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DISSERTATION

Injuries in the immature hippocampus caused by hyperoxia and its prevention by minocycline

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BCA	Bicinchoninic acid
BNDF	brain-derived neurotrophic factor
bp	Base pair
BSA	Bovine serum albumin
CA	cornu Ammonis
cDNA	Complementary deoxyribonucleic acid
CNP	2’3’ cyclic nucleotide phosphodiesterase
CNS	central nervous system
DAPI	4’,6-diamidino-2-phenylindole
DG	dentate gyrus
DNA	Deoxyribonucleic acid
EtBr	Ethidium bromide
FAM	6-carboxy-fluoresceine
GA	gestational age
GD	gestational day
HCl	*hydrochloric acid*
HI	hypoxic ischemia
HPRT	Hypoxanthine-guanine phosphoribosyl-transferase
IgG	Immunoglobulin G
ILCOR	International Liaison Committee on Resuscitation
i.p.	Intraperitoneal
LPS	lipopolysaccharide
MBP	myelin basic protein
MMLV- RT	Moloney murine leukemia virus reverse transcriptase
NDS	normal donkey serum
NGF	nerve growth factor
NGS	normal goat serum
NTs	Neurotrophins
OGD	oxygen-glucose deprivation
OPCs	oligodendrocyte progenitor cells
P(PD)	Postnatal day

PAGE	Polyacrylamide gel electrophoresis
PaO2	average arterial oxygen tension
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	paraformaldehyde
PK	proteinase K
Pre-OLs	Preoligodendrocytes
PVL	periventricular leukomalacia
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
ROS	reactive oxygen species
ROX	6-Carboxyl-X-rhodamine
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulfate
SEM	standard error of the mean
SGA	subgranular zone
Sox 2	Sex-determining region Y (SRY)- Box2
Sox 10	Sex-determining region Y (SRY)-Box10
Sox 11	Sex-determining region Y (SRY)-Box11
TAE	Tris-acetate-EDTA
TAMRA	6-carboxy-tetramethylrhodamine
TBS buffer	Tris-buffered solution
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
8-oxo-dG	8-oxo-7,8-dihydro-2′ -deoxyguanosine

Abstract

Preterm infants of very low birth-weight often suffer from delayed neurological development. Factors affecting immature neural cells are described as inflammation/infection, hypocarbia, and hyperoxia, and the degree of neurological deficits after preterm delivery may vary depending on the type of external stimulus and on the brain region being affected. Whether injury of the hippocampus is responsible for part of neurological sequelae in preterm infants is not clear yet.

In my study, I aimed to investigate the mechanisms by which hyperoxia may damage oligodendroglial precursor cells (OPCs) and immature neurons of the hippocampus. I used an established rat pup model providing 24h exposure to 80% O_2 from P6 to P7 and analyzed apoptotic cell death, neuronal and oligodendroglial cell proliferation, and microglial activation in the dentate gyrus and in the hilus rat at P7 and after recovery in room air at P9 and P11 by immunohistostaining. To identify pathways for pharmacological protection, I used minocycline in hyperoxic rats because of its known anti-apoptotic, anti-inflammatory, and anti-oxidant properties.

Hyperoxia significantly diminished proliferation of immature DCX+ neurons and of NG2+ OPCs and reduced maturation of oligodendroglia to CC1+ stages. Minocycline treatment in rats during hyperoxia improved neural cell proliferation and oligodendroglial maturation, and also chemical factors regulate for development of neurons and oligos, such as SOX2, SOX11and Oligo2 and SOX10. Moreover, increased production of reactive oxygen species (ROS) and nitrotyrosine after hyperoxia was blocked by minocycline. Remarkably, hyperoxia did not cause apoptotic cell death or microglial activation in the hippocampus.

Based on our results, hyperoxic injury in the immature hippocampus is characterized by impaired cell proliferation and development of immature neurons and oligodendroglia. These toxic effects were prevented by minocycline. Since neither apoptosis nor microglial activation was found in the hippocampus of hyperoxic rats, the beneficial effects of minocycline can mainly be attributed to its anti-oxidant properties.

Zusammenfassung

Sehr unreife Frühgeborene mit einem Geburtsgewicht von unter 1500 g (= VLBW, very low birth-weight) leiden oft an einer Schädigung der neurologischen Entwicklung. Die unreifen neuralen Zellen können dabei von äußeren Faktoren beeinträchtigt werden, wie etwa perinatale Infektion/Inflammation, Hypokarbämie, und Hyperoxämie. Das Ausmaß der neurologischen Schädigung hängt dabei unter Anderem von der Art des schädigenden Stimulus und von der betroffenen Hirnregion ab. Ob Veränderungen des Hippocampus für einen Teil der neurologischen Folgen bei Frühgeborenen verantwortlich sind, ist bislang unklar.

Das Ziel meiner Arbeit war, eine Schädigung von Oligodendroglia und unreifen Neuronen des Hippocampus durch postnatale Hyperoxie zu beschreiben und die beteiligten Mechanismen zu identifizieren. Ich habe zu diesem Zweck ein etabliertes Hyperoxie-Modell verwendet, bei dem neugeborene Ratten an Tag 6 (P6) bis Tag 7 (P7) 24h 80% O_2 ausgesetzt waren und danach zur Erholung über zwei bzw. vier Tage an Raumluft gesetzt wurden bis zum Alter P9 bzw. P11. Mittels Immunhostochemie habe ich apoptotischen Zelltod, neuronale und oligodendrogliäre Proliferation und mikrogliäre Aktivierung im Gyrus dentatus und im Hilus des Hipocampus bestimmt. Um Signalwege zu identifizieren, die für die Protektion des Hippocampus zu aktivieren sind, habe ich Minozyklin in hyperoxischen Ratten angewendet und auf dessen anti-apoptotische, anti-inflammatorsiche und anti-oxidative Eigenschaften hin untersucht.

Hyperoxie reduzierte die Proliferation von DCX positive Neuronen und von NG2+ oligodendrogliären Vorläuferzellen und verminderte die Reifung von Oligodendroglia hin zum CC1 positiven Stadium. Die Behandlung von Ratten mit Minozyklin während der Hyperoxie-Exposition verbesserte die Proliferation von Neuronen und Oligodendroglia signifikant. Zudem war die Expression von spezifischen neuronalen (Sox2, Sox11) und oligodendrogliären (Olig2, Sox10) Transkriptionsfaktoren durch Minozyklin erhöht. Die Induktion von oxidativem Stress durch Hyperoxie, die ich anhand von ROS (“reactive oxygen species”) und Nitrotyrosin Konzentrationen bestimmt habe, konnte durch Minozyklin aufgehoben werden. Bemerkenswerter Weise hatte Minozyklin keinen Einfluss auf Apoptose oder Mikroglia-Aktivierung.

Diese Ergebnisse zeigen, dass Hyperoxie die Proliferation und Reifung neuraler Zellen im postnatalen Hippocampus beeinträchtigt. Darüber hinaus konnten die schädlichen

Auswirkungen von Hyperoxie abgeschwächt oder vermieden werden. Da weder Apoptose noch Mikroglia-Aktivierung durch das Medikament beeinflusst wurden, scheint vor allem die anti-oxidative Wirkung von Minozyklin für die Protektion des Hippocampus gegen Hyperoxie relevant zu sein.

1. Introduction

1.1. The developing brain in preterm infants and oxygen

1.1.1. The developing brain in preterm infants

Preterm birth is defined by the WHO as all births before 37 completed weeks of gestation or less than 259 days since the first day of a woman's last menstrual period. Preterm birth can be further sub-divided based on gestational age: extremely preterm (<28 weeks), very preterm (28 - <32 weeks) and moderate preterm (32 - <37 completed weeks of gestation), and moderate preterm birth may be further split to focus on late preterm birth (34 - <37 completed weeks) [1]. It is estimated that preterm birth accounts for 7.4% - 13.5% of live births, including 5.2% extremely preterm births, in 2010, approximately 15 million babies were premature, making up more than 1 in 10 of the world's babies. Preterm birth is common and has been increasing in recent years, especially between 1990 and 2001 with a 19.4% increase in developed regions [2, 3].

While the mortality of premature infants has decreased markedly due to advances in neonatal intensive care, neurologic impairment that manifest throughout life continues to increase. Up to 10% of infants who survive very preterm birth will develop spastic motor deficits such as cerebral palsy [4, 5] and a further 25-50% will suffer cognitive, behavioral, attentional, and socialisation deficits [6-8]. Preterm birth with neurologic sequelae can also impose a heavy burden both in considerable emotional and economic costs to families and public-sector services, such as health insurance, education, and other social support systems. In 2005 it is reported the average first-year medical costs, including in- and out- patient care, were about 10 times greater for preterm ($32,325) than for term infants ($3,325); the average length of stay was 9 times longer for a preterm newborn (13 days), compared a baby born at term (1.5 days). Overall, the annual social economic burden associated with preterm birth in the United States was at least $26.2 billion in 2005[9]. Hence, these effects do cause misfortune for preterm infants themselves and exert a heavy burden on families, society and the health system.

In the brain of an extremely preterm infant (23 - 28 weeks), significant developmental changes are especially noticeable in the cerebellum and in the periventricular zone, both of which host a rapidly evolving germinal matrix. The germinal and subventricular

layers are sites of migrating neurons and preoligodendrocytes which are vulnerable to injury. Because of the dense vascular network and infection injury, there is a high risk for intracranial bleeding, including cerebellar haemorrhage and germinal matrix intraventricular haemorrhage [10].

The vulnerability of the immature brain, namely in preterm infants of less than 32gestational weeks, is mainly characterized by maldevelopment of the white matter, especially in the periventricular zone [11]. The most notable encephalopathy in premature infants is periventricular leukomalacia (PVL) which can be either focal or diffuse. The focal form consists of necrosis and can be macroscopic in size, and evolve to multiple cystic lesions over several weeks which is known as “cystic PVL”; more commonly, after several weeks focal necrosis evolves into glial scars but is not seen by neuroimaging and is known as “non-cystic PVL” [12]. Various factors including hypoxia-ischaemia and infection or inflammation often co-exist and lead to excitotoxity and accumulation of reactive oxygen and nitrogen species. Such that the ultimate result of either event would be cell death at early stages of development, oligodendroglia dysmaturity and neuronal/axonal deficits, and consequent impairment of myelination [11-13].

In the brain of late preterm infants between 32 and 36 weeks, cortical volume and myelinated white matter increase dramatically as term approaches. However, oxidative stress, excitotoxicity and over-expression of glutamate receptors still play a significant role in late-preterm brain injury [14]. These multiple factors are also involved in synaptogenesis and dendritic arborization [15].

In general, growth and maturation of the mammalian brain can be divided into six phases: (a) neural cell genesis followed by (b) neuronal migration, (c) glial cell proliferation, (d) axonal and dendritic proliferation, (e) synaptogenesis and appearance of electrical activity, and (f) axonal myelination [10]. However, these developmental steps as well as biochemical and functional changes are not independent but can occur at different times in various brain regions together. Such that the main brain growth spurt is a reorganization event. During this sensitive developmental period, the immature brain could be stimulated by multiple compounding factors in the intra-uterine and extra-uterine environment, some of which represent high risk factors that may induce brain injury and subsequent neurologic sequelae, such as hypoxic ischemia,

inflammation, hyperoxia, hypoglycemia, hyperbilirubinemia, and chorioamnionitis [15, 16]. Excitotoxicity, oxidative stress and inflammation are the three main downstream mechanisms that cause injury to the developing brain; neuropathological hallmarks include vulnerability of oligodendrocytes, activated microglia, astrogliosis, and neuronal and/or axonal damage [17]. White matter abnormalities in premature infants are associated with psychomotor delay, cerebral palsy, and also neurosensory impairment such as speech, hearing, and vision problems; gray matter abnormalities have been correlated to an increased risk of severe cognitive delay, and also psychomotor delay, and cerebral palsy, even though to a lesser extent than white matter abnormalities [12, 15, 17].

When modeling brain maldevelopment caused by factors that accompany premature birth, it is important to consider the distinct developmental stages at which different species are born. Sheep and guinea pigs are characterized as prenatal brain developers [18, 19]. In contrast, in rodent crucial developmental steps of the brain occur within the first postnatal weeks [20]. In humans the time of greatest vulnerability of the brain starts at about mid-pregnacy and extends well into the third postnatal year [12]. In order to understand the vulnerability of the immature brain to external stimuli, it is important to consider the stages of brain development. In human the hippocampus develops after 10 weeks, but in rats this happens after GD19 (see Tab 1) [21], such that it is nominated to take a rat pup models instead of human hippocampus study.

HUMANS (WEEKS)	3.5-4.0	4.1-5.2	5.3-5.7	5.8-6.6	6.7-7.0	7.1-7.4	7.5-7.9	8.0-9.9	10.0-11.9	12.0-14.9	15.0-18.9	19.0-23.9	24.0-27.9	28.0-31.9	32.0-35.9	36.0-40.0
RATS (DAYS)	GD11	GD12	GD13	GD14	GD15	GD16	GD17	GD18	GD19	GD20	GD21-22	PND 0-3	PND 4-7	PND 8-11	PND 12-15	PND 16-19
Spinal cord																
Medulla																
Pons																
Cerebellum																
Mesencephalic tegmentum																
Mesencephalic tectum																
Thalamus																
Hypothalamus																
Pallidum																
Striatum																
Amygdala																
Neocortex and limbic cortex																
Entorhinal cortex																
Subiculum																
Hippocampus CA1-3																
Hippocampus dentate granule cells																

Table 1: Brain maturation in humans and rats.

The table shows the estimated timeline of neurogenesis in humans and in rats. Development of the hippocampus in humans overlaps with that in rats from GD19 to PD16-19 [21].

2.1.2. Oxygen toxicity

Approximately 10% of newborns require respiratory support and oxygen supplementation immediately after birth to adequately perform postnatal adaption to begin breathing at birth, and about 1% require extensive resuscitation [22]. In the late19th century, oxygen was first given to babies as a respirator gas, however, principles on the use of oxygen in neonate treatment were controversial in recent decades. It was only in 2005 that the International Liaison Committee on Resuscitation (ILCOR) first mentioned that high oxygen concentrations during the neonatal period are associated with damage to various organs, including the eyes (e.g., retinopathy of prematurity), the lung (e.g., bronchopulmonary dysplasia), and the brain (e.g., cerebral palsy), and suggested that room air is as effective as 100% oxygen for the resuscitation of most infants at birth [23]. Current guidelines recommend a cautious if not restrictive use of oxygen supplementation for resuscitation in term and preterm infants [22] and include discussion of strategies favoring the use of room air instead of oxygen supplementation.

Birth always causes hyperoxic conditions in relation to conditions *in utero*, where the average arterial oxygen tension (PaO2) is rather low, with about 32 mmHg in the umbilical vein and 22 mmHg in the descending aorta [21]. Under physiologic conditions, fetal hemoglobin enables oxygen saturation to reach up to 90% and arterial oxygen tension up to 40 mmHg [21]. However, immediately after birth into room air, arterial oxygen tension may increase up to 65–80 mmHg, representing a seven-fold increase for preterm infants, even without supplemental oxygen [24]. This supraphysiological oxygen concentration hence creates a relative hyperoxia to the immature brain of preterm infants. In cases in which mechanical ventilation or oxygen supplementation or both have to be used to ensure adequate tissue oxygenation and pulmonary vasodilation, this hyperoxic condition may be even more pronounced. This hyperoxic condition may be even more pronounced. Hyperoxia in immature neonates is reported to produce pronounced oxidative stress and expose their underdeveloped anti-oxidant defense system to a particular challenge [21]. Premature infants are particularly sensitive to the deleterious effects of oxygen, and this has been attributed in part to their

immature defense mechanisms against oxidative stress, to oxygen-induced vasoconstriction leading to reduced brain perfusion, and to the susceptibility of the rapidly developing brain itself due to environmental changes [21].

The detrimental effects of oxygen are well described in studies about the emergence of retinopathy of prematurity and bronchopulmonary dysplasia. In recent years, more and more experimental and clinical data suggest that high oxygen in preterm infants may be a factor of poor neurological impairment, too [25, 26]. However, the role of oxygen in the etiology of brain injury has not been extensively characterized, so that there is an urgent need to understand pathological mechanisms related with neurobiological, structural and functional levels. In previous studies, our group demonstrated hyperoxia (up to 80% oxygen) as a powerful trigger of widespread cell death in the immature rodent brain associated with oxidative stress, increased intracranial expression of proinflammatory cytokines, decreased levels of neurotrophins, and induction of matrix metalloproteinases [27-30]. The hyperoxia-induced apoptotic process can be divided further into two pathways depending on the involvement of mitochondria and related biochemical cascades. The intrinsic pathway coincided with the upregulation of cytochrome c and caspase 3 [31], while the extrinsic pathway is mainly triggered by ligation of death receptors Fas/CD95 [32]. Subsequently, the group of Schmitz and co-workers characterized hyperoxia-induced cellular changes in oligodendroglia and astroglia in the developing rodent brain. Hyperoxia resulted in a maturation-dependent reduction in myelin basic protein (MBP) expression and caspase-dependent cell death in cultured preoligodendrocytes (pre-OLs, O4+O1-MBP-) and immature oligodendrocytes (O4+O1+MBP-), but not in mature oligodendrocytes (O4+O1+MBP+) [32]. Furthermore, mice pups exposed to 48 h of 80% oxygen from P6 to P8 exhibited a marked loss of NG2+O4- OPCs and a diminished population of mature (i.e., CC1+) oligodendrocytes. As a consequence, a reduction of myelin basic protein expression was found until P12 which returned to control levels during further recovery at P15 [33]. A wild type mouse model also showed that neonatal hyperoxia caused ultrastructural changes, including: myelination abnormalities (i.e., reduced myelin thickness and abnormal extra myelin loops) and axonopathy (i.e., altered neurofilament phosphorylation, paranodal defects, and changes in node of Ranvier number and structure) [34]. In a running wheel system for motor learning analysis, mice after 48h hyperoxia from P6 to P8 exhibited higher velocity of wheel running at P30 compared to

control mice kept in normal room air, which was interpreted as hyperactive behavior [35]. After replacement of the regular running wheel by a complex running wheel with different distances between the wheel rungs, the learning capacity of mice after hyperoxia didn't adapt to this motor challenge. Hence, postnatal hyperoxia may affect motor activity and motor skills long term.

2.2. Hippocampus

2.2.1. Hippocampus in the healthy brain

The hippocampus, Greek name for seahorse, is a bilateral limbic structure, and is phylogenetically one of the oldest parts of the central nervous system (CNS). Due to its unique structure for layouts and interconnectivity, this intriguing region of the mammalian brain fascinated scientists in many fields, from psychology to molecular biology. The hippocampus is implicated in many expressions of an integral role in declarative memory, spatial memory, learning, emotion and synaptic plasticity. Indeed, the hippocampus have been suggested to play a critical role in neurodegenerative and psychiatric disorders [36].

The human hippocampus is a small structure, about the size and shape of a crooked pinkie finger, lying under the cerebral cortex. The hippocampal formation is composed of three regions of the cornu Ammonis (CA), the dentate gyrus (DG) and the subiculum (Fig 1). The CA region is divided into three subfields, CA1, CA2 and CA3. The fascia dentata is comprised of two blades, the suprapyramidal and the infrapyramidal blades. The hilus is located between these two blades.The primary cell of the hilus is the mossy cell. The DG and CA are structured in layers or *strata*. From inside-out, the *strata* of the CA are: the *stratum moleculare*, *stratum lacunosum* (or *lacunosum-moleculare*), *stratum radiatum*, *stratum lucidum*, *stratum pyramidale*, *stratum oriens* and the *alveus*. From inside-out, the *strata* of the DG are: the polymorphic layer, the *stratum granulosum* and *stratum moleculare*. The polymorphic layer of the DG is located within the *hilus*. The principal layers of the DG and CA are the *stratum granulosum* and *pyramidale*, or granule and pyramidal cell layers, respectively. These dense layers contain the cell bodies of the granule and pyramidal cells.

The Ammon's pyramidal cells, the earliest neurons, generate from the neuroepithelium and then migrate radially to their terminal sites within the hippocampus. In the

subsequent formation of the dentate, precursor cells of the dentate granule cells migrate from the embryonic ventricular and subventricular zones to the hippocampal formation. These cells populate a proliferative zone along the hilar side of the granule cell layer or what is termed the subgranular zone [37]. In general, this zone is established between 14 and 30 days following birth with a continued production of neurons in the adult mammal. Briefly, in the hippocampus, new neurons are generated in the innermost region of the dentate granule cell layer and subgranular zone. Granule neurons are generated from a population of continuously dividing progenitor cells. The neurons migrate from the subventricular zone into the granule cell layer where they differentiate, extend axons, and express neuronal marker proteins [38].

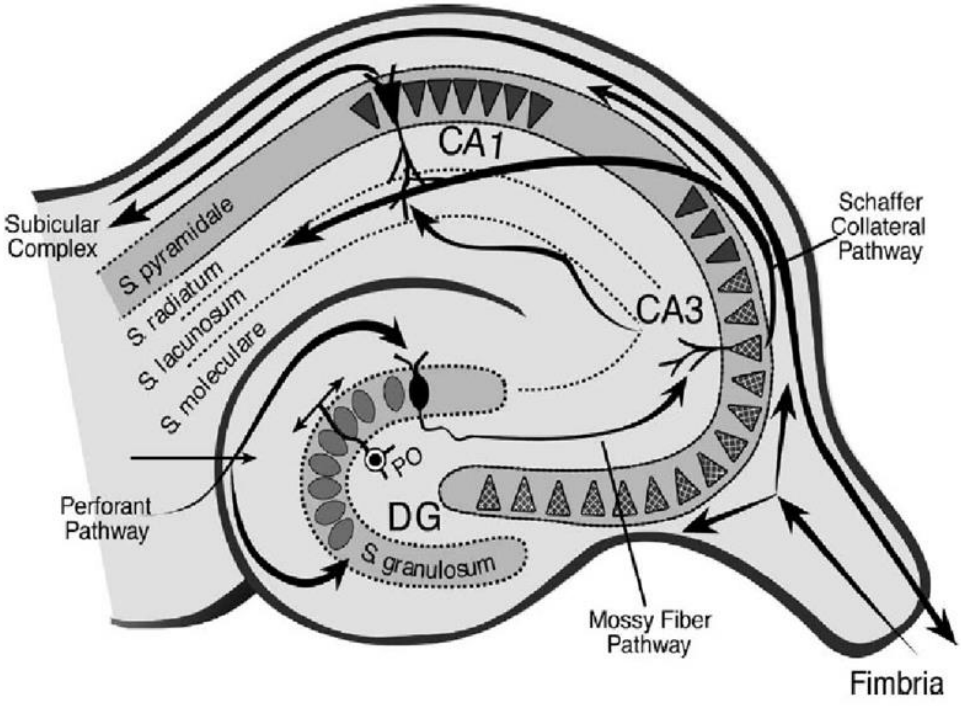


Figure 1: Schematic representation of the primary neuronal regions of the hippocampus and the main projections.

The two major neuronal cells of the hippocampus - pyramidal and granule cells - are primarily organized in tri-synaptic mono-directional pathway [39].

2.2.2. The vulnerability of the hippocampus

Adequate maturation of neurons and their integration into the hippocampal circuit is crucial for normal cognitive function and emotional behavior, and disruption of this

process could cause disturbances in mental health. For years, the hippocampus has been identified as a major target site for numerous initiators of damage to physiological activity, disease processes, or neurotoxic exposures. Numerous experimental animal models of hippocampal damage have been developed over the years to address the issue of hippocampal vulnerability involved in learning disabilities and memory deficits.

Various studies have been conducted on the vulnerability of the hippocampus following hypoxia-ischemic insult. Neuronal loss and widespread gliosis with degenerating oligodendrocytes are two common patterns in the hippocampus [40]. Following exposure to 8% oxygen for 15 min to P4 rats as a model of hypoxia, necrotic neuronal cells were observed in the cortex and the hippocampus [15]. Exposure 8% oxygen for 3 hours to P1 pigs, a combination of hypoxia and ischemia resulted in predominant neuronal loss in the cerebral cortex and in pyramidal neurons of the CA1, CA2 and CA3 regions of the hippocampus [41]. Moreover, combined 75 minutes' 8% hypoxia with permanent ligation of the left common carotid artery at P7 led to more neurogenesis and microglia and less reduced oligodendrocyte synthesis in the injured hippocampus than hypoxic injury alone [42]. With regard to hippocampal function, HI in animal models induced brain injury with cognitive and sensorimotor deficits in learning and memory tasks [41, 43]. Compared to healthy children, the school-aged children who had experienced neonatal hypoxia or/and ischemia and consequent bilateral hippocampal injury, had got impairment deficit, with the specific details of episodes being lost [44]. Long-term outcomes in infants and children, such as cognitive memory impairment and psychiatric disorder schizophrenia, are closely related to hippocampal damage resulting from hypoxia or asphyxia [45]. The cohort investigated by Cooper and co-workers showed hippocamps volumes of 40 children who had experienced hypoxia significantly below normal control values, memory scores significantly below the standard population means, and memory quotients significantly below those predicted by their full scale IQs [46]. Overall, there are extensive data indicating a particular susceptibility of the developing hippocampus to cellular and functional impairment in response to toxic environmental stimuli.

Pro-inflammation reaction represents another established risk factor for injury of the immature brain. In animal studies, it has been demonstrated that multiple infectious and inflammatory stimuli may induce a range of toxic effects on the developing brain [47, 48]. LPS-induced-inflammation in preterm animals resulted in decreased numbers of cortical

and hippocampal early myelinating oligodendrocytes with persistently impaired myelination at 28 days [49]. Oligodendroglial damage is accompanied by microglial and astroglial activation [50], neuronal loss, and also by axonal injury in the CA1 region of the middle dorsal hippocampus [51]. Moreover, neonatal LPS exposure resulted in learning deficits in the passive avoidance task and less anxiety-like (anxiolytic-like) responses in the elevated plus-maze task in P71 rats [51]. Neonatal exposure to bacterial exotoxins such LPS causes persistent injuries to the hippocampus and results in long-lasting learning disabilities [52].

Hypoglycemic brain damage, like ischemia, always affects the hippocampus and cerebral cortex, inducing neuronal necrosis and progenitor cell proliferation. Pathology studies in humans and animals show that hypoglycemia induced extensive neuronal loss in the hippocampus, including areas CA1 and DG. At 4 weeks after hypoglycemia, proliferative activity in the SGZ diminished below baseline in an experiment compared with control rats, with a subsequent reduction of neuroblasts [53]. Extensive neuronal loss with hypoglycemia could lead to neurological sequelae with cognitive decline. According to clinical studies, significant learning and memory deficits correlate to the frequency of hypoglycemia in children with diabetes and infants of diabetic mothers [54-56].

Recent evidence by experimental and clinical data suggests that the risk of neurological and cognitive sequelae in neonates rises with increases in oxygen tension. Brain regions affected by hyperoxia include caudate nucleus, nucleus accumbens, layers II and IV of the frontal, parietal, cingulate and retrosplenial cortices, white matter tracts within the forebrain as well as hippocampus [27]. Acute oxygen supplyment not only decreased proliferation of neuronal progenitors, but also interfered with neuronal differentiation and maturation in the granule cell layer and polymorphic layer of the hippocampus [57]. In a distinct model applying exposure to 80% oxygen form birth until day 5, hyperoxia diminished neurogenesis and caused apoptosis in the CA1 region and in the dentate gyrus of the hippocampus [58]. This is also supported by studies by Andrea Porzionato and co-workers, in which rats exposed to 60% or 95% oxygen for 2 weeks showed reduction of volume and of total cell numbers in the DG compared to control litters kept in room air [59]. In this chronic neonatal hyperoxia model, mice had abnormal neurobehavior at 12-14 weeks of age, deficits primarily in spatial and recognition memory, associated with smaller hippocampus sizes [25].

2.3 Minocycline

Minocycline is a widely used as second-generation semisynthetic tetracycline antibiotic with known anti-inflammatory, anti-apoptotic, and anti-oxidative properties. The drug has been used for decades to treat infections caused by a variety of gram-negative and gram-positive organisms. With good blood–brain barrier penetration, rapid absorption and adequate tissue levels within hours after oral or parenteral administration, minocycline has also been reported to exert neuroprotective effects in various experimental models using adult animal models, including acute stroke [60], Parkinson's disease [61], traumatic brain injury [62, 63], and spinal cord injury [64] models. However, whether minocycline may be used for neuroprotection in neonates is debated controversially.

So far, minocycline is not licensed for the use in children and neonates. It may cause stunting of bone growth because of its property of binding calcium phosphate readily and selectively taken up by developing teeth and bones of fetus and children. Despite the described adverse effects of tetracyclines, minocycline still gets great attention as a potential neuroprotectant in neonatal models of brain injury, such as hypoxia, hyperbilirubinemia, and perinatal inflammation models. Lechpammer et al. [65] reported that treatment with minocycline significantly attenuated the hypoxia-ischemia-induced white matter injury in rats. Protective effects of minocycline against HI-induced brain injury induced high numbers of neurons and oligodendrocytes, increased myelin, prevention of axonal damage, and improved formation of neuronal dendrites [66] . the protective effect of minocycline was largely related with its ability to reduce in HI-induced microglial activation and oxidative stress [66, 67]. This is underlined by Fan LW et al [68], who demonstrated that minocycline inhibited LPS-induced damage, such as enlarged ventricles, deficits in the hippocampus, including loss of oligodendrocytes and tyrosine hydroxylase neurons, damage to axons and dendrites, and hypomyelination, which is associated with suppression of microglia activation. Minocycline also has a neuroprotective effect in a rat model of acute bilirubin encephalopathy in which it greatly reduced bilirubin-induced neurotoxicity due in part to an inhibition of p38 mitogen-activated protein kinase activity [69]. With regard to neurobehavior, treatment with minocycline significantly alleviated the HI- and /LPS-induced brain injury on neurobehavioral performance including sensorimotor and locomotor function, anxiety and cognitive ability [66, 67].

In the Schmitz group, using primary cultures of rat OPCs exposed to oxygen-glucose deprivation (OGD), it was demonstrated that minocycline exerts direct protective effects on OPC against OGD [35]. Minocycline protected oligodendroglial lineage cells, attenuated OGD-induced oxidative stress and apoptosis, prevented the OGD-induced downregulation of the transcription factors Sox10 and Olig2 and of myelin-specific genes including 2’3’ cyclic nucleotide phosphodiesterase (CNP) and myelin basic protein (MBP) [35]. In hyperoxic rats, minocycline administration diminished apoptotic cell death and improved proliferation and maturation of OPCs. Minocycline blocked changes in microglial morphology in the white matter and blocked the IL-1β release induced by hyperoxia [57]. Moreover, long term impairment of white matter diffusivity in MRI/DTI in P30 and P60 animals after neonatal hyperoxia was attenuated by minocycline [57].

1. Materials and Methods

2.1. Materials

Table 2: List of equipments

Centrifuge 5417R	Eppendorf, Hamburg
electrophoresis chamber	Bio-Rad, Munich
LEICA DM 2000	Leica Microsystems, Wetzlar
Mastercycler, ep gradient S	Eppendorf, Hamburg
Megafuge 1.0 R	Thermo Scientific, Bonn
Microplate Reader, model 550	Bio-Rad, Munich
Microtome HM400	Microm International, Walldorf
Molecular Imager® ChemiDoc™ System	Bio-Rad, Munich
NanoDrop® Spektrophotometer, ND-1000	NanoDrop Technologies, Wilmington, USA
OxyCycler	BioSpherix, Lacona, NY, USA
Real-time PCR System StepOnePlus	Applied Biosystems, Darmstadt
Thermomixer Comfort	Eppendorf, Hamburg
Trans-Blot® SD Cell	Bio-Rad, Munich
UV recorder UV solo	Biometra, Göttingen
Video Copy Processor	Biometra, Göttingen

Table 3: List of chemicals

4-20% mini precast Tris-glycine gels	Bio-Rad, Munich
10x DreamTaq™ green buffer	Fermentas, St. Leon-Rot
50x TAE Electrophoresis buffer	Fermentas, St. Leon-Rot
Agarose	Sigma-Aldrich, Steinheim
Aqua ad iniectabilia	B. Braun, Melsungen
BSA	Sigma-Aldrich, Steinheim
Chloroform	J.T. Baker, Griesheim
Complete Protease Inhibitor Cocktail Tablets	Roche Diagnostics, Mannheim
DNase I	Qiagen, Hilden
dNTP Mix (10mM)	Promega, Mannheim
dNTP Mix (2 mM)	Fermentas, St. Leon-Rot
Donkey serum	Sigma-Aldrich, Munich
DreamTaq DNA Polymerase	Fermentas, St. Leon-Rot

Ethanol	Merck, Darmstadt
Ethidium bromide (10 mg/ml)	Roth, Karlsruhe
Glucose	Roth, Karlsruhe
Glycerin	Roth, Karlsruhe
Glycine	Merck, Darmstadt
Goat serum	Sigma-Aldrich, Munich
HCl	Sigma-Aldrich, Munich
Isopropanol	Merck, Darmstadt
Ketamin 10%	Bela Pharm, Vechta
β-Mercaptoethanol	Roth, Karlsruhe
Methanol	J.T. Baker, Griesheim
Minocycline	Sigma-Aldrich, Steinheim
M-MLV Reverse Transcriptase	Promega, Mannheim
M-MLV RT 5x Reaction buffer	Promega, Mannheim
Paraformaldehyde	Merck, Darmstadt
PBS	GIBCO®, Invitrogen, Darmstadt
peqGOLD RNApure	PEQLAB Biotechnologie, Erlangen
Ponceau S Solution	Sigma-Aldrich, Steinheim
Protease inhibitor cocktail tablets	Roche Diagnostics, Mannheim
Proteinase K	Roth, Karlsruhe
Random Primer (500µg/ml)	Promega, Mannheim
RIPA buffer (pH 7,4)	Sigma-Aldrich, Steinheim
RNase A	Sigma-Aldrich, Steinheim
RNase-free distilled water	Qiagen, Hilden
RNasin® Ribonuclease Inhibitor (40U/µl)	Promega, Mannheim
Rox high (50x)	PEQLAB Biotechnologie
Kapa Probe Fast qPCR MM (2x)	PEQLAB Biotechnologie, Erlangen
Skimmed milk powder	Sigma-Aldrich, Steinheim
Sodium *citrate*	Sigma-Aldrich, Steinheim
Sodium dodecyl sulfate	Sigma-Aldrich, Steinheim
Tissue Freezing Medium	Triangle Biomedical Sciences, Durham, USA
Tris base	Roth, Karlsruhe
Triton X-100	Roth, Karlsruhe
Tween® 20	Serva, Heidelberg

Vectashield HardSet Mounting Medium (DAPI)	Vector Laboratories, Burlingame, CA, USA
Xylazin, Rompun 2%	Bayer-Schering, Leverkusen

Table 4: List of antibodies and kit

anti-CC1 mouse mAb	Millipore, Temecula, CA, USA
anti-DCX goat pAb	Santa Cruz Biotechnology
anti-Iba1 rabbit pAb	Wako Chemicals, Neuss
anti-Ki67 rabbit pAb	Leica Biosystems, Nussloch
anti-*NG2 Chondroitin Sulfate Proteoglycan* mouse mAb	Millipore, Temecula, CA,USA
anti-8-oxo-dG monoclonal mouse	*Trevigen*, Gaithersburg, MD, USA
FITC-conjugated donkey anti-goat IgG	Jackson ImmunoResearch, Suffolk, UK
FITC-conjugated goat anti-mouse IgG	Jackson ImmunoResearch, Suffolk, UK
FITC-conjugated goat anti-rabbit IgG	Jackson ImmunoResearch, Suffolk, UK
TRITC-conjugated goat anti-rabbit IgG	Jackson ImmunoResearch, Suffolk, UK
In Situ Cell Death Detection Kit, Rhodamine	Roche Diagnostics, Mannheim
BCA Protein Assay Kit	Thermo Scientific, Rockford, USA
Oxyblot™ Protein Oxidation Detection Kit	*Chemicon*/*Millipore, Schwalbach*

Table 5: List of solutions and buffer

Agarose gel (1%/ 2%)	1%/2% Agarose (w/v) in 1x TAE buffer
3% donkey blocking solution	1% BSA (w/v)+0,3 % Triton X-100 (v/v)+3% normal donkey serum (v/v) in 1xPBS
1% donkey carrier solution	1% BSA (w/v)+0,3% Triton X-100 (v/v)+1% normal donkey serum (v/v) in 1xPBS
10x electrophoresis buffer	1,92 M Glycine+250 mM Tris-HCl (pH8,1)+1% SDS (w/v)
10% goat blocking solution	1% BSA (w/v)+0,3% Triton X-100 (v/v)+10% normal goat serum (v/v) in 1xPBS
1% goat blocking solution	1% BSA (w/v)+0,3% Triton X-100 (v/v)+1% normal goat serum (v/v) in 1xPBS
2x Laemmli buffer(pH 6,8)	0,1% Bromophenol blue+50% Glycerine+5% β-Mercaptoethanol+2% SDS (w/v)+ 0,125 M Tris-HCl
Paraformaldehyde (4%)	4% (w/v) Paraformaldehyde in PBS (pH 7,4)

Proteinase K solution	0,0005% Proteinase K (v/w) in PBS (pH 7,5)
RNase A solution	0,01% RNase A (v/w)+ 150 mM NaCl+15 mM sodium citrate
Skim Milk (4%)	4% Skim Milk (w/v) in TBST
10x TBS buffer (pH 7,4)	0,2 M Tris-HCl (pH 7,4)+ 1,5 M NaCl
TBST	10 M Tris-HCl (pH 7,4)+ 150 mM NaCl+0,1% Tween® 20 (w/v)
Transfer buffer (pH 9,2)	39 mM Glycine+48 mM Tris+20% Methanol (v/v)
0,3% Triton X-100 solution	0,3% Triton X-100 (v/v) in PBS

Table 6: List of Synthetic oligonucleotides and locus

gene		**oligonucleotide sequences 5´- 3´**	
-Actin	forward	CCCTAAGGCCAACCGTGAAAAAGATG	NM_007393.3
	reverse	GAACCGCTCGTTGCCAATAGTGATG	
Sox2	forward	ACAGATGCAGCCGATGCA	NM_001109181
	reverse	GGTGCCCTGCTGCGAGTA	
	probe	**FAM** CAGTACAACTCCATGACCAG **TAMRA**	
Sox10	forward	CCGCACCTCCACAATGCT	NM_019193.1
	reverse	GGTACTTGTAGTCCGGATGGTCTTT	
	probe	**FAM** TTGCTGAACGAGAGTGACAA **TAMRA**	
Sox11	forward	AGCGAGAAGATCCCGTTCATC	NM_053349.1
	reverse	GGGTCCGTCTTGGGCTTTT	
	probe	**FAM** CATGGCTGATTATCCCGACT **TAMRA**	
Olig2	forward	TGCGCAAGCTCTCCAAGAT	NM_001100557.1
	reverse	TCTCGCTCACCAGTCTCTTCATC	
	probe	**FAM**CGAAACTACATCCTGATGCT **TAMRA**	
HPRT	forward	GGAAAGAACGTCTTGATTGTTGAA	NM_012583.22
	reverse	CCAACACTTCGAGAGGTCCTTTT	
	probe	**FAM**-CTTTCCTTGGTCAAGCAGTACAGCCCC-**TAMRA**	

Table 7: List of Molecular weight marker

50 bp DNA-ladder	Fermentas, St. Leon-Rot
Precision Plus Protein™ Standard Dual Color	Bio-Rad, Munich

2.2. Neonatal rat hyperoxia model and minocycline administration

The studies were approved by the Institutional Animal Care and Use Committee at Charity Medical University, Children's National Medical Center, and National Institutes of Health guidelines. P6 wild-type rats were subjected to hyperoxia and subsequently used for immunohistochemistry, PCR analysis and Western Blot. Litters composed of male and female neonatal mice were divided into hyperoxia and control groups. Pups exposed to hyperoxia were placed, along with their mothers, in a chamber containing 80% O2 for 24 h from P6 to P7. The control pups of each litter were kept in room-air with a second lactating mother. Half of hyperoxia and control groups were chosen randomly and injected minocycline at a dosage of 45 mg/kg i.p at the beginning of exposure to hyperoxia (P6), and after 12h exposure to hyperoxia. The other half was treated with the same volume of PBS. Thus, pups were randomized into hyperoxia group, hyperoxia plus minocycline group, and control group. Each experimental group consisted of 4 pups. During further 48h (P9) and 96h (P11) recovery in room air, all pups previously exposed to hyperoxia were reunited with their biological mother until sacrifice (Fig 2).

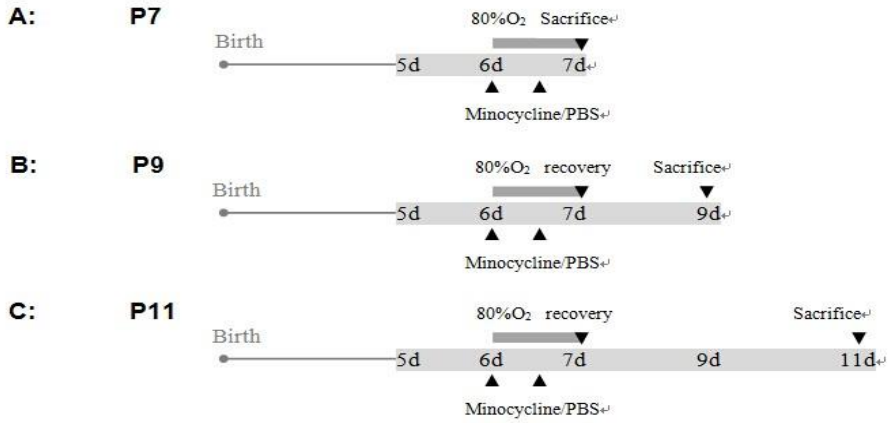


Figure 2: Study protocol.

Litters were randomly divided into hyperoxia and control groups and injected with minocycline 45mg/kg or PBS i.p. at the beginning, and 12h after the beginning of hyperoxia exposure.

2.3. Tissue Preparation

Rats at P7, P9, and P11 were anesthetized following German practice guidelines for animal experiments, and transcardially perfused with PBS and then 4% PFA. After decapitation, the olfactory bulb and cerebellum were removed. For molecular studies, brain hemispheres were snap-frozen in liquid nitrogen and stored at -80°C until further analysis. For immunohistochemistry, brains were post-fixed with 4% PFA overnight at

4°C and afterwards kept in PBS. Then frozen by Tissue Freezing Medium and mounted on a Microm HM400 microtome. Sections were cut (20 µm) and stored at -20°C.

2.4. Immunohistochemistry

2.4.1. Neurons, immature and mature oligodendrocytes, microglia

Brain sections were blocked at RT for at least 1h in specific blocking solution (Tab.8). Primary antibodies, here in particular DCX as a marker for neurons, NG2 chondroitin sulfate proteoglycan for immature oligodendrocytes, CC1 for mature oligodendrocytes, and Iba1 as a marker for microglia, were diluted using appropriate carrier solutions (Tab.8). After washing with PBS, brain sections were incubated in primary antibody with anti-DCX polyclonal goat, polyclonal mouse antibody to NG2 chondroitin sulfate proteoglycan, *monoclonal mouse anti-CC1*, and polyclonal rabbit anti-Iba1 at 4°C overnight. Rinses were performed in carrier solution at RT, with three changes of solution every 10 min. Secondary fluorescein-conjugated donkey anti-goat IgG, fluorescein-conjugated goat anti-mouse IgG, or fluorescein-conjugated goat anti-rabbit IgG was applied with the respective carrier solution of the first antibody and incubated at room temperature in the dark for 4h or 1h (Tab.8). Finally, sections were mounted with Vectashield HardSet Mounting Medium with DAPI.

Table 8: Dilution, blocking and carrier solutions for specific antibodies

Antibody(dilution)		**carrier solution**	**blocking solution**	**secondary antibody(dilution)**	
anti-DCX	1:200	1% donkey carrier solution	3% donkey blocking solution	FITC-conjugated donkey anti-goat IgG	1:500
anti-NG2	1:500	1% goat carrier solution	10% goat blocking solution	FITC-conjugated goat anti-mouse IgG	1:200
anti-CC1	1:200	1% goat carrier solution	3% goat blocking solution	FITC-conjugated goat anti-mouse IgG	1:200
anti-Iba1	1:500	1% goat carrier solution	10% goat blocking solution	FITC-conjugated goat anti-rabbit IgG	1:200

2.4.2. Proliferation cells

Proliferation was determined by double immunofluorescence staining with either DCX or NG2 (see 2.4.2). After blocking for 1h, brain sections were incubated with polyclonal rabbit anti Ki67 antibody (1:500) at 4°C overnight. After washes, incubated with TRITC

goat anti rabbit as secondary antibody for 1h. Finally, after three washings, the sections were mounted with Vectashield HardSet Mounting Medium with DAPI.

2.4.3. Nucleic acid oxidation

Slides were washed twice with PBS, incubated at 37°C for 1h with 100µg/ml RNase A in solution to prevent cross-reaction with RNA adducts and washed twice in PBS. Proteins were removed from DNA by digestion with PK at RT for 20min. To increase the antibody accessibility to the antigen, a denaturation step with 4N HCl for 5min was followed by neutralization with TRIS base for 5 min. Then blocked with 10 % goat blocking solution at RT for 1h and incubated with anti-8-oxo-dG (1:250) overnight at 4°C. After washes, incubated with secondary goat anti-mouse antibody for 30min at 37°C and washed with PBS. Sections were mounted with Vectashield HardSet Mounting Medium with DAPI.

2.4.4. DNA fragmentation assay

Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) assays were performed according to the manufacturer's directions. Sections were permeabilized by 0.3% Triton X-100 in PBS for 1h, rinsed with PBS, and incubated with TUNEL solution for 1h at 37°C. After three washes in PBS, tissue was mounted on slides, allowed to dry, and placed in Vectashield HardSet Mounting Medium with DAPI.

2.4.5. Visualization of cell populations

Sections with DG and hilus were viewed blinded under fluorescent light using a Leica DM 2000 microscope equipped with 100x (for representative microphotographs) and 200x (for quantitation) magnification and analyzed employing Leica Application Suite-LAS software. Counted in DG and hilus separately or measure intensity in hippocampus for 8-oxodG-positive cells (200x magnification), per animals in 4 to 6 sections.

2.5. Gene expression analysis

2.5.1. RNA isolation and quantification

Frozen brain was divided (30-80mg) and added with 800µl RNA-pure™ containing phenol and guanidine isothiocyanate to facilitate immediate inhibition of ribonucleases. To ensure a homogeneous suspension for the isolation of total cellular RNA, brain samples were pipetted up and down and incubated for 5min. Then, 160µl of chloroform was added to the homogenate, incubated for 10min at 4°C and centrifuged at 12,000g

at 4°C for 7min. Three different zones were formed: the lower organic phase, the interphase contained the DNA and proteins, and the upper aqueous phase contained RNA. The aqueous phase was transferred into a new tube without touching the other phases. Then one equivalent volume of ice-cold isopropanol (-20°C) was added and incubated at 4°C for 15min to precipitate the RNA. The precipitated RNA was pelletized by centrifugation at 12,000g for 10min at 4°C. The pellet was washed twice in 1ml ice-cold 75% ethanol and centrifuged at 12.000g for 10min at 4°C. Pelletized RNA was dehumidified for 20min at RT. Dried pelletized RNA was resuspended in 20-50µl RNase-free distilled water and heated at 55°C for 3min. The RNA samples were stored at -80°C. Samples were placed on ice and the RNA content was examined using the NanoDrop® Spectrophotometer at wavelengths of 260nm and 280nm.

2.5.2. Quality control of total RNA

The integrity of the isolated total RNA was verified by capillary electrophoresis. 500ng of isolated RNA was added with RNase-free distilled water to 10µl and 1µl of 10x DreamTaq™ green buffer. 11µl of RNA sample and 7µl of DNA-ladder were loaded into 1% (w/v) agarose gel containing 0.00003% (v/v) EtBr. An electric current of 80V was applied to the gel in 1x TAE-buffer. RNA bands were visualized and photographed using the UV recorder UV solo and Video Copy Processor. The size of the RNA product of 18S and 28S rRNA was determined by comparing it with the DNA-ladder.

2.5.3. Reverse transcription polymerase chain reaction (RT-PCR)

2µg of total RNA in every sample were diluted in RNase-free distilled water to 22.5µl and placed into a PCR reaction tube. After added 1µl of random primers, the RNA samples were incubated in the master cycler for 10min at 70°C to linearize RNA strands and cooled down at 4°C to prevent recreation. Following, 11.5µl of a reaction mixture (Tab.9) was added and samples were incubated at 37°C for 30min. Then heated for 5min at 75°C in order to stop reaction and to inactivate DNase. The reverse transcription of mRNA was then initiated with 1µl MMLV- RT and 0.5µl RNasin® and the cDNA was synthesized at 42°C for 1h. The polymerase was inactivated by heating at 95°C for 5min. The cDNA samples were stored at -20°C.

Table 9: Reaction mix of DNase I treatment

components	Volume [µl]	final concentration

M-MLV-RT 5x reaction buffer	8	1x
dNTP mix (each 10mM)	1	0,3mM
DNase (2U/µl)	2	4U
RNasin® (40U/µl)	0,5	20U

2.5.4. Control-PCR

Successful cDNA synthesis was verified with a control PCR for expression of the "housekeeping" gene ß-actin. A 25 µl PCR reaction was made up by adding 2µl of cDNA to the 23µl master mix batch (Tab.10). Negative control comprised the same supplements exclusive cDNA but with RNase-free water.

Table 10: Master mix for the control-PCR

components	Volume [µl]	final concentration
10x DreamTaq™ buffer	2,5	1x
2mM dNTP mix	2,5	0,2mM
β-Actin forward oligonucleotide	2,5	0,05µM
β-Actin reverse oligonucleotide	2,5	0,05µM
DreamTaq™ DNA polymerase	0,2	1U
RNase free water	12,8	-

The PCR was carried out in the master cycler with the 25µl batches under following conditions (Tab.11). The quality of PCR products was verified by capillary electrophoresis in a 2% Agarose gel with 0.00003% (v/v) EtBr. 12µl of cDNA sample and 7µl of DNA-ladder were loaded to the agarose gel. The electrophoretic separation was carried out at 80V in 1x TAE buffer and cDNA bands were visualized and photographed by the UV recorder UV solo and Video Copy Processor. The quality of cDNA was verified by comparing the molecular size of the cDNA product with the DNA-ladder. Bands of the pure cDNA or contamination with genomic DNA were visible.

Table 11: PCR Reaction process of control-PCR with β-aktin

reaction	temperature [°C]	time [min]
initial denaturation	94	5
subsequent denaturation	94	1
annealing	70	1

40 cycles

elongation	72	1
final elongation	72	10

2.5.5. Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed with fluorescence-labeled TaqMan™ probes, which allows specific cDNA fragments to be amplified and quantified through every cycle. All probes were labeled at the 5' end with a reporter dye FAM and at the 3' end with the quencher dye TAMRA. *HPRT* was used as an internal standard. The FAM spectral data was collected from reactions in separate tubes using the same stock of cDNA to avoid spectral overlap among FAM/TAMRA and limitations of reagents. Each PCR run was done in triplicate. After adding 12µl ROX High to 300µl Probe Fast qPCR MM. One standard assay for the RT-PCR was prepared according to following schedule (Tab.12).

Table12: Standard assay for RT-PCR

components	Volume [µl]
Probe Fast qPCR MM (2x) with ROX High (50x)	5
oligonucleotide mix (each 10µM)	2,5
probe (5µM)	0,5

23 to 230ng of cDNA template (Tab.6) was diluted in RNase-free water to 3µl. The 3µl cDNA and 8µl standard assay were added to the 96 well reaction plate and centrifuged for 10s at 4.000g. Negative control comprised same supplements exclusive cDNA but with RNase free water. Under the following conditions (Tab.13), the reaction was carried out with the StepOnePlus™ Realtime PCR System and fluorescent data were converted into cycle threshold (C_T) values. Relative quantification of the marker gene expression was performed according to the $^{\Delta\Delta}C_T$ method [70] and is given as a percentage of the expression of the *HPRT* product.

Table 13: PCR Reaction process of qRT-PCR

reaction	temperature [°C]	time
Uracil-N-glycosidase activation	50	2min
Initial denaturation/AmpliTaq polymerase activation	95	10min
denaturation	95	15s
annealing/ elongation	72	10min } 40 cycles

2.6. Protein biochemical analysis

2.6.1. Protein isolation and quantification

Frozen brain tissue (30-80mg) was homogenized in 300µl RIPA buffer containing protease inhibitor, incubated for 20min at 4°C and centrifuged at 3.000g for 10min at 4°C. The supernatant containing mitochondrial and cytosolic proteins was centrifuged at 17.000g for 20min at 4°C. The resulting supernatant containing the cytosolic proteins was collected and stored at -80°C. The cytosolic protein concentration was determined by the BCA Protein Assay reagent kit. The absorbance was measured photometrically in the Microplate Reader equipped with a 590 nm filter. The cytosolic protein concentration of brain lysate was determined based on the standard curve.

2.6.2. SDS-PAGE

The SDS-PAGE was used to fractionate proteins according to their molecular size. 20µg of cytosolic protein extract was diluted in Aqua dest. and 2 x Laemmli loading buffer were added. The protein approach was heat-denaturated at 95°C for 5min and cooled down at 4°C. Protein marker and protein samples were loaded on 4-20% mini precast Tris-glycine gels vertically aligned to the electrophoresis chamber and filled with 1 x electrophoresis buffer. The electrophoresis was terminated when the gel end.

2.6.3. Western blot (Semi-Dry blotting)

The gels were transferred onto PVDF membranes at 4°C overnight and blocked in 4% skimmed milk in TBST. OxyBlot™ commercial kit was used for detection of reactive oxygen species in protein samples of P7 rats. Positive signals were quantified by a ChemiDoc™ XRS+ system and the Image Lab™ software (Bio-Rad). The molecular size of proteins was verified by the Precision Plus Protein™ Standard Dual Color.

2.7. Analysis and statistics

For statistical analysis of immunohistochemistry and PCR results, the t test was used. For analysis of multiple sets of immunocytochemistry, ANOVA analysis was performed overall, followed by post-hoc t test for paired groups. All values are given as mean and standard error of the mean (SEM). All graphic and statistical analyses were performed using the Graph Pad Prism 5.0 software. A *p*-value lower than 0,05 was considered to be significant.

3. Results

3.1. Neuronal responses in the hippocampus

3.1.1. Minocycline protects proliferating neurons of the immature hippocampus against oxygen toxicity.

A unique characteristic of the hippocampus is that, unlike most brain regions, it continues to generate new neurons after birth and into adulthood. After their generation in the subgranular zone of the dentate gyrus, new neurons migrate into the granule cell layer and become structurally and functionally integrated into existing hippocampal circuitry. In my experiments, I attempted to label immature, proliferating neurons in the hippocampus by immunohistochemistry with DCX and investigate whether hyperoxia has negative effects on cell numbers and on proliferation activity of DCX-expressing neurons. As a second approach, I wanted to test whether newborn rats receiving minocycline during hyperoxia have improved cell numbers and proliferation of DCX-expressing neurons.

DCX is a microtubule binding protein directing neuronal migration into the cerebral cortex. It is transiently expressed for presumably 2–3 weeks by mitotic neuronal precursor cells and early postmitotic neurons. DCX is most abundantly expressed in the subgranular layer of the dentate gyrus. Immediately after exposure to 24hours of 80% oxygen, numbers of DCX+ neurons seemed to decrease in both regions of the hippocampus (hyperoxia 218.65 ± 4.61 cells/field vs. control 235.83 ± 14.61 cells/field in the layer, hyperoxia 165.33 ± 22.29 cells/field vs. control 220.54 ± 11.15 cells/field in the hilus) (Fig 3A,D,J,K), with a significant decrease in the hilus ($P<0.05$) (Fig 3K). Minocycline administration led to a significant increase of DCX+ neurons in hilus (hyperoxia with minocycline 253.21 ± 23.03 cells/field, $P<0.05$) (Fig 3A,G,K), but only to a small increase in the DG layer without statistical significance (hyperoxia with minocycline 232.83 ± 8.91 cells/field) (Fig 3A,G,J). At P9, rats showed a reduction in DCX+ cells after exposure to hyperoxia within both the DG and the hilus (hyperoxia 217.55 ± 13.81 cells/field vs. control 251.94 ± 8.09 cells/field in the layer, hyperoxia 199.96 ± 4.16 cells/field vs. control 228.88 ± 3.88 cells/field in the hilus) (Fig 3B,E,J,K). At P9, minocycline significantly attenuated neuronal damage in both regions (hyperoxia with minocycline 237.33 ± 8.55 cells/field in the layer, 236.84 ± 15.68 cells/field in the hilus) (Fig 3H,J,K). The impact of hyperoxia on DCX+ neurons in the hippocampus

persisted during recovery until P11 (hyperoxia 206.31 ± 10.71 cells/field vs. control 234.13 ± 7.85 cells/field, P<0.05 in DG layer; hyperoxia 164.29 ± 12.30 cells/field vs. control 201.33 ± 3.05 cells/field, P<0.01 in hilus) (Fig 3C,F,J,K). Benefits of minocycline were still visible at that age, too (hyperoxia with minocycline246.00 ± 7.17 cells/field, P<0.05 in the layer; 199.71 ± 9.02 cells/field, P<0.05 in the hilus) (Fig 3F,I,J,K). These data clearly indicate that postnatal oxygen toxicity causes neuron loss in the hippocampus which can be significantly attenuated by pharmacological intervention.

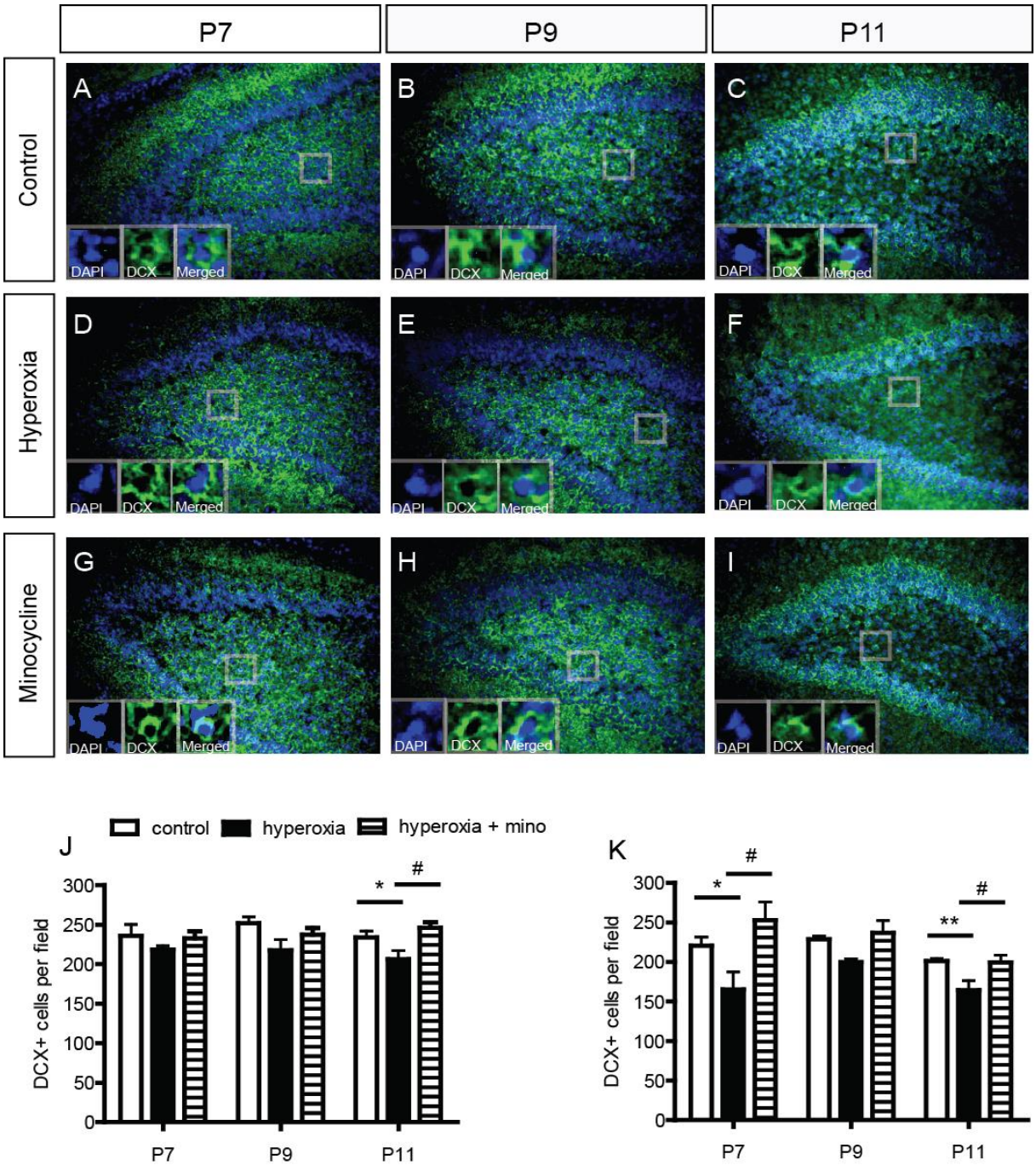


Figure 3: Minocycline attenuated hyperoxia-injury in neurons

Immunostainings of immature neurons labeled with DCX in the hippocampus after 24 hours hyperoxia with or without minocycline treatment at P7, and after 2 and 4 days recovery in room air at P9 and P11, respectively (A-I). Numerical densities of DCX+ cells in normoxic (white columns) rats and in rats subjected to hyperoxia without (black columns) or with minocycline (shaded columns) in DG layer (J) and hilus (K). (n = 4-5 for each group, unpaired t-test with *p<0.05; **p<0.01; for control vs. hyperoxia, and #p<0.05 for hyperoxia vs. minocycline).

In P7 hyperoxic rats compared to control litters, the number of proliferating neurons as detected by co-labeling with immunostianings using DCX and Ki67 antibodies was significantly decreased within the hilus (hyperoxia 7.07 ± 0.58 cells/field vs. control 13.65 ± 2.23 cells/field, P<0.05 in the hilus) (Fig 4A,D,K). Similar results were obtained in the DG (hyperoxia 5.52 ± 0.60 cells/field vs. control 10.00 ± 1.08 cells/field, P<0.01 in the layer) (Fig 4J). In hyperoxic rats treated with minocycline, the number of DCX+Ki67+ cells was higher in both hippocampal regions region (hyperoxia with minocycine 9.17 ± 0.75cells/field, P<0.01 in the layer; 11.29 ± 1.13 cells/field, P<0.05 in the hilus) (Fig 4D,G) compared to rats after hyperoxia without minocycline treatment (Fig 4J,K). Interestingly, after recovery in room air at P9 and P11, rats previously exposed to hyperoxia still showed decreased numbers of DCX+Ki67+ neurons both in the hilus (hyperoxia 6.28 ± 0.32 cells/field vs. control 10.56 ± 1.07cells/field, P<0.01 at P9; hyperoxia 2.31 ± 0.57 cells/field vs. control 5.63 ± 0.10 cells/field, P<0.05 at P11) (Fig 4B,C,K) and in the DG (hyperoxia 4.58 ± 0.23 cells/field vs. control 7.04 ± 0.54c ells/field, P<0.01 at P9; hyperoxia 2.20 ± 0.29 cells/field vs. control 5.39 ± 1.12 cells/field, P<0.05 at P11) (Fig 4J), indicating persistent damage in this cell population even after withdrawal of the toxic stimulus. Proliferating DCX neurons in minocycline treated rats were protected at those ages, too (hyperoxia with minocycline 3.67 ± 0.66 cells/field, P<0.05 in the layer; 3.88 ± 0.49 cells/field, P<0.05 in the hilus) (Fig 4H,I,J,K). These results indicate that acute hyperoxia induced the loss of neurogenesis in the hippocampus. Moreover, minocycline treatment prevented or attenuated the hyperoxia-induced loss of neurogenesis in the immature hippocampus.

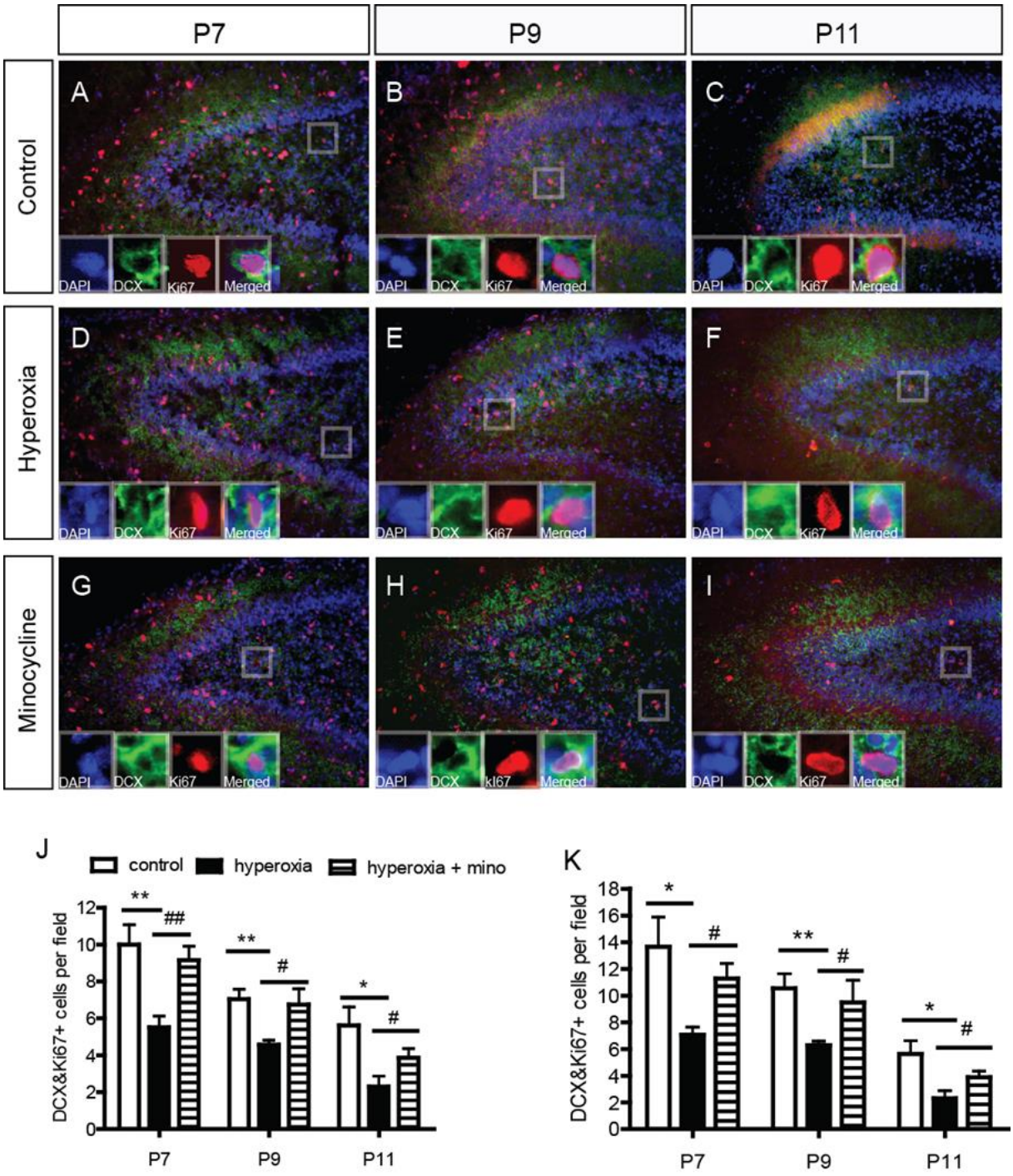


Figure 4: Numbers of proliferating DCX+ neurons in the hippocampus are decreased by hyperoxia and improved by minocycline.

Immunostainings of immature, proliferating neurons expressing DCX and Ki67 at P7 and after recovery in room air for 2 and 4 days at P9 and P11, respectively (A-I) are shown. In P7 rat pups after hyperoxia, numbers of DCX+Ki67+ neurons were significantly decreased both in the DG and in the hilus (A,D,J,K). In contrast, in P7 rats treated with minocycline, numbers of DCX+Ki67+ were similar to those of control rats always kept in room air (A,G,J,K). Reduction of proliferating DCX+ neurons by hyperoxia was also observed after recovery in room air at P9

(B,E) and at P11 (C,F) (J,K). Protection against hyperoxia by minocycline was confirmed at those ages (H-K). (n = 4-5 for immunohistochemistry, unpaired t-test with *p<0.05; **p<0.01; for control vs. hyperoxia, and #p<0.05; ##p<0.01; for hyperoxia vs. minocycline).

3.1.2. Minocycline improves expression level of neuronal factors in the immature hippocampus exposed to hyperoxia.

The Sox family of transcription factors are well-established regulators of cell fate decisions during development. Here, I analyzed molecular evidence of Sox2 and Sox11mRNA during hyperoxia and administration of minocycline.

At P7, there were no differences in Sox2 or Sox11 gene expression between the experimental groups (hyperoxia 105.51% ± 3.88 and hyperoxia with minocycline 118.64% ± 11.82 in Sox2; hyperoxia 102.41% ± 4.12 and hyperoxia with minocycline 100.09% ± 8.70 in Sox11) (Fig 5A,B). However, a significant delayed downregulation of both transcription factors by hyperoxia was found at both P9 and P11 (at P9, hyperoxia 39.52% ± 9.43 in Sox2, P<0.05; hyperoxia 70.74% ± 6.82 in Sox11, P<0.05; at P11, hyperoxia 69.51% ± 5.17 in Sox2, P<0.05; hyperoxia 59.74% ± 8.26 in Sox11, P<0.05) (Fig 5A,B). Prevention of neuronal damage through minocycline administration was confirmed in these analyses by improved Sox2 and Sox11 gene expression at both ages P9 (in Sox2, hyperoxia with minocycline 108.26% ± 12.18, P<0.05; in Sox11 hyperoxia with minocycline 110.93% ± 15.71, P<0.05) and P11 (in Sox2, hyperoxia with minocycline 103.71% ± 17.54; in Sox11 hyperoxia with minocycline 92.23% ± 12.06, P<0.05) which did not differ from control levels (Fig 5A,B).

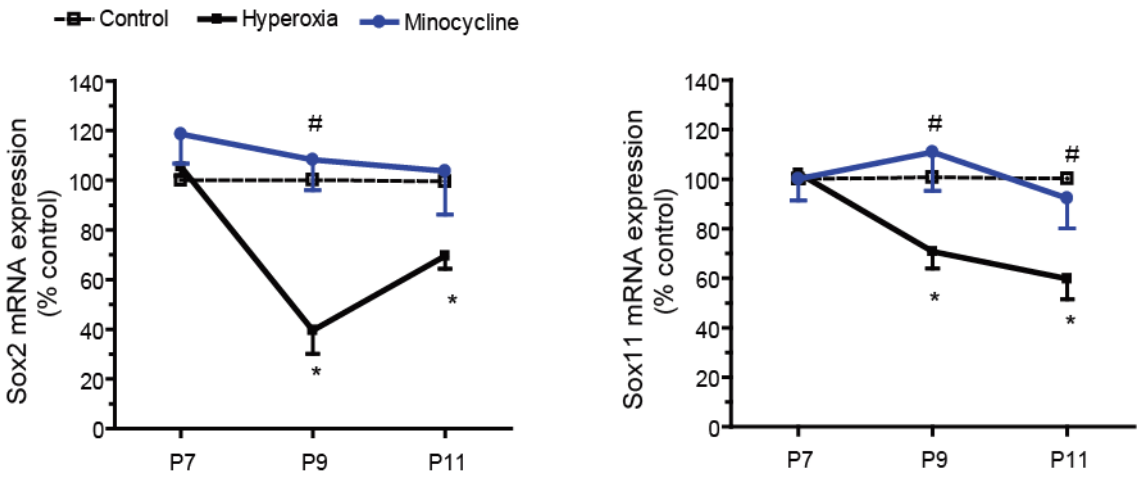


Figure 5: Expression of neuronal transcription factors

Results of gene expression analysis of transcription factors Sox2 and Sox11 which are important for neuronal development are shown. Analysis with qPCR revealed downregulation of both transcription factors after hyperoxia which was blocked by minocycline (Fig.5A,B). (n = 6 for gene expression analysis, unpaired t-test with *p<0.05 for control vs. hyperoxia, and #p<0.05 for hyperoxia vs. minocycline).

3.2. Oligodendroglial population in immature hippocampus

Cells of oligodendroglial lineage at different maturational stages can be distinguished using specific cell markers. To identify immature oligodendroglia in my experiment, rat brain sections were immunohistochemically labeled with NG2, expressed by OPCs, mature oligodendroglia were identified with anti-CC1 antibody (Fig 6).

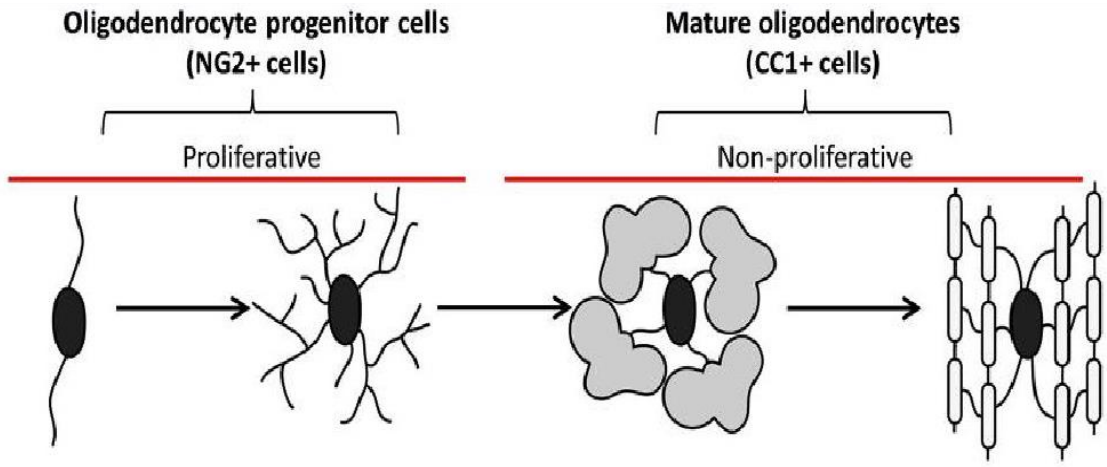


Figure 6: Scheme showing maturational changes in oligodendroglial lineage cells

In my experiment, oligodendrocyte progenitor cells and mature oligodendrocytes were identified with the expression of NG2 and CC1 markers, respectively [71].

3.2.1. Responses of oligodendroglial precursor cells to hyperoxia in the hippocampus

In the mammalian hippocampus, oligodendroglial lineage cells are another cell population that are capable of proliferation. OPCs have been described as a constitutive reservoir of oligodendrocytes in remyelination after myelin damage [72]. For my

experiment, I analyzed total numbers of NG2+ OPCs in the hilus of newborn rats subjected to hyperoxia with or without minocycline treatment (Fig 7). In P7 hyperoxic rats, there was a slight decrease in the number of NG2+ cells within the hilus when compared with controls (hyperoxia 171.95 ± 26.92 cells/field vs. control 193.17 ± 26.29 cells/field) (Fig 7A,D,J). In hyperoxic rats treated with minocycine, the number of NG2+ cells did not improve (hyperoxia with minocycline 176.58 ± 14.00 cells/field) (Fig 7D,J) Notably, after recovery in room air at P9, rats previously exposed to hyperoxia showed a significant reduction in the numbers of NG2+ OPCs (hyperoxia 154.83 ±1 2.30 cells/field vs. control 213.76 ± 8.59 cells/field, $P<0.05$) (Fig 7B,E,J). In rats receiving minocycline, reduction of proliferating OPCs by hyperoxia was fully blocked (hyperoxia with minocycline 184.01 ± 9.29 cells/field, $P<0.05$) (Fig 7H,J). Apparently, minocycline mediated protection of OPCs at a time beyond the actual exposure to toxic hyperoxia. However, at P11, OPCs stimulated by hyperoxia decreased slightly (hyperoxia 153.79 ± 11.34 cells/field vs. control 174.08 ± 6.19 cells/field), and did not recover after treatment with minocycline (hyperoxia with minocycline 156.38 ± 4.98 cells/field) (Fig 7C,F,J).

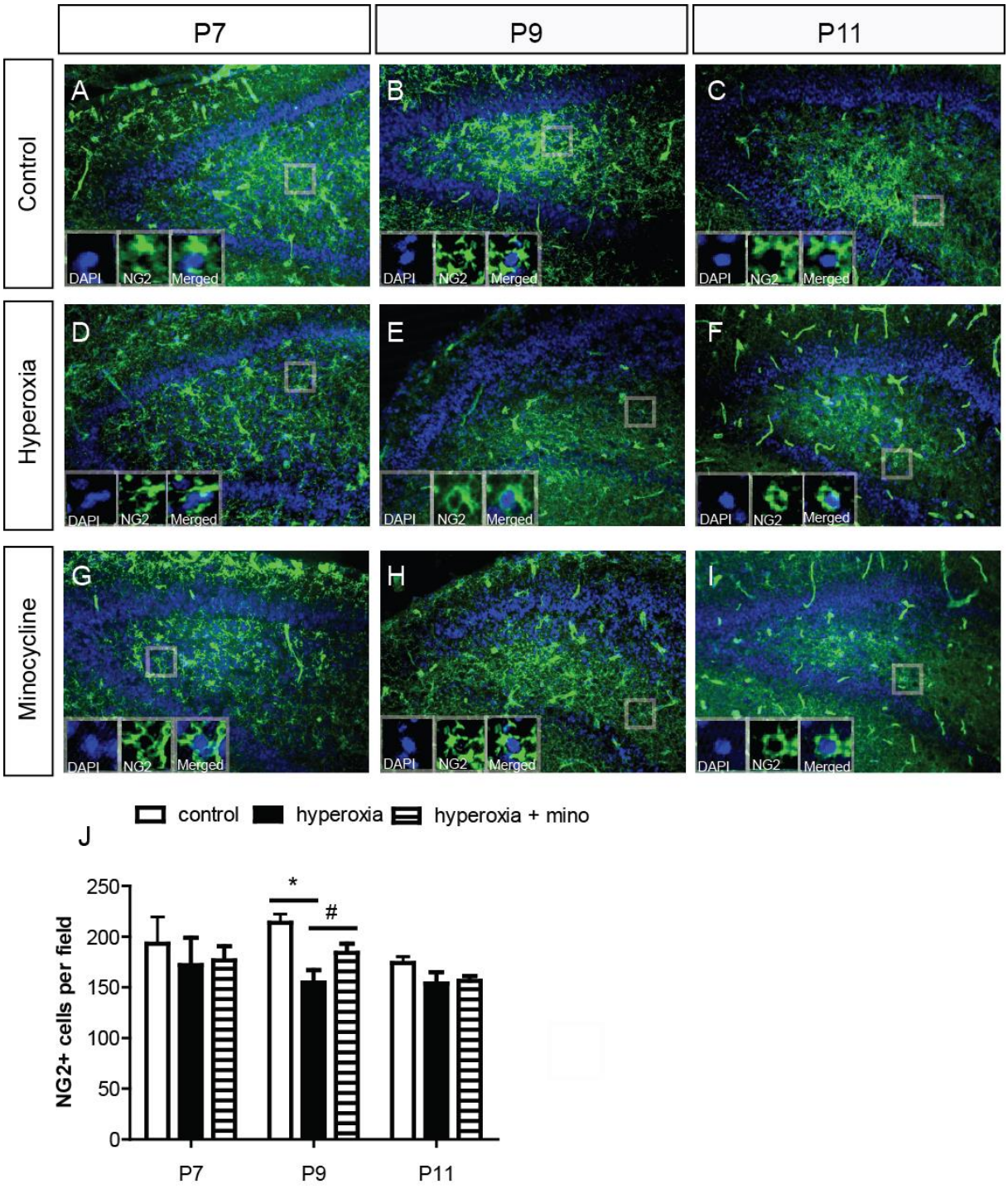


Figure 7: Minocycline protects NG2+ oligodendroglial progenitor cells of the immature hippocampus against hyperoxia.

Immunostainings of OPCs labeled with NG2 antibodies at P7 and after recovery in room air for 2 and 4 days at P9 and P11, respectively (A-I) are shown. In P9 rat pups after hyperoxia, numbers of NG2+ cells were significantly decreased in the hilus (B,E,J). In contrast, in P9 rats exposed to hyperoxia but treated with minocycline, numbers of DCX+Ki67+ are significantly improved (H,J). At P11, no difference in numbers of OPCS was detectable between the

experimental groups any longer (I,J). (n = 4-5 for immunohistochemistry, unpaired t-test with *p<0.05; for control vs. hyperoxia, and #p<0.05; for hyperoxia vs. minocycline).

Analysis of NG2 positive OPCs was also performed in the hilus of the hippocampus with Ki67 for proliferation (Fig 8). Compared to controls, hyperoxic rats showed a marked reduction in NG2+Ki67+ OPCs at P7 (hyperoxia 4.90±1.45 cells/field vs. control 9.54 ± 1.09cells/field, P<0.01) (Fig 8A,D), and also after recovery in room air at P9 (hyperoxia 4.13 ± 0.38 cells/field vs. control 5.82 ± 0.49cells/field, P<0.05) (Fig 8B,E) and P11 (hyperoxia 1.69 ± 0.41 cells/field vs. control 3.03 ± 0.50 cells/field, P<0.05) (Fig 8C,F). In rats receiving minocycline, exposure to hyperoxia did not affect the number of NG2+Ki67+ OPCs at P7 (hyperoxia with minocycline 10.17 ± 1.08 cells/field, P<0.05) (Fig 8G,J). However, this protective effect was not seen after recovery at P9 or P11, at which NG2+Ki67+ cell numbers were lower than in controls (4.80 ± 0.47 cells/field at P9; 2.00 ± 0.30 cells/field at P11) (Fig 8H-J). These results indicate that hyperoxia alters the growth of the NG2+ oligodendroglia population. Prevention by minocycline, however, is only partial. Interestingly, the extent of OPC protection by minocycline varies with different subregions of the hippocampus.

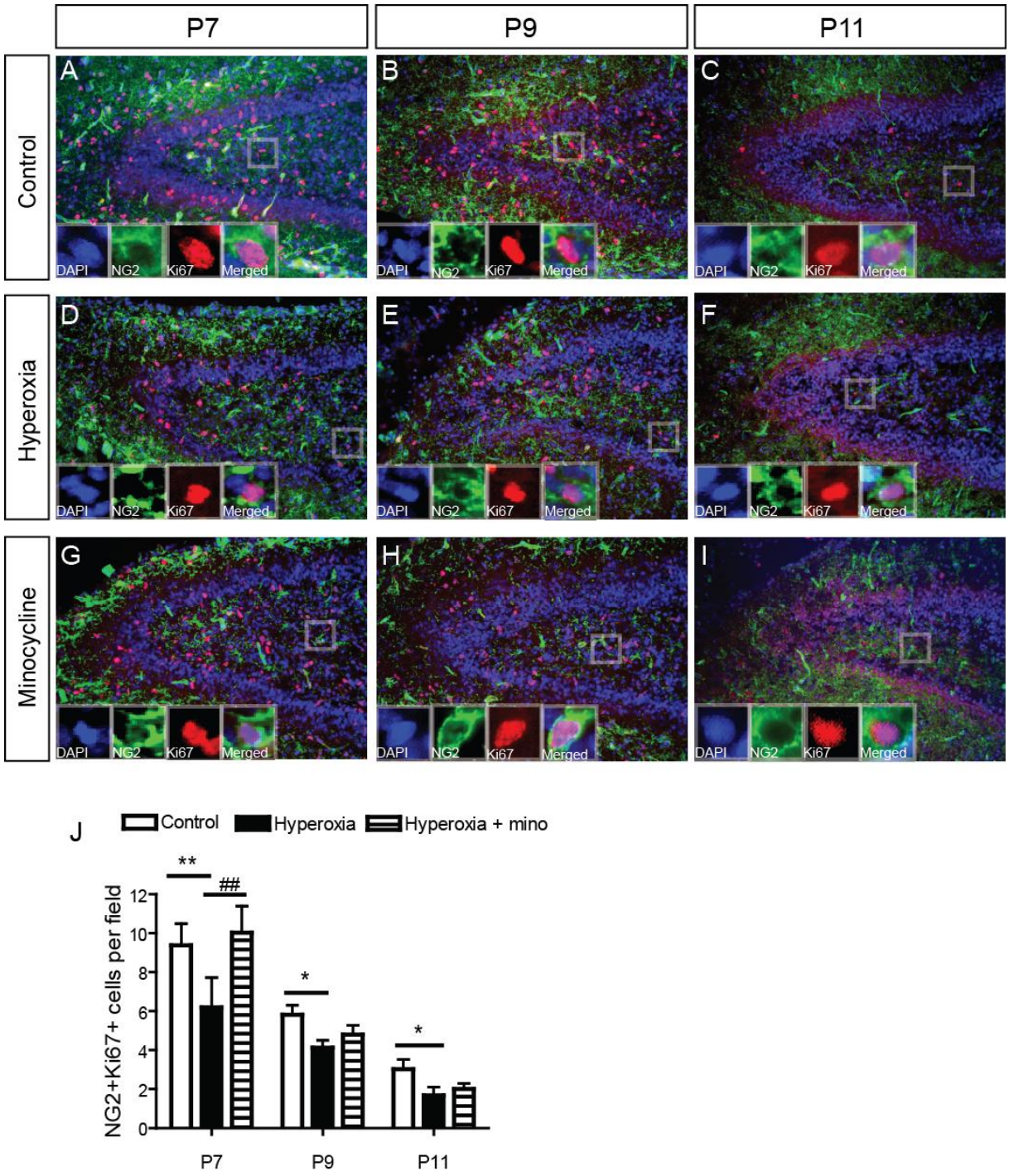


Figure 8: Minocycline protects NG2+ oligodendroglial progenitor proliferation of the immature hippocampus against hyperoxia.

The number of NG2+Ki67+ proliferating OPCs in the hippocampus of P7 rats after 24h hyperoxia is significantly decreased by 38% of those compared to control litters kept in room air (A,D). This decrease at P7 is abolished by treatment with minocycline (G,J). The hyperoxia-induced deficit of proliferating OPCs is also present at ages P9 (E) and P11 (F) during recovery in room air (J). At these ages, there was no significant protection detectable in those rats

treated with minocycline during preceding hyperoxia. (n = 4-5 for each group, unpaired t-test with *p<0.05; **p<0.01; for control vs. hyperoxia, and #p<0.05 for hyperoxia vs. minocycline).

3.2.2. Maturation of hippocampal oligodendrocytes after hyperoxia

I analyzed the effect of hyperoxia and minocycline on oligodendroglial maturation by quantification of CC1-expressing oligodendrocytes in the hippocampus (Fig 9). In correspondence with the immature developmental stage at P7, the population of CC1+ oligodendrocytes in the DG was relatively small in control rats at that age but was growing during further maturation until P9 and P11 (Fig 9A-C). However, this developmental expansion of CC1+ cells in the DG was diminished in rats after hyperoxia, thus leading to a significant deficit of mature oligodendrocytes at P11 (hyperoxia 17.13 ± 3.33 cells/field vs. control 15.83 ± 0.69 cells/field at P7; hyperoxia 25.30 ± 1.35 cells/field vs. control 27.22 ±1 .78 cells/field at P9; hyperoxia 25.88 ± 0.25 cells/field vs. control 33.87 ± 4.54 cells/field, P<0.05 at P11) (Fig 9F,J). This maturational delay related to hyperoxia was not found in rats receiving minocycline (hyperoxia with minocycline: 18.94 ± 1.73 cells/field at P7; 23.25 ± 2.14 cells/field at P9; 29.75 ± 3.20 cells/field, P<0.05 at P11) (Fig 9I,J). In the hilus, mature oligodendrocytes were found at a relatively high density in control rats at P7, yet the cell population was still growing until P11. Exposure to hyperoxia led to a reduction of CC1+ oligodendrocytes in the hilus at all ages analyzed, i.e. P7, P9, and P11 (hyperoxia 47.67 ± 2.43 cells/field vs. control 64.58 ± 0.90 cells/field, P<0.001 at P7; hyperoxia 42.07 ± 3.87 cells/field vs. control 64.89 ± 7.18 cells/field, P<0.05 at P9; hyperoxia 50.23 ± 2.66 cells/field vs. control 71.84 ± 8.13 cells/field, P<0.05 at P11), whereas minocycline prevented or attenuated this deficiency (hyperoxia with minocycline: 61.46 ± 1.37 cells/field, P<0.01 for P7; 64.90 ± 5.41 cells/field, P<0.05 for P9; 60.71 ± 3.32 cells/field, P<0.05 for P11) (Fig 9G-I,K). These data clearly indicate that postnatal hyperoxia causes a delay of oligodendroglial maturation in the hippocampus which can be attenuated by pharmacological intervention with minocycline.

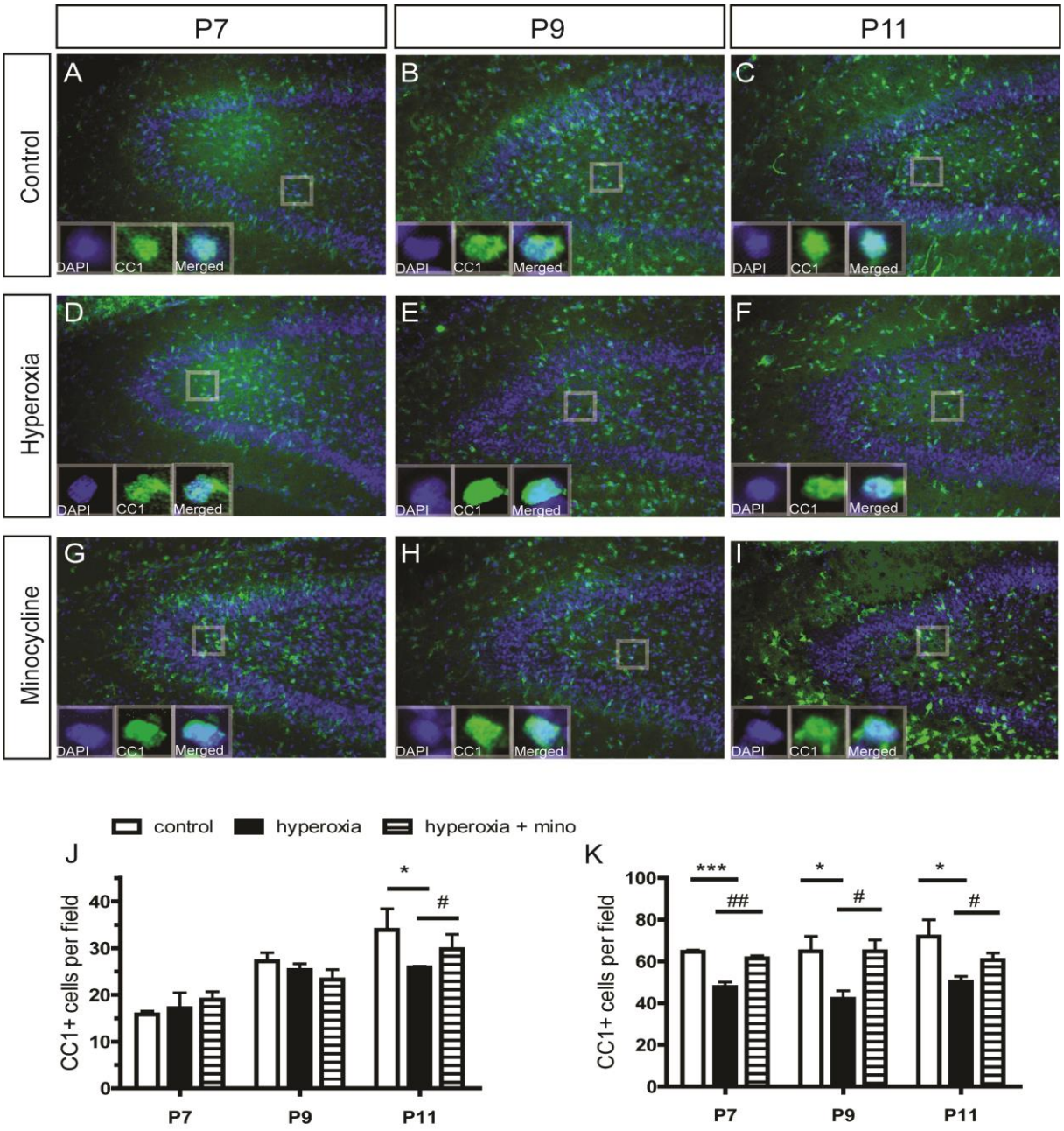


Figure 9: Hyperoxia-induced changes in mature oligodendrocytes in the hippocampus with and without minocycline treatment.

Mature oligodendrocytes in the hippocampus were labeled using CC1 antibody for immunohistochemistry (A-I). In DG the CC1+ oligodendrocytes in rats after exposure to hyperoxia significantly reduced at age P11 (A-F, J). This maturational delay is attenuated by minocycline (G-I, J). In the hilus, the maturation of oligodendrocytes is also inhibited by hyperoxia, and the loss of CC1+ cells is significant at all three ages investigated (K). Again, rats receiving minocycline clearly benefit from drug treatment. (n = 4-5 for each group, unpaired t-test with *p<0.05; **p<0.01; ***p<0.001 for control vs. hyperoxia, and #p<0.05; ##p<0.01 for hyperoxia vs. minocycline).

3.2.3. Oligodendrocyte transcription factor response in hippocampus

The transcription factor Olig2 is a basic helix-loop-helix transcription factor expressed by OPCs and mature oligodendrocytes in the CNS. SOX10 is part of the SOXE family of transcription factors, which are known to regulate oligodendrocyte fate. They both play a major role in oligodendroglial development and differentiation. Therefore, we used real-time PCR to determine the level of mRNA expression of these two essential transcription factors (Fig 10).

The expression of Sox10 decreased immediately after 24h high oxygen and maintained to be significantly downregulated both after 2 days at P9 and after 4 days at P11. (hyperoxia 43.71% ± 10.97, P<0.05 for P9; hyperoxia 69.51% ± 5.17, P<0.05 for P11). Benefits of minocycline on Sox10 expression were significant during both at P9 and at p11 (hyperoxia with minocycline 88.92% ± 14.12, P<0.05 for P9; hyperoxia 103.71% ± 517.54 for P11) (Fig 10A). However, reduction of Olig2 expression occurred in hyperoxia rats at P9, which was not found in pups with minocycline (hyperoxia 73.12% ± 4.21, P<0.05; hyperoxia with minocycline 93.84% ± 3.58, P<0.05) (Fig.10B). Interestingly, Olig2 expression increased after hyperoxia with and without minocycline at P11 (hyperoxia 135.86% ± 6.12, P<0.05; hyperoxia with minocycline 134.35% ± 11.80) (Fig.10B).

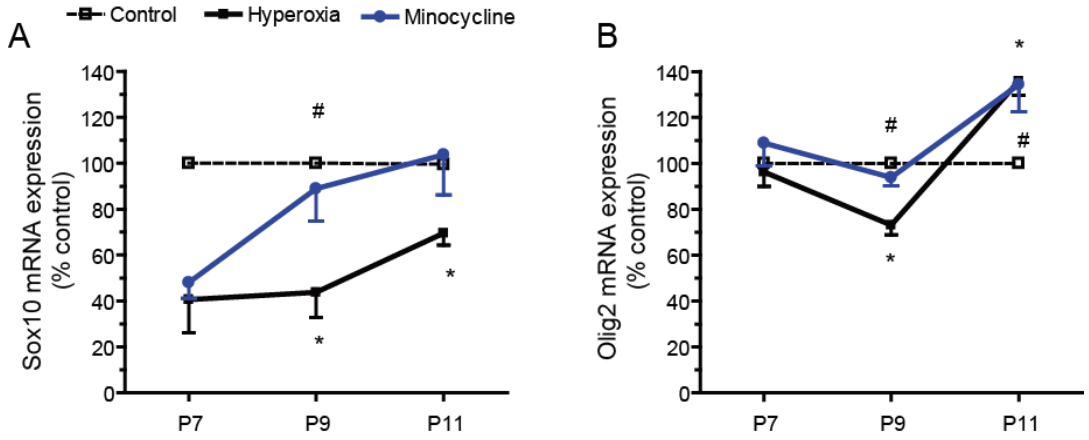


Figure 10: Expression of oligodendroglial transcription factors

Gene expression analysis of Sox10 and Olig2 after hyperoxia which was blocked by minocycline (Fig.10A,B). (n = 6 for gene expression analysis, unpaired t-test with *p<0.05for control vs. hyperoxia, and #p<0.05 for hyperoxia vs. minocycline).

3.3. Hyperoxia does not induce apoptosis in the immature hippocampus.

Exposure of the immature brain to high oxygen concentrations for 24 to 48 hours has previously been found to cause apoptotic cell death in cortical layers of rat pups [27] and in white matter oligodendroglia of mice [33]. I investigated whether neural cells of the hippocampus also undergo increased apoptosis after exposure to hyperoxia (Fig 11). In our TUNEL stainings of hippocampal slices of P7 control rats, physiological numbers of TUNEL positive cells were very low (control: 0.55 ± 0.31 cells/field in the layer; 1.23 ± 0.43 cells/field in the hilus) (Fig 11A). Notably, 24h hyperoxia-exposure did not induce an increase in apoptotic, TUNEL+ cells at P7 (hyperoxia: 0.82 ± 0.60 cells/field in the layer; 1.28 ± 0.42 cells/field in the hilus) (Fig 11B). Accordingly, any anti-apoptotic properties of minocycline did not play a relevant role in terms of its neuroprotective use in this study (hyperoxia with minocycline: 0.34 ± 0.20 cells/field in the layer; 0.76 ± 0.33 cells/field in the hilus) (Fig 11C,D).

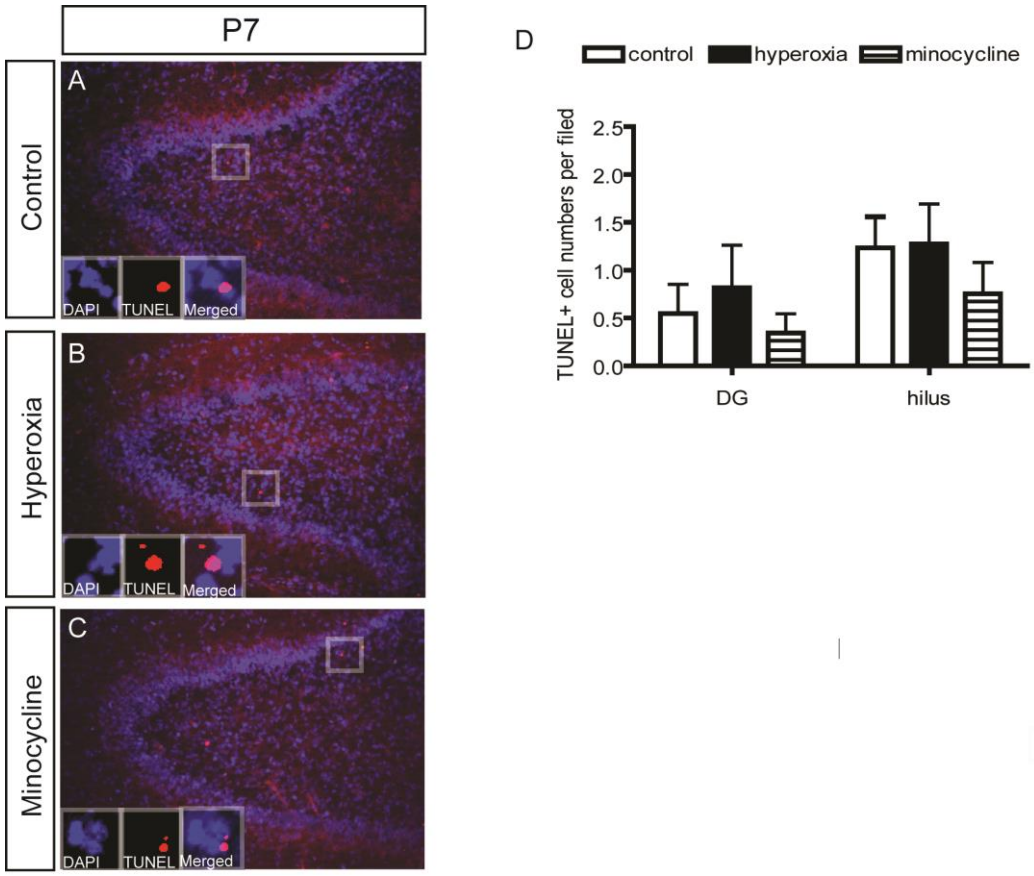
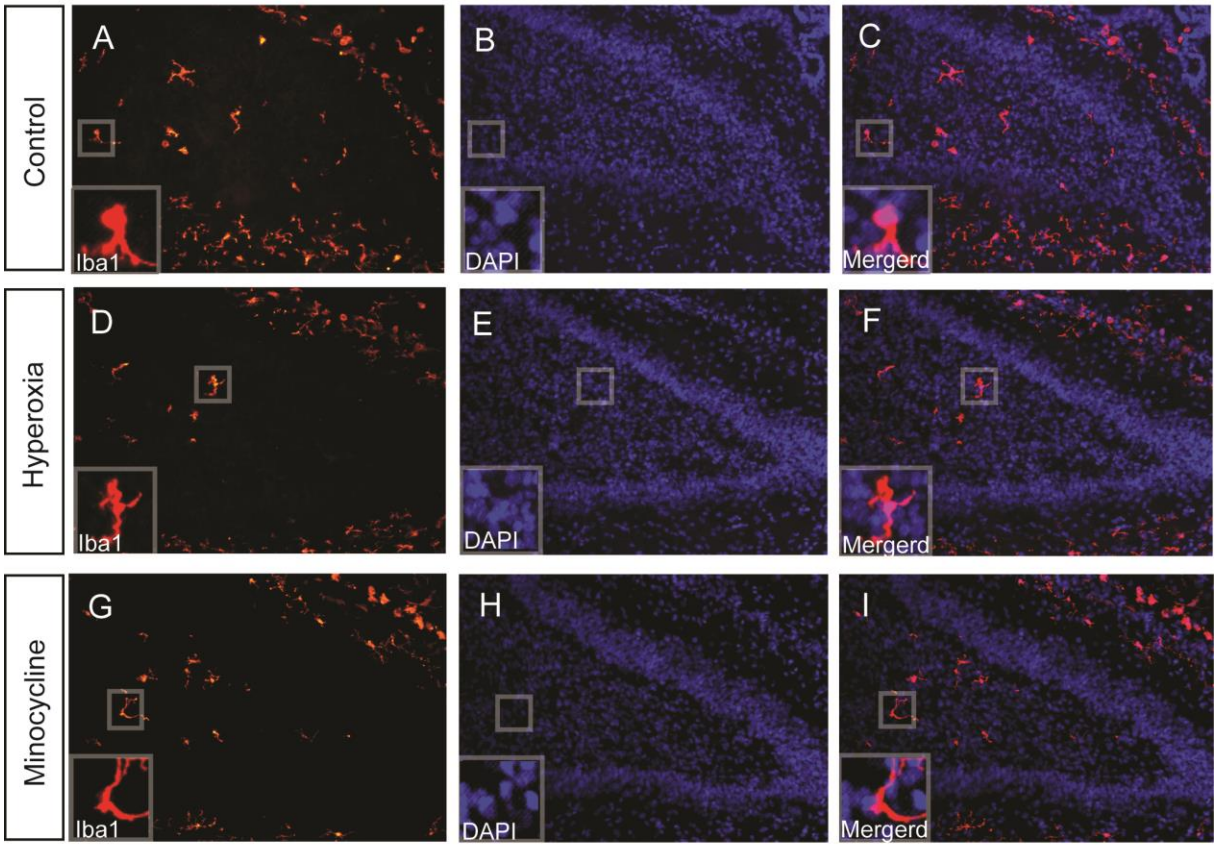


Figure 11: Hyperoxia does not induce an increase in the number of TUNEL+ cells in the hippocampus

Apoptotic cells in rat hippocampus at P7 were labelled using fluorescent TUNEL stainings. TUNEL+ cell numbers were not higher in rats after 24h hyperoxia than in control litters in room air (A,B,D). Minocycline did not affect apoptotic cell numbers, either (C,D).

3.4.Microglial response after exposure to hyperoxia

Microglial inflammatory activation is often reported to accompany brain injury and may deteriorate brain damage as a secondary toxic response. In a distinct hyperoxia model applying high oxygen concentrations for a duration of seven days, an increase in microglial numbers has been found in various brain regions [73]. To investigate whether microglial inhibition by minocycline may also play a protective role in the hippocampus exposed to hyperoxia, I examined microglial activation in the hippocampus using Iba1 immunohistochemistry (Fig 12). Therefore, numbers of Iba1+ microglia were determined in the DG and in the hilus in P7 rat brains. However, hyperoxia did not induce an increase in microglia in the hippocampus in those regions. In contrast, our analysis indicated a decrease of microglial numbers in the hippocampus of P7 hyperoxic rats (hyperoxia 1.99 ± 0.72 cells/field vs. control 4.79 ± 0.66 cells/field, $P<0.05$ in the layer; hyperoxia 6.44 ± 0.10 cells/field vs. control 7.67 ± 0.47 cells/field, $P<0.05$ in the hilus) (Fig 12A-F,J). The picture was similar in rats receiving minocycline during hyperoxia (hyperoxia with minocycline: 2.02 ± 1.08 cells/field in the layer; 6.99 ± 0.88 cells/field in the hilus) (Figure 12G-J). Based on our Iba1 analysis in the hippocampus, hyperoxic damage of neural cells and also the aforementioned protection by minocycline is unlikely to involve microglial changes.



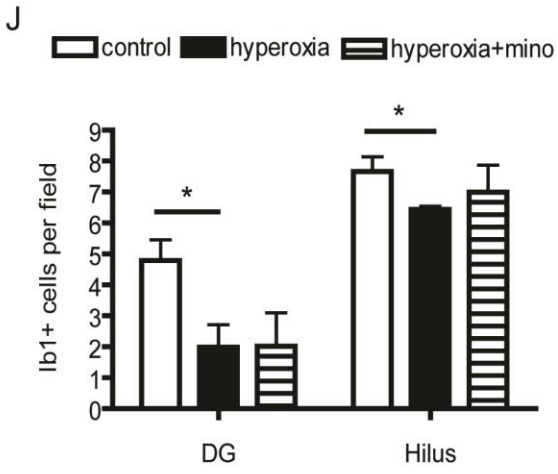


Figure 12: Microglia of the hippocampus do not exhibit signs of activation.

Microglial cell numbers were analyzed in immunohistochemically using Iba1 antibody. In comparison to control litters, P7 rats exposed to hyperoxia had decreased Iba1+ cell numbers (A-F,J) (#P<0.05). Shape of microglia appeared similar in both groups with a preponderance of ramified morphology.

3.5. Oxidative stress in the hippocampus caused by high oxygen is attenuated by minocycline.

Oxidative stress is a well described potential trigger of injury in the immature brain and can be evoked by various toxic conditions such as energy failure after hypoxia-ischemia [74] and perinatal infection/inflammation [75]. To describe possible DNA damage caused by oxidative stress induced by hyperoxia, I used immunohistochemical analysis of 8-hydroxy-desoxyguanosin (8oxo-dG) in P7 rat brains after 24h exposure to 80 % oxygen (Fig 13). In comparison to control litters in room air, hyperoxic rats had significantly increased 8oxo-dG fluorescence intensity in hippocampal cells (hyperoxia 13.40 ± 0.49 pixel intensity/field vs. control 10.42 ± 0.80 pixel intensity/field, $P < 0.01$) (Fig 13A-F,J). Anti-oxidant properties of minocycline were evident by significant reduction of 8oxo-dG fluorescence intensity in rats with minocycline treatment during exposure to hyperoxia (hyperoxia with minocycline 11.64 ± 0.40 pixel intensity/field, $P<0.05$) (Fig 13G-J). ROS production was detected in Western blots using OxyBlot™ kits with brain protein samples of P7 rats (Fig 13K,L). A significant increase of ROS productions in hyperoxic rats was prevented by administration of minocycline ($P<0.01$) (Fig.13K,L).

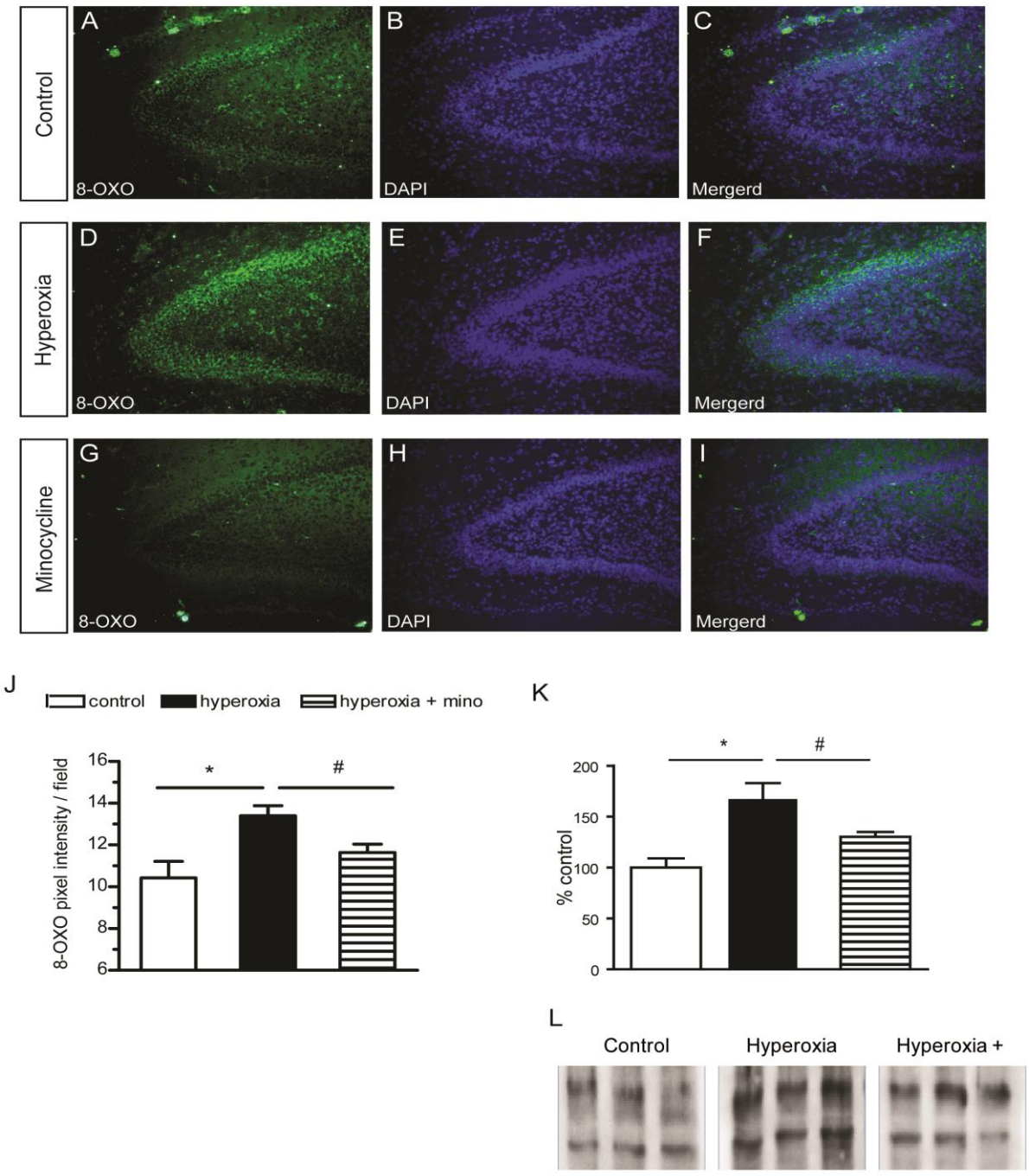


Figure 13: Hyperoxia causes oxidative stress in the hippocampus which is prevented by minocycline.

Oxidative stress as a mechanism of injury in the immature hippocampus was measured in rat brains using immunohistochemistry of 8oxo-dG for detection of oxidative DNA damage (A-J) and Western blot for analysis of ROS with OxyBlot^{TM} (K,L). Oxidative stress was induced in P7 rat hippocampus by 24h exposure to hyperoxia (D-F) as indicated by significantly higher 8-oxo-dG pixel intensity, which was blocked by minocycline (G-I, J). The anti-oxidant effect of

minocycline administration during hyperoxia (Hyperoxia+) was confirmed in OxyBlot^{TM} analysis (K,L). (n = 5 for each group, unpaired t-test with *p<0.01 for control vs. hyperoxia, and #p<0.05 for hyperoxia vs. minocycline)

4. Discussion

In this study I have investigated cellular mechanisms of hyperoxic damage in the immature hippocampus during a critical developmental time window. Moreover, I examined the pharmacological protection by minocycline during and beyond the acute phase of exposure to hyperoxia. I demonstrated that hyperoxia exposure in newborn rat pups decreased neuronal proliferation and expression of neurotrophins. Hyperoxia caused DNA oxidization in hippocampal cells and affected development and maturation of oligodendroglial lineage cells. Minocycline treatment in rats during hyperoxia improved neural cell proliferation and oligodendroglial maturation, and also transcription factors that regulate the development of neurons and oligodendrocytes, such as Sox2, Sox11and Oligo2 and Sox10. Since neither apoptosis nor microglial activation were found in the hippocampus of hyperoxic rats, the beneficial effects of minocycline can largely be attributed to its anti-oxidant properties.

4.1. Neuronal differentiation and proliferation

Neurogenesis is a multi-step process involving a symmetrical division of multi potent undifferentiated neural stem cells to give rise to progenitor cells that differentiate into one distinct lineage, i.e. neuronal, astroglial or oligodendroglial. They then finally migrate, and become fully functional neurons that are integrated into the brain circuitry [76]. Development of neural progenitor and stem cells is known to be regulated by oxygen tension levels with lower levels promoting and higher levels inhibiting proliferation [77, 78]. In the present study, we demonstrate that acute hyperoxia is able to reduce neurogenesis in the neonatal brain which can be traced by a significant decrease of expression of proliferation markers in immature neurons and also by transcription factor expression. However, short-term treatment with minocycline significantly attenuated hyperoxia-induced toxicity.

DCX is a microtubule binding protein that is required for normal neocortical and hippocampal development in humans. It is most abundantly expressed in the subgranular layer of the dentate gyrus. DCX normally represents an endogenous marker of neurogenesis in immature, migrating and differentiating neurons, and acute changes of DCX expressing immature neurons may have a long-term impact on neuron development and maturation. In this study, decreased neurogenesis in the DG and the hilus occurring at P7 immediately after hyperoxia persisted during recovery in room air

until P11. As early as 1986, neuronal damage in newborn rats after three hours of 100% hyperoxia had been reported in the subiculum of the hippocampus, thalamus, reticular nuclei of the brain stem and the granular cells of the cerebellum [79]. In following publications, it was reported that chronic hyperoxia caused decreased neuronal immunostaining intensity in the forebrain, including CA1 and dentate gyrus [58]. Moreover, Kaindl et al. observed reduction of DCX+ neuron density in the forebrain and also of protein levels after 12 hours hyperoxia, however, at the ages of P14 and P35, the levels of DCX seem to have been restored [80]. In humans, the period of fastest brain growth is observed during the last 3 months of a full-term pregnancy. In the brain of early preterm infants before the age of 34 gestational weeks, there is a crucial period of neuronal migration, necrosis and gliosis which is of particular vulnerability to toxic environmental changes. In rats and mice, this brain growth spurt occurs before postnatal days 14 [21]. Hence, using the time point of hyperoxia exposure from P6 to P7 and analysis at P7, P9, P11, it is possible to investigate whether postnatal hyperoxia interferes with brain development at important stages that are relevant in humans, too. In our study, reduced proliferation of immature DCX positive hippocampal neurons was found at P7 immediately after 24h exposure to hyperoxia. Remarkably, reduced proliferation persisted even at 2 days and 4 days while animals were placed in room air for recovery. The fact that reduced neuronal proliferation persisted over several days even after withdraw of hyperoxic conditions underlines the susceptibility of hippocampal neurons to oxygen toxicity.

Specific expression patterns of neural transcription factors such as Sox2 and Sox11 have been shown to maintain postnatal neuronal proliferation and differentiation in the developing and adult nervous system [81]. In our study in the immature brain, these two transcription factors were significantly downregulated after 24h hyperoxia, indicating that an unphysiologically high oxygen concerntration impairs neural mitosis and differentiation potential. The Sox genes are divided into different groups termed A to H are characterized by an SRY box, a 79 amino acid motif that encodes the DNA binding domain of an HMG-type (high mobility group). A growing body of evidence documents that they play a critical role in the embryonic development and cell fate determination. Sox2, a member of the Sox B1 subgroup, is characteristic of the proliferating, undifferentiated precursors [82] and highly expressed in the developing nervous system, predominantly in the subventricular zone and hippocampus dentate gyrus [83]. Sox11 is

a member of the SoxC subgroup which is expressed restrictively in DCX positive cells and postmitotic neuroblasts within the dentate gyrus [84] and play function as key players to regulate neuronal differentiation [31]. Loss of Sox2 in the developing brain resulted in a reduction of neurosphere-forming cells[85], while knockdown of Sox11 led to a diminished neuronal differentiation [31]. In other experimental *in vivo* and *in vitro* studies, hyperoxia is also related to alteration of neurotrophins expression [86, 87], such as brain-derived neurotrophic factor (BDNF), p75 neurotrophic receptor, and decreased activation of neurotrophin-regulated pathways.

Summing up, unphysiological high oxygen concentration seems to be a vital environmental stimulus interfering with neuronal proliferation and differentiation in the developing brain. Our results on the neuronal marker and transcription factors, confirm that in the immature brain high oxygen concentration can impair genesis and development of neurons.

4.2. Oligodendrocyte lineage cell proliferation and maturation

In the central nervous system, oligodendrocytes are the resident cell type responsible for the production of myelin. Apart from formation and maintenance of myelin, oligodendrocyte lineage cells paly an important roles in contributing to neuronal survival and development, and to neuronal transmission and synaptic activity [88]. In the present study, we demonstrate that acute hyperoxia affected oligodendroglial maturation in the developing brain through reduction of NG2+ OPC proliferation cells and CC1+ mature oligodendrocytes stages.

OPCs are generated in the ventral neuroepithelium of the neural tube in early embryonic life, and represent a highly relevant proportion of the glia in the developing and adult CNS (between 5% and 10%) [51, 89]. OPCs are thought to be capable of migratory and proliferative potential throughout the lifepan. These proliferative cells migrate into the developing white and grey matter, exit the cell cycle, undergo terminal differentiation into mature oligodendrocytes and elaborate myelin sheaths [90, 91]. During the oligodendrocyte lineage development, a panel of biochemical and immunochemical markers is available to monitor cell differentiation as these cells mature into oligodendrocyte. The NG2 proteoglycan-expressing (NG2) cells, a type 1-transmembrane protein, are thought as one type of the OPCs [92]. Mature

oligodendroglia were identified with anti-CC1 which is be used to label the cell body of mature oligodendrocyte.

NG2+ OPC cells are abundant across various white and gray matter regions. In the hippocampus, NG2+ cell proliferation was found in CA regions and also in hilus, but only few NG2+ cells were reported to be in the granule cells layer [93]. Jean and co-workers observed that NG2+ cells are more frequently associated with hilar interneurons than with granule cells, as they acquire more mature membrane properties, moreover, hilar interneurons and associated NG2+ cells are similarly integrated into the local network, receiving excitatory inputs from both granule cells and CA3 pyramidal neurons [94]. I also demonstrated in our study that far less NG2+ cells were located in the granule cell layer than in the hilus. Our results showed that hyperoxia induced a reduction of OPCs in the hilus, which was most pronounced at P9. Hyperoxic injury to OPC proliferation started at P7 immediately after hyperoxia and persisted both at 2 days and 4 days during room air recovery. This contrasts to the manifest repair in cell numbers found in oligodendroglia of the white matter during these ages after hyperoxia [57]. This difference during recovery could be explained by either a higher susceptibility of immature neurons to hyperoxic injury compared to oligodendroglia or by differences between the two distinct tissue regions' repair capacity. In the white matter tracts, new oligodendroglial cells may invade the injured tissue by migration from the subventricular zone and compensate cell numbers locally at the site of injury. Such mechanisms have been described by Fagel and co-workers [95] for the white matter and frontal cortex . According to my findings, this mechanism of repair does not seem to take place in the hippocampus.

With regard to the maturational process, exposure to high oxygen delayed the expansion of the mature oligodendroglial cell population both in the hilus and in the DG of the hippocampus. In control rats, there is a marked increase of CC1+ mature oligodendrocytes in the hilus from P7 to P9 and furthermore to P11, which is in corresponds with the maturation progress at those developmental ages. However, the growth of the oligodendrocyte population did not occur to a similar magnitude in rats previously exposed to hyperoxia. Instead, there were fewer CC1+ cells at all ages as compare to control litters. In the DG of controls, the picture was different from that in the hilus, with high numbers of mature oligodendrocytes being present at P7 already without a marked increase at the later ages. This observation in controls points towards

a variable pace of oligodendroglial maturation in these two subregions of the hippocampus. Hyperoxic rats showed diminished oligodendrocytes in the DG, too, but consistent with DG oligodendrocytes being more mature, the difference between them and the controls seemed to be less pronounced than in the hilus. This is consistent with previous finding in the white matter, in which hyperoxia decreased expression of CC1+ cells and of the myelin compound myelin basic protein [33, 96]. Votter and co-workers also described that exposure to 60% oxygen for up to 5 days significantly delayed myelination and caused oligodendroglial dysmaturation in the immature white matter [73]. As reported elsewhere [97], MBP-immunoreactive oligodendrocytes in the DG were visible both in the hilus and in the molecular layer, but myelin synthesis in the granule cell layer was apparently appearing at later time points than that in the hilus and the molecular layer. These observations suggest that oligodendrocytes get mature earlier in hilus than in granule cell layer. Such that in our study, there were more mature oligodendrocytes in the hilus than in the granule cell layer at the same postnatal age. At P7 in the hilus most of oligodendroglia cells had matured, but in granule cell layer they still continued to grow.

Some specific transcription factors such as the high-mobility-group protein Sox10 or the basic helix-loop-helix proteins Olig2 have already been reported to regulate oligodendrocyte development [51, 82]. In our study of the immature brain, hyperoxic injury was detected by Sox10 expression showing diminished levels at all ages investigated, i.e. P7, P9, and P11. The expression of Olig2 in rats subjected to hyperoxia was also slightly reduced at P9. These results correlate to the aforementioned cellular changes, which could be interpreted as a regulatory deficiency in developing oligodendroglia. Recent molecular and genetic studies demonstrated that Sox10 and Olig2 play distinct roles in OL lineage progression. *In vivo*, ectopic expression of Olig2 and Sox10 was sufficient for maturation of oligodendrocytes and myelin gene expression in the developing spinal cord [98]. The absence of *Sox10* activity dramatically delayed oligodendrocyte differentiation in the spinal cord [99]. Disruption of Olig2 expression caused failure to generate new cells of the oligodendroglia lineage [100] while overexpression of Olig2 induced the differentiation of neural stem cells into mature oligodendrocytes [101].

Oligodendrocyte lineage development is a complex and vulnerable process that also occurs in close relation to neuronal differentiation and axon development. The data on

hyperoxia in the immature brain confirm that oxygen toxicity impairs genesis and maturation of oligodendrocytes based on cell proliferation, transcription factor expression, and differentiation of OPCs to mature oligodendrocytes.

4.3 Oxidative damage and apoptosis

The developing brain is particularly susceptible to injury caused by oxidative stress. There is growing evidence that oxidative stress is involved in the pathogenesis of developing brain injuries, such as infection/inflammation, hyperoxia, hypoxia-ischemia or epilepsy [102]. There is close interaction between the production of reactive oxygen species and cellular biomolecules, in particular, lipids, proteins, and DNA. 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) is commonly used as a marker of oxidative stress-derived DNA damage. It reflects DNA repair enzyme activity and local antioxidant capacity [103, 104]. In the experiments of this thesis, hyperoxia markedly increased the intensity of 8-oxo-dG positive cells by almost 50% in the hippocampus. In recent years, hyperoxia has been well described as a powerful trigger for neural cell death associated with oxidative stress in the immature brain, because apoptotic pathways can be mediated by intrinsic stress signals, such as DNA damage and oxidative stress [105, 106]. Short exposures to non-physiologic oxygen levels changed the balance of the ROS-dependent thioredoxin/peroxiredoxin system in the developing rat brain [31]. Moreover, accumulated oxidative stress induced by hyperoxia was also defected through an increase of oxidized glutathione and of malondialdehyde, and breakdown of lipids [29].

Microglia are the resident phagocytic cells in the central nervous system which perform many diverse functions which are involved in brain development, maintenance of the neural environment, injury, and repair. Microglia, which commonly maintain a ramified quiescent state in the mature brain, represent 20% of all glia in the brain. In addition to providing a toxic stimulus, microglia activate themselves to fully active macrophages. The most common active forms in the developing brain are intermediate and amoeboid morphologies. Microglial activation induced by external stimuli in the immature brain have been reported to play a pivotal role in various CNS regions, including cerebellum, hippocampus and retina [107, 108]. In a postnatal rat injury model using hypoxia-ischemia, the peak of microglial response was found in the ipsilateral hippocampus of injury [109]. In a rat model for asphyxia with cardiac arrest, activated microglia and pro-

inflammatory cytokine release were increased in the hippocampus [108]. After exposure to lipopolysaccharide to mimic perinatal infection/inflammation, marked microglial inflammatory responses were observed, including release of pro-inflammatory cytokines [110, 111]. Depending on the duration of exposure, hyperoxia may cause either pronounced microglial activation in the white matter including an increase of cell numbers in the tissue [112], or only partial responses with induction of only a few inflammatory markers [113]. Injury of the immature brain is often accompanied by microglial activation which can either contributed to neural damage or mediate protective actions by anti-inflammatory responses. However, in my studies, staining intensity and morphology of microglia in Iba1 immunohistochemistry were not altered in the hippocampus of newborn rats exposed to hyperoxia. Numbers of microglia were not increased by hyperoxia either. Hence, it is not possible to determine whether microglia paly a detrimental or a benefical role in the context of my study in the hippocampus. It should rather be assumed that microglia do not contribute to hippocampal injury caused by hyperoxia. This is in contrast to the report by Ferrazzano et al, according to which inhibition of microglia in juvenile brains may be less effective or possibly worsen the outcome after HI [109]. A recent study about acute neonatal focal stroke indicated that they exert a protective effect by endogenous defense mechanisms [114].

Even though in several studies it was described that hyperoxia induced apoptotic cell death in the developing brain, in my study, the numbers of Tunel positive cells in hyperoxia rats are very low in both DG and hilus. In comparison to control litters in room air, there was no increase found in apoptotic cell numbers in the hippocampus of hyperoxic animals**.** Similar results were found in a rat model applying 95% oxygen in newborn rats in which accumulated oxidative stress during early development eventually induced increased apoptosis in basal forebrain and striatum, but unfortunately not in hippocampus [115]. This can be interpreted to mean that neural cells in the immature hippocampus have a lower apoptotic susceptibility (or a higher anti-apoptotic activity) compared to other brain regions.

Our results underline the remarkable differences in the immature hippocampus compared to other brain regions with regard to mechanisms involved in the injury during and after exposure to hyperoxia. In a former study in hyperoxia in rodents, increased apoptosis of neural cells and/or microglial activation have been identified to occur as a mechanism of injury in white matter tracts, cortex, and basal ganglia [27, 33, 57, 73]. In

contrast, based on this study, these destructive responses are unlikely to contribute to injury of the hippocampus, whereas oxidative stress seems to be the predominant cause of cell toxicity resulting in low proliferation and delayed maturation. A higher functional resistance of the postnatal hippocampus compared to other regions might in fact be also reflected by the clinical MRI studies in former preterm infants in which no significant or only minor correlation of hippocampal volumes with memory and learning performance were reported [116, 117].

4.4 Minocycline

Minocycline, a tetracycline derivative, is well known to exert neuroprotective effects unrelated to its antimicrobial action in developing brain. I examined the protective effects of low dose minocycline administration in neuronal and oligodendroglial genesis on postnatal immature hippocampus in response to 24h exposure to 80% oxygen. Treatment with minocycline successfully restored the number of proliferating neurons in the hippocampus and blocked the downregulation of neuronal marker expression even after 4 days recovery after hyperoxia. OPC proliferation and oligodendroglial maturation as well as transcription factors were preserved at control levels in those hyperoxic rats that received minocycline. Hence, minocycline had a protective effect on the development of oligodendroglial lineage cells of the hippocampus in the immature brain. The observed beneficial effects may be based on the anti-oxidant properties of minocycline, since there was a significant recovery of 8-oxo-dG intensity in the hippocampus hyperoxic rats with minocycline.

Minocycline has been reported to exert a variety of non-antibiotic properties, including regulation of immune cell activation and proliferation, anti-oxidant activity, inhibition of apoptosis and so on. The effects of minocycline on proliferation have been shown for various cell types. In adult animal models, the capacity of minocycline to inhibit proliferation of endothelial cells, microglia, and lymphocytes has been documented [35, 63-65, 67, 118]. In the newborn rodent brain, many reports have also supported a protective role in neurons and oligodendroglia. For example, minocycline administration during the first postnatal week increased microglial proliferation and modified the phenotype of microglia into thicker and shorter processes and a larger some in cortical layer [119]. Furthermore, in a hypoxia-ischemia rat pup model, minocycline significantly ameliorated the loss of mature oligodendrocytes and dopaminergic neurons [15]. In my

studies presented here, minocycline at 45mg dose caused a cytoprotection in oligodendroglial cells and neuronal proliferation in the injured hippocampus after hyperoxia exposure. Mechanistically, the protective activity of minocycline can be due to a direct interaction of mitochondria including voltage-dependent anion-selective channel [120]. Besides, PARP-1 inhibitor, which is an enzyme that partially regulates cell cycle progression, provides a potential mechanistic relation between minocycline and the regulation of proliferation [32, 59]. In addition, minocycline may also have antioxidant functions. Minocycline revealed direct anti-oxidant properties with radical-scavenging potency due to the chemical structure, multi-substituted phenol ring, similar to Vitamin E.

In studies on behavioral outcome after neonatal injury, minocycline improved neurodevelopmental performance after neonatal HI in rats. More specifically, minocycline administration ameliorated HI-induced injury on neurobehavioral performance in sensorimotor and locomotor function, anxiety and cognitive ability in P21 rats [15]. However, there are no studies available yet that would substantiate the benefits of minocycline on neurodevelopmental performance into adulthood. However, long term impairment of white matter diffusivity in MRI/DTI in P30 and P60 animals after neonatal hyperoxia was attenuated by minocycline [113].

According to the results obtained in my studies, minocycline is a useful drug to further identify and characterize the protective pathways that need to be activated to improve neuronal and oligodendroglial development after postnatal injury.

4.5. Summary

The core of my findings presented in this work is that minocycline as a pleiotropic neuroprotective agent is effective in markedly attenuating or even blocking hyperoxia-induced injury in the immature hippocampus by its anti-oxidant capacities.

Our analysis shows that exposure to 24h 80% hyperoxia during the critical development of 6 day old rat pups significantly decreased the expression of immature neuronal marker and neuronal proliferation. Neuronal factors crucial for neurogenesis and neuronal development were downregulated by hyperoxia. The hyperoxia-induced damage to oligodendroglia was found by decreased numbers of proliferating oligodendroglia and by downregulation of transcription factors that are related to

oligodendroglial development. DNA damage induced by higher levels of oxidative stress induced was detectable by increased levels of 8-oxodG.

Remarkably, low dose of minocycline administration attenuated or inhibited all of these toxic effects induced by hyperoxia. Protection provided by minocycline in cellular oxidative stress target might be an important cue for understanding the anti-oxidative mechanism of minocycline. Based on these data of the hyperoxia model, minocycline as a drug seems to be a promising agent for neuroprotective strategies in preterm infants.

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Affidavit

“I, Yuwei Zhao, certify under penalty of perjury by my own signature that I have submitted the thesis on the topic “Damage in the immature hippocampus caused by hyperoxia and its prevention by minocycline”. I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated.The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My interest in any publications to this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date Signature

My curriculum vitae is not published for privacy reasons in the electronic version of my thesis.

Publication

At present, there is no publication for this study.

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