochemical analyses were performed in brain sections of mice pups at postnatal day 0.5, which had been *in utero* electroporated with a Sirt1 silencing plasmid (small hairpin (sh)Sirt1) at E15.5. The advantage of this technique is that Sirt1 is only lost in single cells, mainly of the SVZ. Therefore the differentiation and migratory capacity of those SVZ resident stem cells can be evaluated, excluding side effects which might be caused by Sirt1 deficiency in neighbouring cells / other CNS cell types.

4.1.1.4 Migration of Sirt1 deficient neuronal precursor cells during cell maturation

To assess migratory capacity of Sirt1 deficient stem cells, immunohistochemical evaluations were performed in the SVZ. For this analysis, the SVZ was preferred to the SGZ, as the distance of migration is longer in SVZ, with cells wandering from this area into the cortical layers (see Dehay & Kennedy 2007). This allows a more detailed analysis of migratory behaviour.

First it was investigated if also $Sox2^+$ SVZ stem cells were *in utero* electroporated. Therefore the SVZ of electroporated mice was stained with a Sox2 targeting antibody (fig. 18). As $Sox2^+$ cells also expressed GFP, this could prove that $Sox2^+$ stem cells also were electroporated in both experimental groups: Mice electroporated with a scrambled plasmid (control hairpin designed to demonstrate positive electroporation without targeting Sirt1) as well as mice electroporated with shSirt1.

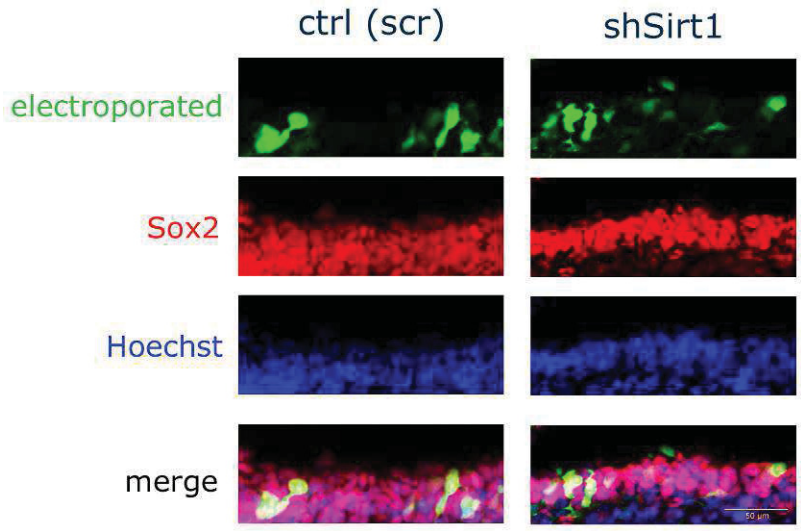

Figure 18: Expression of Sox2 in the SVZ does not differ in mice electroporated with a scrambled plasmid (ctrl (scr); left) and a plasmid silencing Sirt1 expression (small hairpin Sirt1 RNA (shSirt1); right). Immunohistochemical staining of the HC SGZ. Successful electroporation can be assessed by expression of GFP (green), which is transferred via the plasmid. $Sox2^+$ cells (red), nuclei stained by Hoechst (blue). Scale bar displays 50µm.

Next, having proved that *in utero* electroporation also led to Sirt1-deficient $Sox2^+$ SVZ stem cells, it was assessed whether specification, cell fate decision and migratory capacity of new born neurons is changed by loss of Sirt1 in those cells. Investigations focused on NPCs, as these are the main stem cell type of the SVZ. Immunohistochemical analyses revealed that Sirt1 deficiency does not affect migration and integration of SVZ stem cells / new born neurons into the cortical layers (fig. 19).

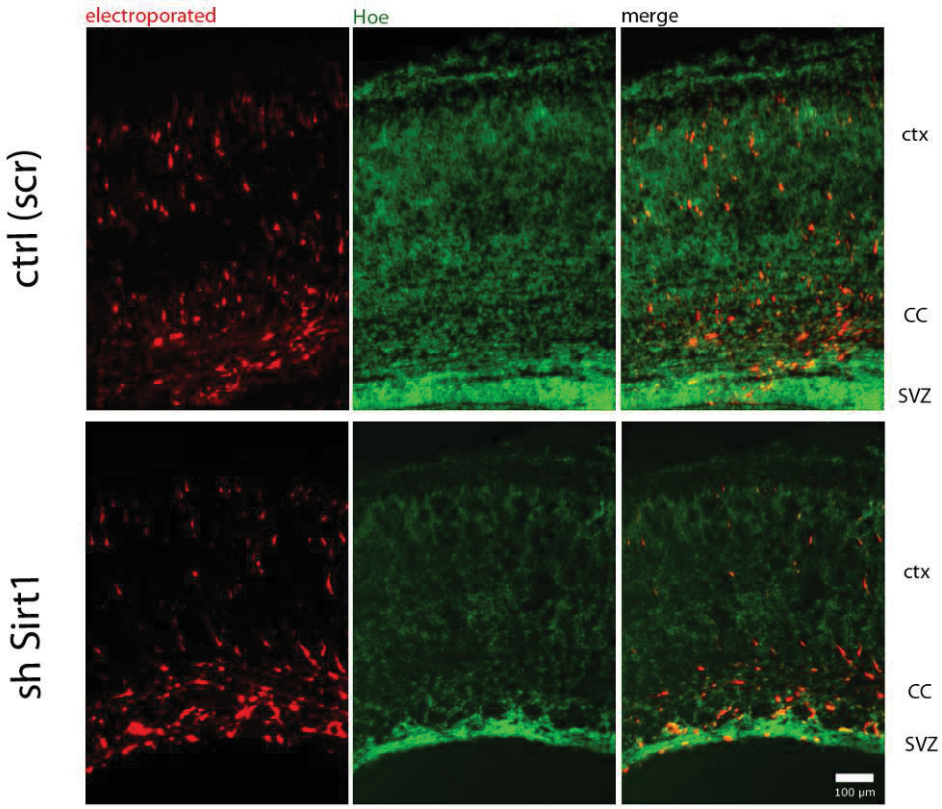

Figure 19: Migratory capacity of SVZ stem cells electroporated with scrambled (ctrl(scr)) and Sirt1 silencing plasmids (shSirt1) does not reveal changes upon Sirt1 loss. Immunohistochemical staining of the coronally cut mouse cortex. Electroporated, GFP expressing cells (red) migrate from the SVZ through the corpus callosum (CC) into the outer layers of the cortex (ctx). Nuclei counterstained with Hoechst (green**).** Scale bar displays 100µm.

Next it was investigated if single cell Sirt1 deficiency led to changes regarding differentiation into oligodendrocytes. Therefore brain sections of electroporated mice were stained with the oligodendrocyte specific markers Ng2 and Olig 2. Differentiation of Sirt1 deficient cells into oligodendrocytes seems to be decreased in Sirt1 deficient cells in the SVZ and corpus callosum, though this data did not reach significance (%$NG2^+/GFP^+$ in SVZ of scr: 3,4; in SVZ of shSirt1: 1,7. %$NG2^+/GFP^+$ in CC of scr: 3,0; in CC of shSirt1: 2,5) (%$Olig2^+/GFP^+$ in SVZ of scr: 10,0; in SVZ of shSirt1: 7,1. %$Olig2^+/GFP^+$ in CC of scr: 11,1; in CC of shSirt1: 9,4) (fig. 20).

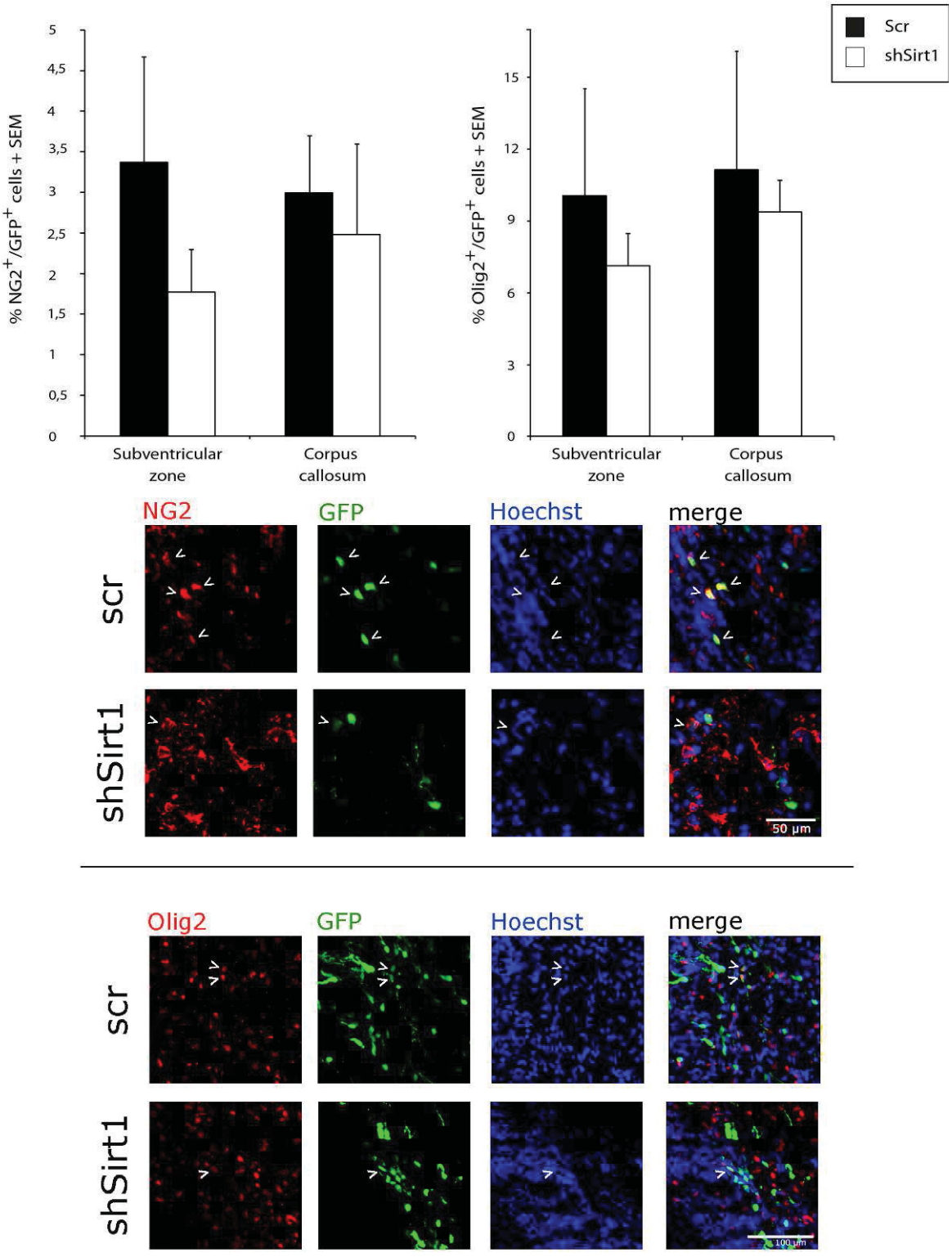

Figure 20: Expression of OPC cell markers in the HC of mice electroporated with a scr plasmid (scr) and a plasmid silencing Sirt1 (shSirt1). n=3 per genotype; Top: evaluations of $NG2^+/GFP^+$ and $Olig2^+/GFP^+$ cells in SVZ and corpus callosum of electroporated mice. Data presented as mean percentage of positive cells of GFP^+ electroporated cells. For each mouse at least 3 brain sections were investigated. Mean percentage per mouse of positive cells was calculated, from which mean per genotype and significance were calculated. Unpaired t-test did not reveal any significance. Bottom: Representative images of immunochistochemical staining of the HC SVZ used for evaluation (NG2 and Olig2 expression (red). GFP (green) shows successfully electroporated cells. Nuclei counterstained with Hoechst (blue). Scale bar displays 50µm for NG2 analysis and 100µm for Olig2 analysis.

In summary, *in vivo* analyses of Sirt1 deficiency revealed an increased amount of actively proliferating, mitotic stem cells in the SGZ compared to wt mice. To test if this effect also occurs in NPC cultures, *in vitro* experiments using mouse cortical NPCs from wt as well as Sirt1 deficient animals were performed.

4.1.2 Sirt1 and adult neurogenesis - *in vitro* data

4.1.2.1 Characterization of neuronal precursor cells in long-term cultures

To characterize long-term mouse cortical NPC cultures, prolonged passaging of these cells was performed. After isolation and culturing for some days in proliferation medium, cells of NPC cultures from wt C57/Bl6 mice divided fast, leading to the creation of cell clusters called neurospheres (fig. 21 (B)). This self renewal capacity is severely decreased with prolonged passaging. The amount of cells being able to create neurospheres is significantly decreased in late (passages 5/6) compared to early passages (passage 1/2) (fig. 21 (A)).

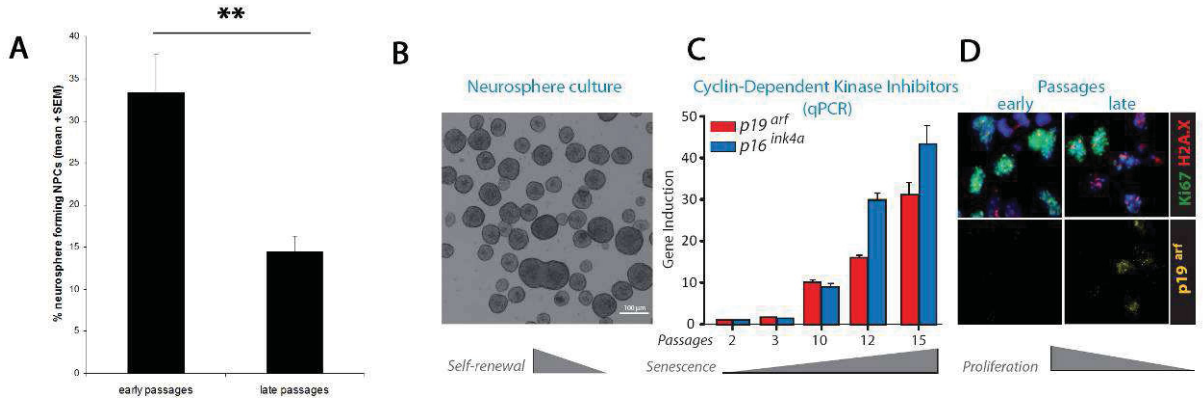

Figure 21: Cellular senescence in NPC cultures. (A) Analysis of neurosphere forming ability of NPCs. Upon prolonged culturing, self-renewal capacities of NPCs, assessed by the amount of NPCs able to create spheres, are lost, visible by a significant decrease of neurosphere forming NPCs from early (passage 1/2) to late passages (passage 5/6). Data presented as mean percentage of cells able to create neurospheres, normalized to whole amount of cells investigated. For each passage at least 3 images of 3 cultures were investigated. Mean percentage per culture was calculated, from which mean per passage and significance were calculated. Unpaired t-test, **p < 0.01. (B) Representative light microscopy image of a neurospheres culture evaluated for (A). (C) Prolonged culturing leads to an upregulation of cyclin-dependent kinase inhibitors $p19^{arf}$ and $p16^{ink4a}$ as markers of DNA repair mechanisms (results of qRT-PCR). This is also evident on immunocytochemical level (D) indicated by the increased immunoreactivity of H2A.X, which is responsible for DNA repair. Therefore,. Those markers proof that upon prolonged passaging DNA damage accumulated. Proliferative capacity, indicated by staining of the protein Ki67, occurring only in mitotic cells, is decreased in late compared to young passages.

In parallel, cells of several passages (pas. 2, 3, 10, 12 and 15) were analysed by qRT PCR: Compared to cells of early passages, cells of late passages showed increased cyclin-dependent kinase inhibitor expression, suggesting an exit from the cell cycle (fig. 21 (C)). In line with these results, immunohistochemical analysis showed decreased Ki67 immunoreactivity in cells of late compared to cells of early passages. As the protein Ki67 only occurs in mitotic cells, it serves as a direct marker of proliferative activity. Additionally, in late passages DNA damage increases, indicated by enhanced immunoreactivity of histone H2A.X and $p19^{arf}$ (fig. 21 (D)). Histone H2A.X and $p19^{arf}$ are known to stabilize DNA, repair DNA damage and suppress tumour formation. This increase in DNA damage is one of the reasons for decreased proliferation, as DNA damage responses are activated, leading to an exit of cell cycle.

To further investigate the effect of Sirt1 on proliferation and differentiation of NPCs, Sirt1 expression in HC NPC cultures was assessed. Sirt1 is expressed in neurospheres. This expression is rather restricted to non-proliferating cells, as there are no cells costained with Sirt1 and the proliferation marker Ki67. During interphase of cell division, Ki67 is located in the nucleus. During mitosis, it is relocated to the surface of the chromosomes. Ki67 is present during all active phases of the cell cycle (G1, S, G2, and mitosis), but is absent from resting cells.

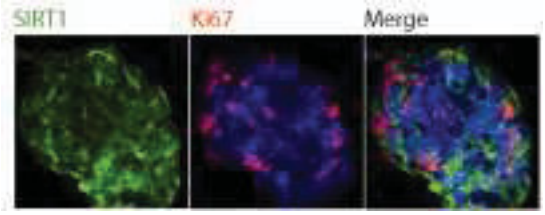

Figure 22: Immunocytochemical staining of a HC derived neurospere. Representative image of fluorecence microscopy of a neurospheres, consisting of NPCs. Sirt1 (green) is not coexpressed with actively proliferating Ki67 staining (red). Nuclei counterstained with Hoechst (blue).

To check Sirt1 expression upon prolonged culturing, Sirt1 gene induction in cells of late passages was compared to early passages. Sirt1 is upregulated in cells of late compared to early passages (fig. 23). It is already known that Sirt1 is also upregulated upon oxidative stress (Prozorovski et al., 2008). As during aging the brain becomes more and more vulnerable to oxidative stress (Mariani et al., 2005; Venkataraman et al., 2013), another experiment was performed to investigate if the upregulation of Sirt1 in late passages might be a result of enhanced oxidative stress.

For this purpose, young NPC cultures were treated with buthionine-sulfoximine (BSO). This compound reduces the levels of glutathione, due to which oxidative stress increases in the cells. Upon BSO treatment, Sirt1 was significantly upregulated, leading to the assumption, that aging induced oxidative stress might be the reason for the upregulation of Sirt1 gene induction in cells of late passages.

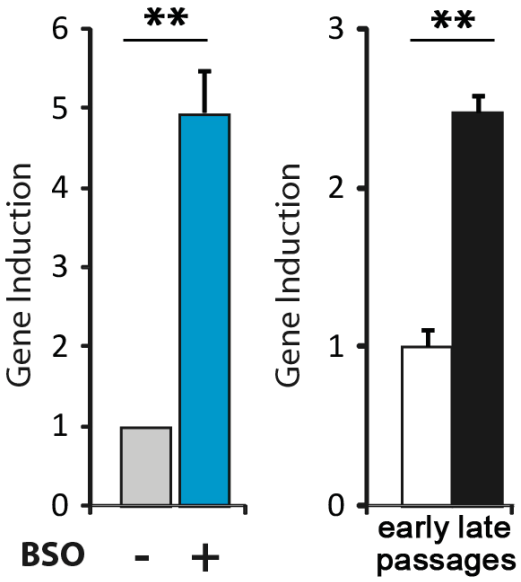

Figure 23: Sirt1 expression in NPC cultures of late passages and under oxidative stress. Oxidative stress induced via BSO treatment led to an induction of the Sirt1 gene (left). An induction is also evident upon prolonged passaging (right). Data of qRT-PCR presented as gene induction normalized to 1 (no induction). Unpaired t-test, **p < 0.01.

The next question was, wether loss of Sirt1 function in NPC culture affects proliferation or differentiation capacities of those cells. To address this question, further experiments were performed using NPC cultures from Sirt1 deficient mice.

4.1.2.2 Hippocampal stem cell maintenance of neuronal precursor cells expressing unfunctional Sirt1

To characterize Sirt1 deficient NPCs, proliferation as well as differentiation assays were performed. NPCs of all three genotypes (Sirt1 wt, $Sirt1^{+/-}$ and $Sirt1^{-/-}$) were capable of giving rise to $CNPase^{+}$ oligodendrocytes, $GFAP^{+}$ astrocytes and NFM^{+} neurons (fig. 24, top right).

Sirt1 deficient NPC cultures showed a higher number of cells creating neurospheres. This effect could be investigated for NPCs derived from $Sirt1^{+/-}$ mice and was more pronounced in cells derived from $Sirt1^{-/-}$, in which whole Sirt1 activity is lost (fig. 24).

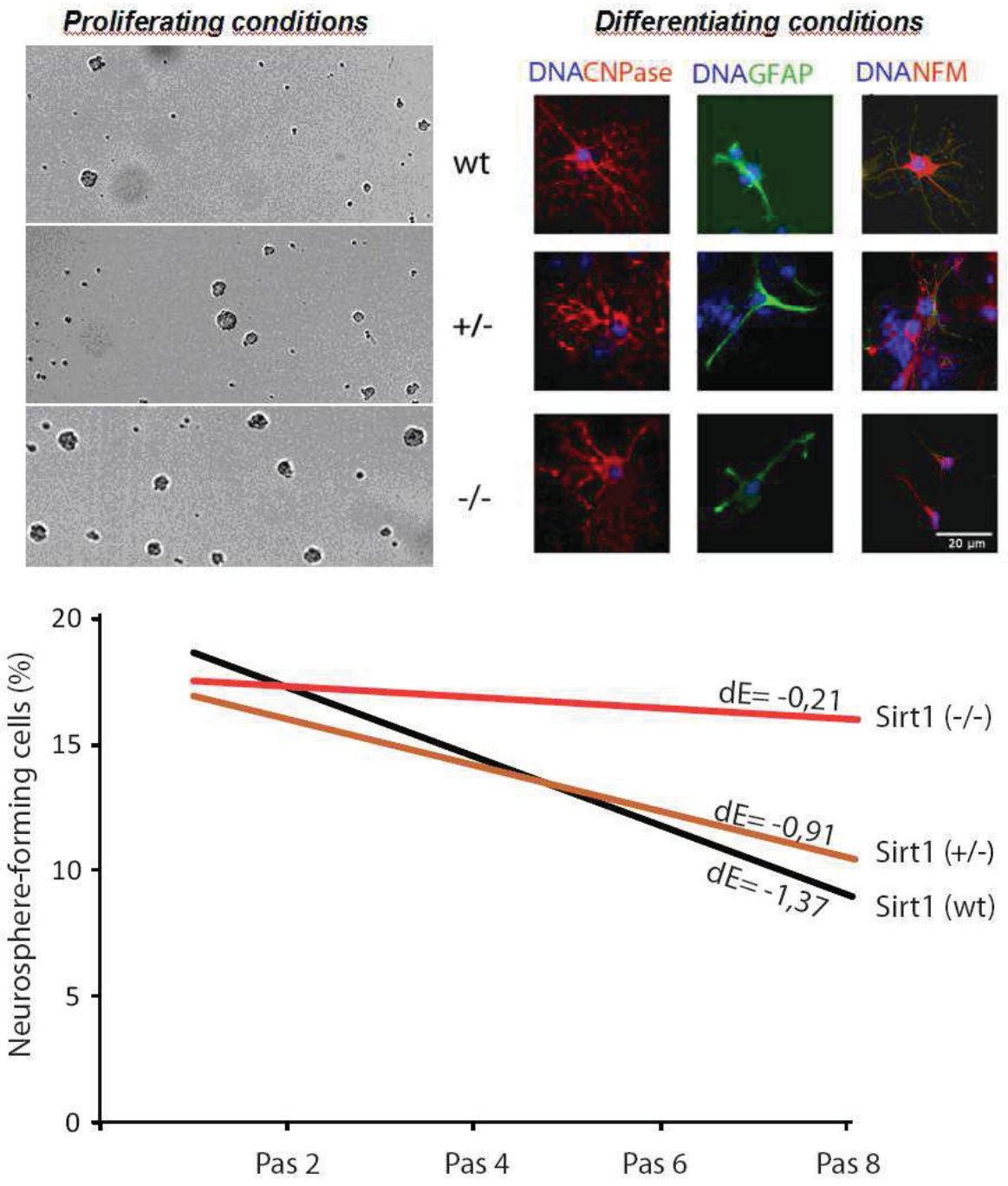

Figure 24: Characterization of Sirt1 deficient NPCs. Top left: Representative light microscopy images of cell cultures of all 3 genotypes ($Sirt1^{+/+}$, $Sirt1^{+/-}$ and $Sirt1^{-/-}$) show increasing numbers of free-floating colonies (neurospheres) with increasing Sirt1 deficiency. Top right: Immunohistochemical staining of NPCs expressing unfunctional Sirt1. $Sirt1^{-/-}$ NPCs are not restricted in their capacity to give rise to all brain relevant cells: 2',3'-Cyclic-nucleotide 3'-phosphodiesterase $(CNPase)^{+}$ oligodendrocytes, $GFAP^{+}$ astrocytes and neurofilament M $(NFM)^{+}$ neurons. Nuclei counterstained with Hoechst (blue). Scale bar displays 20µm. Bottom: Analysis of loss of Sirt1-function on the ability to form neurospheres upon prolonged passaging**.** The maintenance of proliferative capacity of Sirt1-deficient NPCs was pronounced in late passages. Data presented as linear regression of percentage of neurosphere forming NPCs. dE represents delta extinction, which was calculated by Pearson's method. For each genotype at least 3 images of 3 cultures were investigated. Mean percentage per culture was calculated, from which mean per genotype was calculated.

IAdditionally, in this experiment loss of Sirt1 function led to delayed aging of NPCs, assessed by a rescue of proliferative capacity (percentage of neurospheres forming cells) upon prolonged culturing (fig. 24, bottom).

To further assess the differences regarding proliferative capacity upon loss of Sirt1 function, a BrdU incorporation assay was performed. In line with the data from *in vivo* proliferation analysis (fig 17), the BrdU assay showed an increase in actively proliferating $BrdU^+$ NPCs upon Sirt1 deficiency (mean of $Sirt1^{+/+}$: 24% $BrdU^+$ cells, mean of $Sirt1^{+/-}$: 37% $BrdU^+$ cells) (figure 25). These results support the *in vivo* data of this work: Upon systemic or brain specific loss of Sirt1 function, proliferation of stem cells in the HC SGZ was increased.

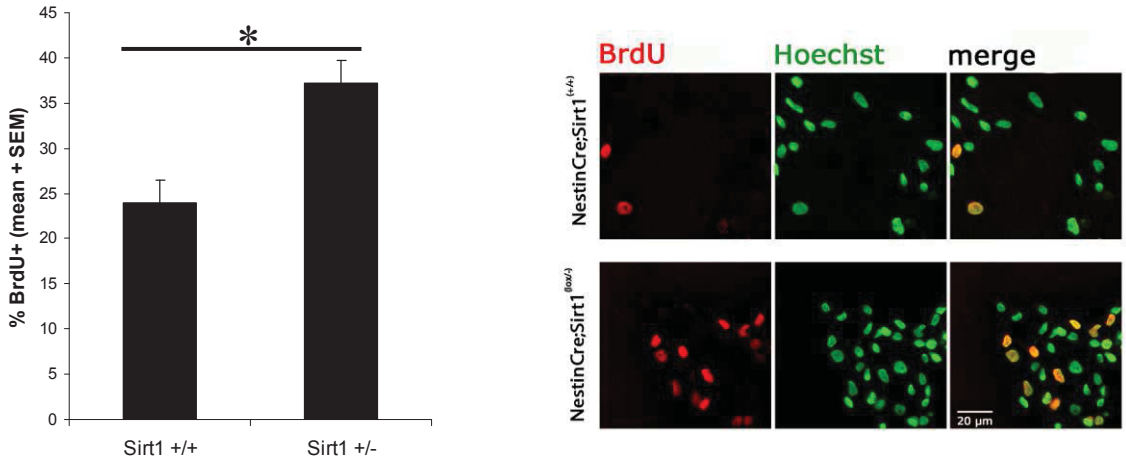

Figure 25: Proliferation capacity of passage 4 hippocampal progenitor cells expressing unfunctional Sirt1. BrdU assay of Sirt1 deficient NPCs has been performed for cells in passage 4 (approx. 2 weeks after isolation) to assess actively proliferating cells. The number of $BrdU^+$ cells was counted and percentage of positive cells regarding total cell number was calculated. The percentage of cells with incorporated BrdU is enhanced in cultures isolated from postnatal Sirt1 deficient mice ($Sirt1^{+/-}$) compared to wt ($Sirt1^{+/+}$) mice. For each genotype at least 3 images of 3 cultures were investigated. Mean percentage per culture was calculated, from which mean per genotype was calculated. Unpaired t-test, *$p < 0.05$. Representative images of immunocytochemical staining of the BrdU assay. BrdU (red) was counterstained with Hoechst, staining nuclei (green). Scale bar displays 20µm.

Next, *in vitro* experiments with Sirt1 deficient stem cells were performed to investigate how they cope with starvation as a metabolic stress condition. Previously, Sirt1 has been shown to be upregulated in the brain upon calorie restriction (Geng et al., 2011).

Starvation was induced by supplementation of the cells with 2-deoxyglucose (DG). In DG the 2-hydroxyl group is replaced by hydrogen, so that it cannot undergo further glycolysis. As it competitively inhibits the production of glucose-6-PO4 from glucose, the cells metabolic pathways are disturbed and they suffer from starvation.

Upon starvation, proliferation of wt NPCs is decreased (fig. 26, bottom, white bars). This correlates with an upregulation of cell cycle inhibitors like p21Cip and p16Ink4 (fig. 26, top left). Notably, Sirt1 was upregulated in those conditions, in line with results from Geng et al., 2001.

Therefore a BrdU assay was performed: Sirt1 deficient cells were grown in the presence of DG. This starvation condition did not affect the proliferation of Sirt1 deficient NPCs (fig. 26, bottom, black bars and representative image (top)).

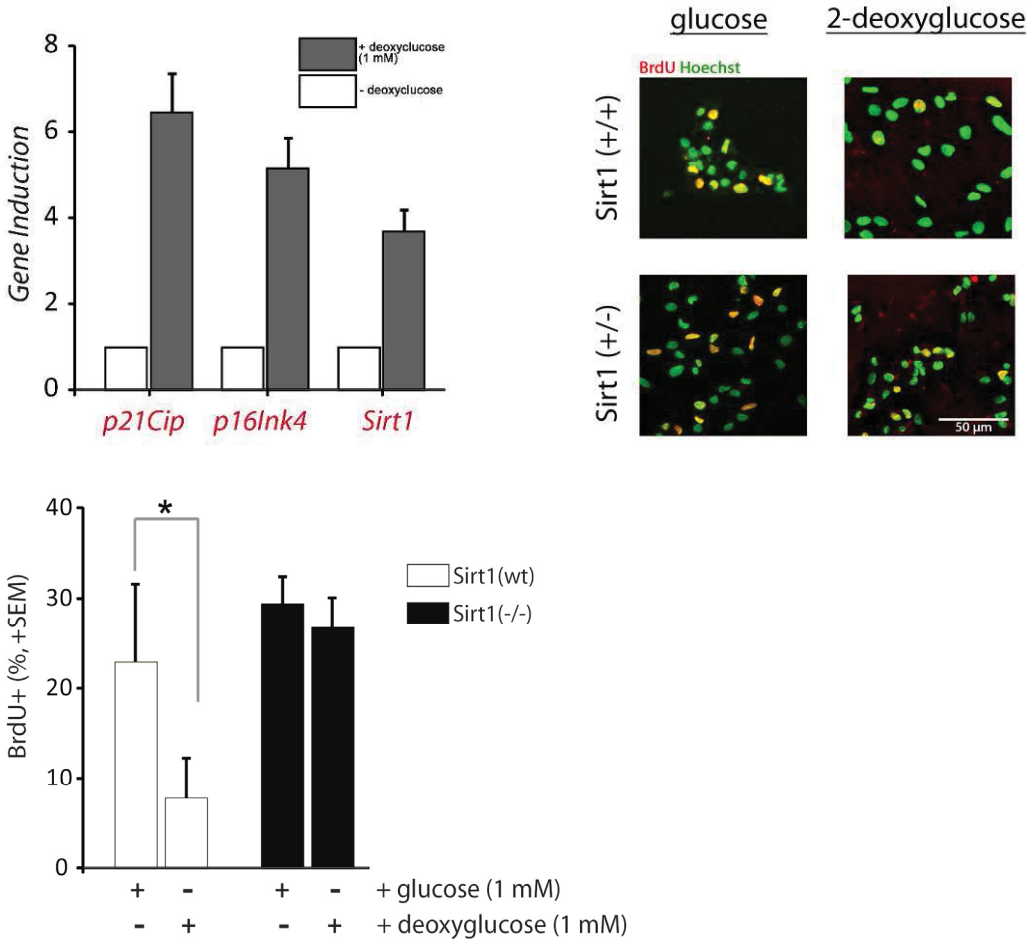

Figure 26: Effects of metabolic stress on Sirt1 deficient NPCs. Top left: In wt NPCs, metabolic stress leads to upregulation of genes indicating cell cycle arrest (p21Cip, p16Ink4) as well as upregulation of Sirt1. Values of qRT-PCR data are given as fold induction normalized to 1 (no induction). Bottom: Proliferation assessed by incorporation of BrdU is not affected upon metabolic stress in NPCs expressing unfunctional Sirt1. For each genotype at least 3 images of 3 cultures were investigated. Mean percentage per culture was calculated, from which mean per genotype was calculated. Values are given as mean of the total number of positive cells in %. Unpaired t-test, *p < 0.05. Top right: Representative image of immunocytochemical staining for BrdU (red) analysis. Nuclei counterstained with Hoechst (green). Scale bar displays 50µm.

Sirt1 deacetylates Nrf2, leading to downregulation of antioxidant genes (Kawai et al., 2011). As it could be found that Nrf2 gene expression is upregulated in aged mice (fig. 9), next experiments dealt with the question, how loss of Nrf2 function affects HC

stem cell homeostasis, neurogenesis and Sirt1 expression in brains of young compared to middle-aged Nrf2 deficient mice.

4.2 Nrf2 and adult neurogenesis

The Sirt1 target Nrf2 is important to challenge oxidative stress. It regulates the transcription of several antioxidant enzymes, like SOD1 (reviewed by Hybertson et al., 2011). Gene expression analysis revealed that loss of Nrf2 function leads to significant downregulation of these genes in the HC of middle-aged mice (gene inductions: SOD1 0,66; SOD2 0,62; Catalase 0,49) (fig. 27, right). To assess whether loss of Nrf2 function has an impact on neurogenesis, expression of pro-neurogenic transcription factors was evaluated: Parallel to the downregulation of antioxidant enzymes, gene induction of pro-neurogenic transcription factors was severely decreased upon loss of Nrf2 function in 12 months old mice (gene inductions: NeuroD1 0,60; Mash1 0,53; Lef1 0,52) (fig. 27, left).

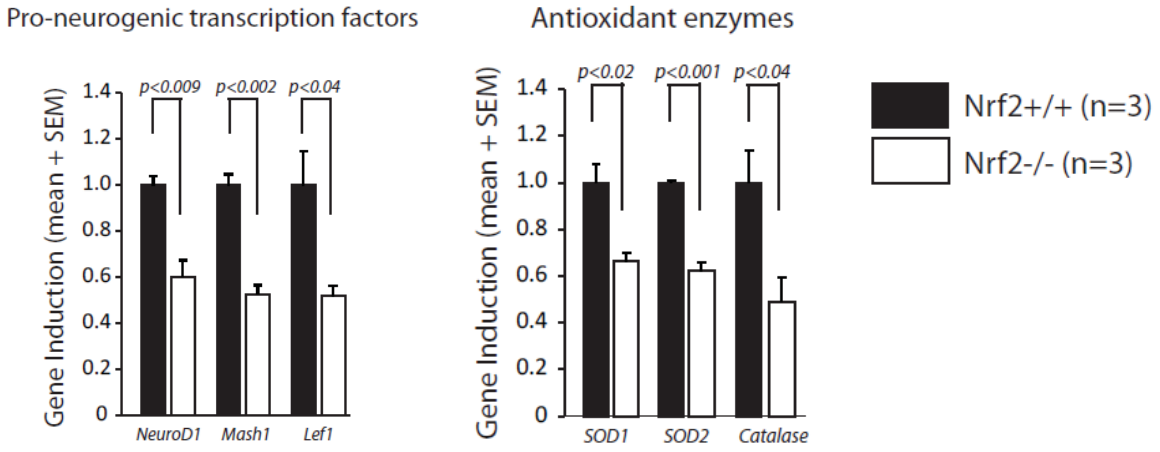

Figure 27: qPCR analysis of gene expression of pro-neurogenic transcription factors (left) and antioxidant enzymes (right) in the HC of 12 months old Nrf2$^{-/-}$ (n=3) compared to wt (Nrf2$^{+/+}$) (n=3) mice. Values are given as fold induction normalized to 1 (no induction). Unpaired t-test, *p < 0.05, **p < 0.01, ***p < 0.001.

Further investigation of neurogenesis in Nrf2 deficient mice was performed using BrdU assay *in vivo*. As shown before (fig. 8), proliferative capacity of HC NPCs is severely decreased upon aging. This decrease was even more pronounced upon Nrf2 deficiency (fig. 28, immunohistochemical images (right) and white bars (right)).

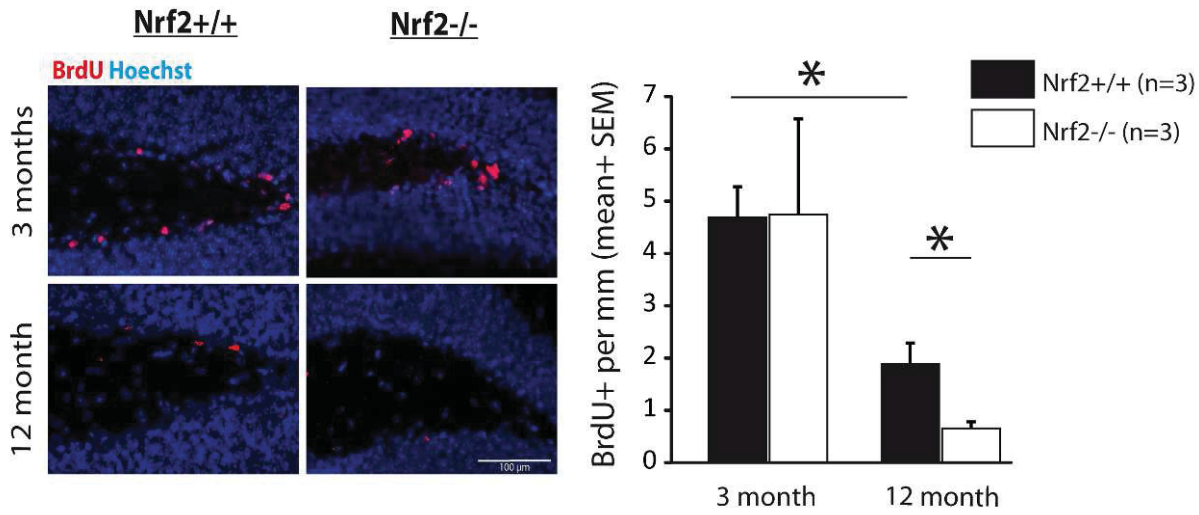

Figure 28: Decreased number of proliferating cells in the SGZ of middle-aged mice expressing unfunctional Nrf2. Histological evaluation of BrdU$^+$ cells in the HC 10 days after first BrdU injection (experimental plan see fig. 8). Left: Representative images of immunohistochemical staining of the HC DG for BrdU$^+$ (red) in 3 months (young) and 12 months (middle-aged) Nrf2$^{-/-}$ and Nrft$^{+/+}$ (wt) mice. Nuclei counterstained with Hoechst (blue). Scale bar displays 100µm. Right: Evaluation of proliferating BrdU$^+$ cells in HC of 3 and 12 months old Nrf2$^{-/-}$ and Nrf2$^{+/+}$ (wt) mice. Values are given as mean of the total number of positive cells per mm of SGZ. For each group at least 4 brain sections of 3 different animals were investigated. Mean per mouse of positive was calculated, from which mean per genotype and significance was calculated. Unpaired t-test, *p < 0.05.

To investigate if Nrf2 deficiency changes differentiation capacities of HC NPCs, histological analyses of several brain cell types were performed using immunohistochemical staining techniques. Young Nrf2 deficient mice show a trend towards decrease of the Sox2$^+$ stem cell pool (mean of young Nrf2$^{+/+}$: 1.196,7 Sox2$^+$ cells per mm^2, mean of young Nrf2$^{-/-}$: 882,5 Sox2$^+$ cells per mm^2), whereas it might be increased in middle-aged mice (mean of middle-aged Nrf2$^{+/+}$: 591,3 Sox2$^+$ cells per mm^2, mean of middle-aged Nrf2$^{-/-}$: 642,5 Sox2$^+$ cells per mm^2) (fig. 29, top). In contrast to this, the numer of DCX$^+$ neurons shows a trend towards increase in young (mean of young Nrf2$^{+/+}$: 9,1 DCX$^+$ cells per mm, mean of young Nrf2$^{-/-}$: 13,9 DCX$^+$ cells per mm) and decrease in middle-aged Nrf2$^{-/-}$ mice (mean of middle-aged Nrf2$^{+/+}$: 3,1 DCX$^+$ cells per mm, mean of middle-aged Nrf2$^{-/-}$: 2,2 DCX$^+$ cells per mm). The number of Olig2$^+$ oligodendrocytes is unaffected by loss of Nrf2 function in both age groups (mean of young Nrf2$^{+/+}$: 399,0 Olig2$^+$ cells per mm^2, mean of young Nrf2$^{-/-}$: 361,4 Olig2$^+$ cells per mm^2) (mean of middle-aged Nrf2$^{+/+}$: 525,7 Olig2$^+$ cells per mm^2, mean of middle-aged Nrf2$^{-/-}$: 530,3 Olig2$^+$ cells per mm^2).

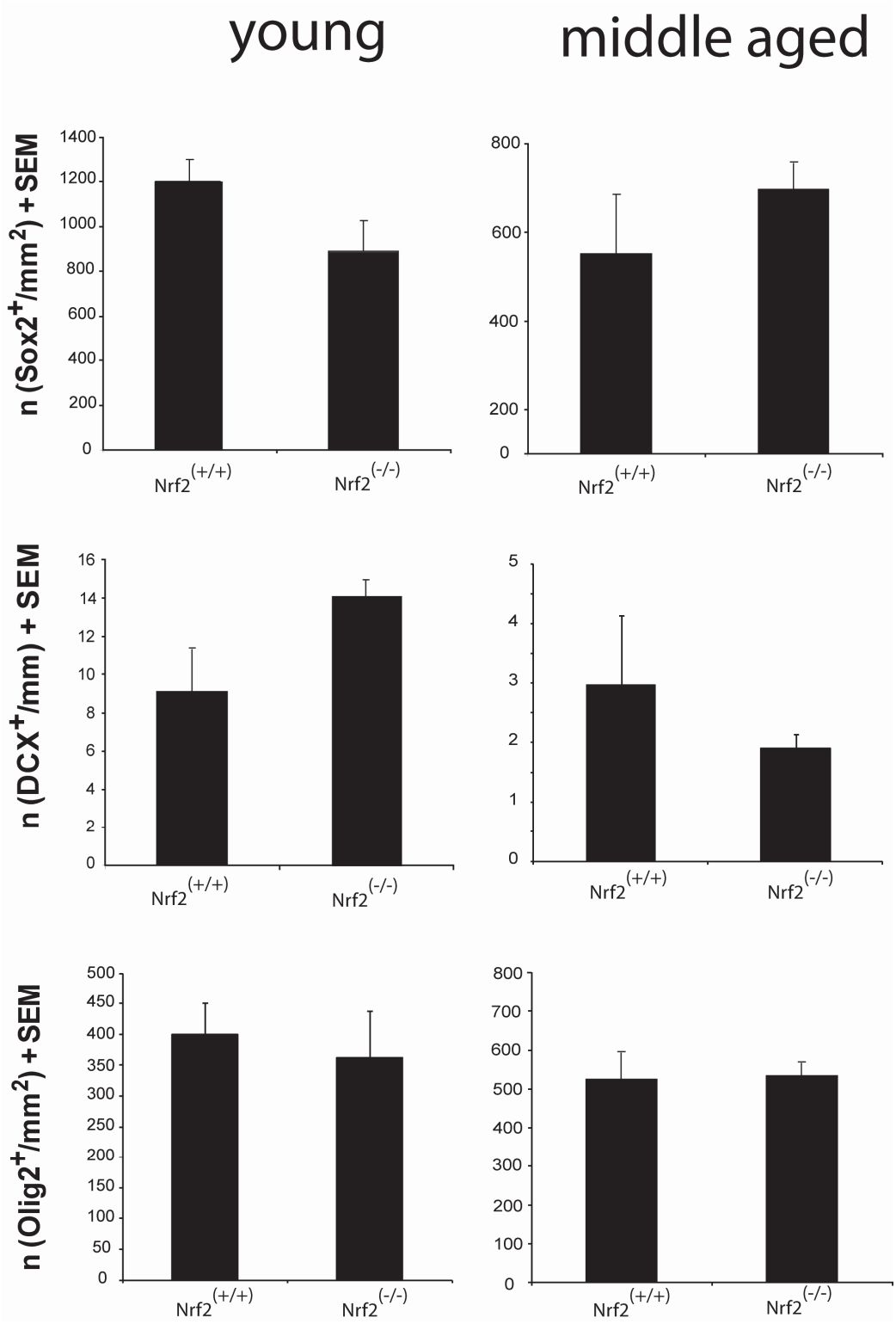

Figure 29: Histological analysis of different cell types in the HC of young (3 months old; left; n=3) vs. middle-aged (12 months old; right; n=3) $Nrf2^{-/-}$ and $Nrf2^{+/+}$ (wt) mice. Top: Analysis of $Sox2^{+}$ stem cell amount. Middle: Analysis of neurons, assessed by DCX^{+} cells. Bottom: Analysis of $Olig2^{+}$ oligodendrocytes. Values are given as mean of the total number of positive cells per mm^2 (Sox2, Olig2) of the DG. The number of immature neurons (DCX) was calculated per mm of SGZ. For each group at least 3 brain sections of 3 different animals were investigated. Mean per mouse of positive cells was calculated, from which mean per genotype and significance was calculated. Unpaired t-test did not reveal any significance.

As Sirt3 also was upregulated in middle-aged compared to young mice (fig. 8), the effects of loss of Sirt3 function on adult neurogenesis were investigated, too. This was especially interesting, as not only Sirt1, but also Sirt3 is important to challenge oxidative stress in the cell (Qiu et al., 2010).

4.3 Sirt3 and adult neurogenesis

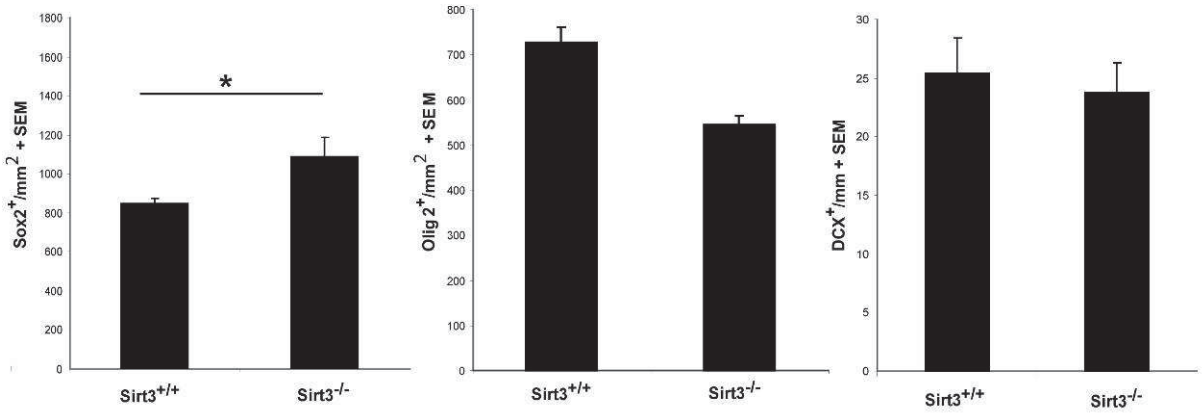

Figure 30: Increased number of hippocampal Sox2$^+$ stem cells in young Sirt3-deficient mice. Histological analysis of neurogenesis in the SGZ of 3 month old Sirt3-deficient (Sirt3$^{-/-}$; n=3) vs wt (Sirt3$^{+/+}$; n=3) mices. Values are given as mean of the total number of positive cells per mm^2 (Sox2, Olig2) of the DG and per mm of SGZ for DCX. For each genotype at least 3 brain sections of 3 different animals were investigated. Mean per mouse of positive cells was calculated, from which mean per genotype and significance was calculated. Unpaired t-test, *p < 0.05.

Upon loss of Sirt3 function in Sirt1$^{-/-}$ mice via deletion of exon 2-3 of the Sirt3 gene, the amount of Sox2$^+$ stem cells in the SGZ of 3 months old mice increases (mean of Sirt3$^{+/+}$: 835,7 Sox2$^+$ cells per mm^2, mean of Sirt3$^{-/-}$: 1.133,7 Sox2$^+$ cells per mm^2). (fig. 30, left). Those stem cells do not seem to differentiate into the oligodendrocyte lineage, as the number of Olig2$^+$ cells shows a trend towards decrease upon lost Sirt3 function (mean of Sirt3$^{+/+}$: 713,3 Olig2$^+$ cells per mm^2, mean of Sirt3$^{-/-}$: 569,4 Olig2$^+$ cells per mm^2) (fig. 30, middle). The number of DCX$^+$ neurons is not affected by loss of Sirt3 function (mean of Sirt3$^{+/+}$: 25,7 DCX$^+$ cells per mm, mean of Sirt3$^{-/-}$: 23,7 DCX$^+$ cells per mm).

5. Discussion

Previous work of our group revealed that mild, non-toxic oxidative stress affects proliferative capacity of embryonic NPCs *in vitro* and *in vivo* (Prozorovski et al., 2008). This effect depends on activation of the NAD^+-dependent deacetylase Sirt1. In the adult brain, accumulation of ROS negatively affects NPCs: In 3–4 months old mice, EC-superoxide dismutase deficiency was associated with significantly decreased HC neurogenesis (Zou et al., 2012). Similar results were obtained from mice deficient for the dusmutases CuZnSOD or MnSOD (Fishman et al., 2009). Contradictory to this, a mouse model for increased oxidative stress (G93A mice with transgenic expression of a G93A mutant form of human SOD1) showed higher HC neurogenesis, compared to wt mice (Ma et al., 2012).

As the effects of ROS and Sirt1 on adult neurogenesis seem to be rather unclear, this study was designed to address those effects. Therefore the behaviour of HC stem / progenitor cells regarding proliferation and differentiation capacities in young and middle-aged animals was examined and the role of Sirt1 in these processes was analysed. Next, HC neurogenesis in adult Nrf2 deficient mice was studied. The Sirt1 target Nrf2 is an important regulator of antioxidant stress responses. In the third part of this work, the maintenance of HC stem cells and generation of neurons in animals deficient for the mitochondrial sirtuin Sirt3, an important enzyme for mitochondrial antioxidant defence mechanisms, was evaluated.

5.1 Comparison of hippocampal neurogenesis in young and adult mice

Using BrdU-pulse analysis *in vivo*, the number of proliferating cells in the SGZ, the neurogenic niche of the adult HC, was quantified. 12 months old adult mice showed a significant decrease in the number of proliferating cells as compared to young, 3 months old mice. This was correlated with a decreased number of $Sox2^+$ cells (high mobility group containing transcription factor), which is expressed in Type-1 and Type-2 precursor cells (Steiner et al., 2006). Decrease in mitotic activity of adult neural stem / progenitor cells has previously been described by other groups: Significantly decreased numbers of $BrdU^+$ cells in the HC of 11 months old mice was found, compared to 1-2 months old mice (Martinez-Canabal et al., 2013). Equal

results were shown in rats by Kuhn and colleagues (21 months vs. 6 months old rats) (Kuhn et al., 1996) and Koltai (26 months vs. 3 months old rats) (Koltai et al., 2011).
In this study, gene expression analysis of antioxidant enzymes revealed a significant upregulation of Sirt1, Sirt3 and Nrf2 in the HC of 12 months old compared to 3 months old mice. This speaks for an activation of anti-oxidant mechanisms in the adult brain, maybe as a result of increased oxidative stress. Supporting these findings, it has been shown that challenging *in vitro* cultures with oxidative stress induces the expression of Sirt1 (in mouse NPCs; Prozorovski et al., 2008) and Sirt3 (in primary rat HC cultures; Weir et al., 2012).
Additionally the results of this work indicate that induction of Sirt1 / Sirt3 in the middle-aged mouse HC (1-year old) may represent an adaptive mechanism against age-associated changes. Interestingly, in aged rats (more than 2 years) the level of Sirt1 is down-regulated, correlating with a drastically decreased number of proliferating cells (Koltai et al., 2011; Quintas et al., 2012). This indicates that Sirt1 is very important for aging processes and its expression changes in different stages of aging, also independently from redox state.

5.2 Impact of Sirt1 on adult neurogenesis

5.2.1 Sirt1 and hippocampal neurogenesis

To investigate the effects of loss of Sirt1 function on HC neurogenesis, three different mouse strains were analysed: 1.) middle-aged (12 months old) $Sirt1^{+/-}$ mice, exhibiting systemic loss of Sirt1 function, 2.) young (3 months old) NestinCre;$Sirt1^{lox/-}$ mice with brain-specific loss of Sirt1 function and 3.) embryos / pups of mice electroporated with shSirt1, in which Sirt1 is exclusively lost in transfected brain cells, meinly stem cells of the SVZ.
In the first two strains, Sirt1 deficiency led to an increase in the $Sox2^{+}$ stem cell pool and higher proliferation rates (assessed by staining for H3Ser19). As in wt mice Sirt1 is expressed in actively proliferating stem cells of the adult SGZ (fig. 11), this result indicates, that Sirt1 *in vivo* is necessary to downregulate stem cell proliferation in the SGZ. This assumption is supported by earlier findings of our group: *In vitro*, activation of Sirt1 in embryonic NPCs inhibited their proliferation (Prozorovski et al., 2008). On the other hand, in human embryonic lung fibroblasts (2BS) Sirt1 activation or

overexpression led to increased proliferation (Huang et al., 2008) This leads to the assumption that the effects of Sirt1 on proliferation vary in different stem cell types of different tissues or species.

Additionally to the findings regarding $Sox2^+$ stem cells and proliferation, systemic Sirt1 deficiency in middle-aged, 12 months old mice led to higher numbers of $Olig2^+$ oligodendrocytes in the SGZ, but did not change the amount of other cell types. Rafalski and colleagues found similar results in their mouse strain with inducible Sirt1 inactivation exclusively in adult mice (NestinCreER;mT/mG;$Sirt1^{lox/lox}$) (Rafalski et al., 2013). Therefore Sirt1 seems to be of high importance for regulation of oligodendrocyte production in the adult brain. On the other hand, the SGZs of the 3 months old mice used for this work showed an upregulation of DCX^+ neurons. Similar results were obtained by Saharan and colleagues, who showed that lentiviral Sirt1 knockdown in the SGZ did not alter the amount and proliferation of NPCs, but led to increased production of neurons (Saharan et al., 2013). The variances of all these data lead to the assumption, that the effects of loss of functional Sirt1 critically depend on the cell types, in which Sirt1 function is lost: In this work, in NestinCre;$Sirt1^{lox/-}$ mice Sirt1 function is lost in the whole brain, while in the strain used by Rafalski Sirt1 deficiency can be induced in adult animals exclusively in NSCs and NPCs. In the strain used by Saharan, Sirt1 was knocked down via injection of a lentivirus in the whole SGZ. Therefore regulation of neurogenesis by Sirt1 seems to depend on different functions of this molecule in different cell types, linked through a complex interplay.

To assess migratory capacity of Sirt1 deficient cells, without side effects of Sirt1 deficiency in other cell types, brains of *in utero* electroporated mouse embryos / pups, which had been transfected with shSirt1 plasmids were analysed. No changes regarding migratory capacity could be found in Sirt1 deficient compared to wt cells, indicating that Sirt1 does not affect this characteristic of differentiating stem cells To further investigate the effects of Sirt1 on NPCs, experiments with primary NPC cultures were performed.

5.2.2 Sirt1 and maintenance of *in vitro* cultured neuronal precursor cell proliferation

In line with decreased proliferative capacity of NPCs in the SGZ of aged mice (discussed above), this work showed for the first time that *in vitro* cultured NPCs develop replicative senescence. This state can be induced by prolonged culturing (decribed for non-neuronal cell types by Campisi 2000 and Hayflick & Moorhead 1961). Using this approach, this work revealed that upon prolonged culturing NPCs develop hallmarks of replicative senescence like decreased proliferation, decreased neurosphere (colony) formation and upregulation of senescence-associated inhibitors of cell cycle $p16^{ink4a}$ and $p19^{arf}$. Therefore, this *in vitro* cell ageing paradigm may serve as a suitable tool for analysis of the effects of particular molecules on maintenance of neuronal stem cell function.

First, using immunofluorescence analysis, it was found that Sirt1 is expressed in adult hippocampal NPCs. In view of the *in vivo* data of this study, suggesting an increase in oxidative stress in the adult SGZ upon aging, NPCs were challenged with oxidative stress via treatment with BSO, an inhibitor of glutathione synthesis. This resulted in increased levels of Sirt1, leading to the assumption that the mechanism, by which Sirt1 is upregulated in NPCs upon aging, might be dependent upon oxidative stress. This assumption is supported by earlier findings of our group, showing an upregulation of Sirt1 *in vivo* after BSO treatment (Prozorovski et al., 2008).

However, using analysis of protein expression by WB Huang and colleagues showed a decreased Sirt1 level in senescent more oxidized 2BS (human fetal diploid lung fibroblast) cell cultures compared to young cells (Huang et al., 2008). This led to the assumption that upon aging, regulation of Sirt1 expression differs in various cell types and tissues.

Next, the effect of loss of Sirt1 function on proliferative capacity of long term cultured NPCs was assessed: Sirt1 deficient NPCs keep up their proliferative capacity for a longer time period of culturing and show an overall increased proliferation rate compared to wt NPCs. These effects are in line with the *in vivo* data presented in this work: Loss of Sirt1 function led to increased proliferation of stem cells in the SGZ. Chua et al found similar results in MEFs. Sirt1-KO MEFs showed an increased resistance to replicative senescence. Additionally, like the NPCs used in this study, Sirt1-KO MEFs showed enhanced proliferative capacity under conditions of mild oxidative stress (Chua et al., 2005). On the other hand, a positive correlation

between the level of Sirt1 and cell proliferation was found for 2BS cells (Huang et al., 2008), human lung fibroblasts (IMR90) and MEFs (Sasaki et al., 2006), suggesting that Sirt1 controls proliferation in different ways in various stem cell types.

To test if the enhanced proliferative capacity of Sirt1-deficient cultures observed in this study is vulnerable to changes in the NPCs environment, the cultures were challenged with metabolic stress via supplementation with DG instead of glucose. In wt NPCs, this supplementation leads to cell cycle arrest, as DG cannot be metabolized by the cells. Sirt1 deficient NPCs did not react to the decrease in nutrient availability but kept their proliferation capacity high. Therefore Sirt1 seems to be an important factor for adaptation of stem cells to nutrient availability. This is in line with the data presented by Ramadori et al., 2008, who showed that Sirt1 functions as regulator of energy metabolism coupling the status of energy availability with cellular functions not only in various peripheral tissues, but also in neurons. Obviously it is of high importance for tissue survival to downregulate proliferation in conditions of low energy or nutrient availability. This is in line with previous publications showing an upregulation of Sirt1 upon calorie restriction in different rodent tissues: In the heart (Yu et al., 2014), pancreas (Chen et al., 2013), liver (Hayashida et al., 2010) as well as in the brain (Geng et al., 2011).

Additional evidence for disadvantages of higher proliferative capacities of brain NPCs come from Oliver & Wechsler-Reya showing that NPCs and brain tumours share many common features and express common sets of markers like Nestin and Sox2. Additionally they share pathways regulating their proliferation, e.g. Shh-signalling (Oliver & Wechsler-Reya, 2004). First hints coming from experiments with *Drosophila melanogaster* intestinal stem cells lead to the opinion that excessive proliferation of stem and progenitor cells leads to accelerated exhaustion of stem cell niches (Rera et al., 2011).

5.3 The effect of lost Nrf2 function on neurogenesis in the adult mouse hippocampus

As Nrf2 also is a Sirt1 target which is of high importance for challenging oxidative stress, additionally effects of lost Nrf2 function on neurogenesis in young (3 months old) and middle aged (12 months old) mice were evaluated: Nrf2 deficiency led to downregulation of pro-neurogenic transcription factors and antioxidant enzymes.

Additionally, immunohistochemical data showed a decreased number of DCX^+ neurons in middle-aged $Nrf2^{-/-}$ mice (fig. 29). These results are in line with observations of Kärkkäinen and colleagues demonstrating that Nrf2 deficiency prevented ischemia-induced increase in newborn neurons in the SGZ. (Kärkkäinen et al., 2014).

In young mice, stem cells seem capable to compensate Nrf2 deficiency, whereas in middle-aged mice this compensatory mechanism seems to be lost. Therefore Nrf2 might be essential to challenge oxidative stress, thereby keeping proliferative capacity in stem cells of the aged mouse HC up. As proliferation of stem cells is enhanced in Sirt1 deficient brains, it is possible that Sirt1 inhibits Nrf2, thereby regulating proliferation of those cells. In line with these data, stem cell proliferation is decreased upon loss of Nrf2 function. Therefore Sirt1 and Nrf2 might set up a complex system to regulate adult HC neurogenesis.

5.4 The effect of lost Sirt3 function on neurogenesis in the adult mouse hippocampus

The last molecule important to challenge oxidative stress in the adult brain investigated in this study was Sirt3. In young Sirt3 deficient mice the amount of $Sox2^+$ stem cells in the SGZ is slightly increased compared to wt mice (fig. 31). Those stem cells might give rise to astroglia, as the number of oligodendrocytes is decreased with loss of functional Sirt3 and the number of neurons is constant. At first view the increase of the $Sox2^+$ stem cell pool upon loss of Sirt3 function might be surprising, as Sirt3-KO mice show substantial acetylation of mitochondrial proteins, and have reduced ATP levels at baseline and during cellular stress (Ahn et al., 2008). This would suggest a lower proliferation rate due to a lower energy availability. However, in the work presented here, the number of $Sox2^+$ stem cells is increased, though this does not proove that those cells are actively proliferating. They might rather be in a dormant state. Additionally, the analyses presented here were performed with young, 3 months old mice. If there are effects of loss of Sirt3 function, those might rather occur from middle-age on, like in mice with loss of functional Sirt1. In line with this suggestion, Koltai et al. found, that Sirt3 levels in the brain increase with aging (Koltai et al., 2011). Therefore loss of Sirt3 in young age may not be of consequence, as Sirt3 levels are already low under normal physiological conditions in young animals.

6. Appendix

Abbreviations

2BS	human fetal diploid lung fibroblasts	EGFR	epidermal growth factor receptor
Adam	A disintegrin and metalloprotei-	ESA	essential for Sirt1 activity
	nase domain-containing protein	Fam	6-Carboxyfluorescein
ANOVA	analysis of variance	FCS	fetal calf serum
AROS	active regulator of Sirt1	FGF	fibroblast growth factor
ATM	Ataxia Telangiectasia Mutated	Foxo	Forkhead-Box-Protein O
ATP	adenosine triphosphate	G-phase	gap-phase
BCA	bicinchoninic acid assay	GAPDH	glyceraldehyde 3-phosphate dehydrogen-
BCL6	B-cell lymphoma 6 protein		ase
BDNF	brain derived neurotrophic factor	GFAP	glial fibrillary acidic protein
bFGF	bovine fibroblast growth factor	H2AX	histone H2AX
bHLH	basic helix-loop-helix	H3	histone 3
BMP	bone morphogenetic protein	H3K9	methylated form of histone 3
BrdU	bromodeoxyuridine	H3Ser10	phosphorylated form of histone 3
BSA	bovine serum albumin	HBSS	Hanks' Balanced Salt solution
BSO	buthionine sulfoximine	HC	hippocampus
CA 1-3	Cornu Ammonis area 1-3	HDAC	Histone deacetylase
Catal	catalase	Hes	hairy and enhancer of split
cDNA	complementary DNA	hGH	human growth hormone
Ck	casein kinase	HIF2α	Hypoxia-inducible factor-2alpha
CNPase	2',3'-Cyclic-nucleotide 3'-phosphodiester-	HSF	Heat shock factor protein
	ase	HSP70	70 kilodalton heat shock protein
CNS	central nervous system	HUR	human antigen R
Cre	cyclization recombinase	Id1	inhibitor of DNA binding 1
CREB	cAMP response element-binding protein	IgG	immunoglobulin G
ctrl	control	IL	interleukin
DARPP32	dopamine- and cAMP-regulated	Ink	inhibitor of CDK
	neuronal phosphoprotein	IPC	ischemic preconditioning
DBC	Deleted in bladder cancer protein	JNK	c-Jun N-terminal kinase
DCX	doublecortin	KO	knockout
DETC	diethyldithiocarbamate	Lef	lymphoid enhancer-binding factor
DG	detate gyrus	LoxP	locus of X-over P1
DG	2-deoxy-glucose	LTP	long term potentiation
DGC	dentate granule cell	Mash	mammalian achaete scute homolog
Dkk	dickkopf	MEF	mouse embryonic fibroblast
DLL	delta like ligand	mRNA	messenger RNA
DMSO	dimethyl sulfoxide	ms	mouse
dNTP	deoxyribonucleotide	NAC	N-acetylcysteine
DSB	double strand break	NAD/NADH	nicotinamide adenine
dT	deoxythymine		dinucleotide
E18	embryonic day 18	NAMPT	nicotinamide phosphoribosyl-
E2F1	E2F transcription factor 1		transferase
EAE	experimental autoimmune encephalo-	NBM	neurobasal medium
	myelitis	NES	nuclear export sequence
EDTA	ethylenediaminetetraacetic acid	NeuN	neuronal nuclear antigen
EGF	epidermal growth factor	NeuroD1	neurogenic differentiation 1

NFM	neurofilament M
NfκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NG	neurogenin
NGS	normal goat serum
NLS	Nuclear localization signal
NPC	neuronal precursor cell
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
NSC	neuronal stem cell
OB	olfactory bulb
Olig2	Oligodendrocyte transcription factor
OPC	oligodendrocyte precursor cell
p-value	probablilty-value
P/S	Penicillin/Streptomycin
$p15^{INK4b}$	Cyclin-dependent kinase 4 inhibitor B
$p16^{INK4a}$	cyclin-dependent kinase 4 inhibitor A
$p18^{INK4c}$	Cyclin-dependent kinase 4 inhibitor C
$p19^{ARF}$	alternate reading frame tumour suppressor
$p19^{INK4a}$	Cyclin-dependent kinase 4 inhibitor C
$p21^{Cip}$	Cyclin-dependent kinase inhibitor
$p38^{MAPK}$	P38 mitogen-activated protein kinases
p44	P44 mitogen-activated protein kinases
p53	Tumour protein p53
p65	Transcription factor p65
PACP	Prostatic Acid Phosphatase
Pas	passage
PAX6	Paired box protein 6
PBS	phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PDGFRα	Alpha-type platelet-derived growth factor receptor
PFA	paraformaldehyde
PGC1α	peroxisome proliferator-activated receptor gamma coactivator 1-α
PTEN	phosphatase and tensin homo-log deleted on chromosome 10
PVDF	Polyvinylidene fluoride
PMSF	phenylmethanesulfonylfluoride
P/S	penicillin / streptomycin
PPARγ	peroxisome proliferator-activated receptor gamma
qPCR	quantitative PCR
RARβ	retinoic acid receptor beta
Rel	relin
RIPA	radioimmunoprecipitation assay
RMS	rostral migratory stream
Rock	Rho-associated protein kinase
ROS	reactive oxygen species
RSV	resveratol
RT	room temperature
rtPCR	real time PCR
S6K1	ribosomal protein S6 kinase beta-1
SAHF	senescence-associated heterochromatin focus
scr	scrambled
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SGZ	subgranular zone
Shh	sonic hedgehog
shSirt1	small hairpin Sirt1 RNA
Sirt	Sirtuin
SOD	Superoxide dismutase,
Sox2	sex determining region Y-box 2
S-phase	synthesis -phase
SUMO	Small Ubiquitin-like Modifier
SUV38H1	Histone-lysine N-methyltransferase
SVZ	subventricular zone
TAMRA	6-carboxytetramethylrhodamine
TLE	transducin like enhancer of split
TNF	tumour necrosis factor
TORC	transducers of regulated CREB
VEGF	vascular endothelial growth factor
Wld	Wallerian Degeneration
wt	wildtype

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Curriculum Vitae

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Expertise

- Molecular biology
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- Light- and fluorescence microskopy
- Cell cultures
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Graduation

Since 10.2010	**Graduation, Heinrich-Heine-University Düsseldorf**
	PhD stipend of Deutsche Forschungsgemeinschaft (DFG), University Clinic Düsseldorf, Molecular Neurology

Professional Experience

10.2009 - 03.2010	**Research associate, University Clinic Göttingen**
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Study

04.2007 - 04.2009	**Master study „Biology of Cells“, University Osnabrück**
	Master’s thesis as external work at Westfälische Wilhelms-Universität Münster, Behavioural Biology and Neurobiology “Effects of Neurotrophic Learning Enhancement via G-CSF and Housing Conditions on Learning and Memory in TgCRND8 Alzheimer Mice" (1.9 Grade B)

04.2004 - 03.2007	**Bachelor study „Biology of Cells“, University Osnabrück**
	Bachelor’s thesis in Neurobiology entitled “Effekt pharmakologischer Beeinflusser der Mikrotubulidynamik auf die Dichte dendritischer Dornen in Hippocampus und Neocortex von Mäusen“ (1.5 Grade A)
10.2002 - 03.2004	**University training to be a teacher, University Osnabrück**
	School subjects: German, English, Social Studies and Science Intermediate examinations in pedagogics and psychology

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German	mother tongue
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07.2008 - 12.2008	**Student research assistant, University Münster**
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Declaration

Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist. Die Dissertation wurde in der vorgelegten der in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, den _____

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(Barbara Koop)