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Proteome changes associated with developmental defects following perinatally acquired brain damage

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Dr. med. Angela Maria Kaindl
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Dekan: Prof. Dr. med. Annette Grüters-Kieslich
1. Gutachter: Prof. Dr. Eugen Boltshauser
2. Gutachter: Prof. Dr. Cobi Heijnen

Science is organized knowledge.

Herbert Spencer

to my parents

and to Robert

Abstract

Injuries to the developing brain contribute considerably to the mortality and the long-term neurologic and neurocognitive morbidity of children. The immature brain reacts differently from the mature brain when exposed to potentially damaging environmental factors or insults. During the so-called brain growth spurt phase, a transient period when the brain is growing most rapidly, several insults may induce widespread apoptotic neurodegeneration in infant rodent brains. It is intruiging to decipher whether, depending upon their timing, lesions of the immature brain carry the potential of influencing developmental events in their natural sequence and redirecting subsequent development.

The aim of the presented research work was to characterize mechanisms that can potentially influence normal development and/or participate in reorganization events following an insult to the developing brain. We have disclosed mechanisms implemented in brain damage and repair following exogenous insults during critical phases of brain development and following treatment with neuroprotective drugs such as erythropoietin. Insults such as hyperoxia/oxidative stress, traumatic brain injury, and an exposure to substances influencing NMDA and/or $GABA_A$ receptor neurotransmission (antiepileptic drugs, sedatives, drugs of abuse) have the potential of inducing an imbalance between neuroprotective and neurodestructive mechanisms that may subsequently cause apoptotic cell death and decrease neurogenesis. In animal models of these conditions, brain proteome changes were characterized at various developmental stages and time points following brain injuries using two-dimensional gel electrophoresis (2-DE) coupled to mass spectrometry (MS) and combined with other histological and biochemical methods. Age-specific acute and long-term changes in brain proteins associated not only with apoptosis and cell proliferation but also with synaptic function, neuronal migration, cell differentiation, and thus neuronal network formation suggest an interference of the exogenous insults with these developmental processes. Treatment with recombinant erythropoietin (rEpo) was found to be neuroprotective following hyperoxia in infant rodents and to lead to a restoration of proinflammatory cytokine and neurotrophin levels, reduction of oxidative stress, and restoration of proteome changes observed when only hyperoxia was applied. The protective effect of rEpo is generated through mechanisms such as a reduction of oxidative stress as well as restoration/reversal of hyperoxia-induced activation of caspases and depression of neurotrophin levels.

We have further addressed whether alterations of the same isoprotein exist in different disease entities. Nodal point proteins may integrate different pathways into one pathway in diseases of the central nervous system, but can also diverge one pathway into several others. This observation offers an explanation for the clinical heterogeneity and phenotype overlap of distinct disorders. Moreover, changes in proteins at central network positions can induce unspecific dysregulations of disease-unrelated proteins. This protein-network concept provides a way to explain the difficulties that arise in an attempt to elucidate the fundamental mechanism underlying a specific developmental disorder.

Zusammenfassung

Erworbene Schädigungen des sich entwickelnden Gehirns tragen beträchtlich zur Mortalität sowie neurologischen und neurokognitiven Morbidität von Kindern bei. Dabei unterscheidet sich die Reaktion des unreifen Gehirns auf exogene Noxen und Erkrankungen von derjenigen des reifen Gehirns. Exogene Faktoren können im unreifen Gehirn während der Phase des raschen Gehirnwachstums („growth spurt phase“), eine extensive apoptotische Neurodegeneration auslösen. Abhängig vom Zeitpunkt des Einwirkens, können Insulte Entwicklungsprozesse akut und chronisch beeinflussen. Das Verständnis der Pathomechanimen und damit die Identifizierung von Angriffspunkten für adjuvante neuroprotektive Therapien ist entscheidend für den Schutz des unreifen Gehirns.

Das Ziel der vorgestellten experimentellen Arbeiten war die Charakterisierung von Proteinen und Stoffwechselwegen, welche die physiologische Gehirnentwicklung beeinflussen können und/oder an Reparaturprozessen erworbener Schäden des sich entwickelnden Gehirns beteiligt sind. Wir fanden Mechanismen, welche potentiell an der Schädigung des unreifen Gehirns und an nachfolgenden Reorganisationsprozessen sowie an der protektiven Wirkung von Substanzen wie Erythropoietin beteiligt sein können. Programmierter Zelltod oder Apoptose wird im sich entwickelnden Gehirn nicht nur physiologisch zur Reduktion überschüssig angelegter neuronaler Zellen beobachtet, sondern ist auch der vorwiegende Modus des Zelltodes durch eine Imbalance zwischen neuroprotektiven und -destruktiven Mechanismen nach Hyperoxie/oxidativem Stress, mechanischem Hirntrauma und Pharmaka, welche die physiologische Neurotransmission über NMDA- oder $GABA_A$-Rezeptoren verändern. In entsprechenden Tiermodellen wurden Proteomveränderungen im unreifen murinen Gehirn zu unterschiedlichen Entwicklungsphasen und Zeitpunkten nach erfolgter Hirnschädigung charakterisiert. Hierzu kamen die Zweidimensionale Gelelektrophorese (2-DE) in Verbindung mit der Massenspektrometrie (MS) und weitere histologische und biochemische Methoden zum Einsatz. Altersabhängige akute und langfristige Veränderungen des Gehirnproteoms, welche mit Apoptose, Zellproliferation, synaptischer Funktion, neuronaler Migration, Zelldifferenzierung und somit Netzwerkbildung in Verbindung stehen, deuten auf eine Beeinflussung dieser Prozesse durch Insulte.

Wir fanden zudem die neuroprotektive Wirkung rekombinanten Erythropoietins (rEpo) bei hyperoxie-induzierter Hirnschädigung. Dabei führt rEpo nicht nur zu einer deutlichen

Reduktion hyperoxie-induzierter Neurodegeneration, sondern inhibiert auch die meisten Veränderungen des Gehirnproteoms, die nach Hyperoxie auftraten. Wir fanden Mechanismen, die der neuroprotektiven Wirkung zugrunde liegen könnten, wie eine Reduktion oxidativen Stresses, eine Normalisierung hyperoxie-induzierter vermehrter Caspaseaktivierung sowie eine Erniedrigung der Neurotrophinkonzentrationen im Gehirn.

Beim Studium verschiedener Erkrankungen wurden oftmals Veränderungen bei denselben Proteinen beobachtet. Wir beschrieben diesbezüglich Knotenpunktproteine, die in Proteinnetzwerken auftreten und sowohl verschiedene Stoffwechselwege in einen zuammenführen als auch einen Stoffwechselweg in mehrere aufteilen können. Diese Beobachtung bietet eine Erklärung für die klinische Heterogenität und für die Überlappung von Phänotypen unterschiedlicher Krankheitsentitäten. Darüber hinaus können Veränderungen von Proteinen in zentralen Netzwerkpositionen unspezifische Veränderungen an Proteinen hervorrufen, die mit einer Erkrankung nicht ursächlich in Verbindung stehen. Dies kann die Schwierigkeiten bei der Identifizierung fundamentaler Mechanismen erklären, die bestimmten Krankheiten zugrunde liegen.

List of Abbreviations

AED	antiepileptic drug
AKT	protein kinase B
APV	2*R*-amino-5-phosphonovaleric acid
ARNP	alcohol related neurodevelopmental disorder
CA	cornu ammonis (hippocampal region)
CNS	central nervous system
2-DE	two-dimensional gel electrophoresis
E	embryonal day
ERK	extracellular signal-regulated kinase
FAE	fetal alcohol effects
FAS	fetal alcohol syndrome
FASD	fetal alcohol spectrum disorder
$GABA_A$	gamma-aminobutyric acid type A
IEF	isoelectric focusing
IL	Interleukin
IL-18BP	interleukin-18 binding protein
IME	intramyelinic edema
IQ	intelligence quotient
IRAK	interleukin-1 receptor-associated kinase
JNK	c-jun terminal kinase
MAPK	mitogen-activated protein kinase
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MS	mass spectrometry
Mw	molecular weight
NMDA	N-methyl-D-aspartatic acid
P	postnatal day
PCP	Phencyclidine
pI	isoelectric point
PI3K	phosphatidylinositol-3 kinase
R	Receptor
Ras	rat sarcoma
rEpo	recombinant erythropoietin
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBI	traumatic brain injury

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1 Introduction

Perinatal brain injury contributes considerably to mortality and long-term morbidity of term and preterm infants.[1] In Germany, perinatally acquired insults cause severe injury to the central nervous system (CNS) in approximately 1,000 children each year.[2]

1.1 Vulnerability of the developing brain

The developing brain reacts differently from the mature brain when exposed to potentially damaging environment factors or insults.[3-8] Short- and long-term deleterious effects resulting from an interference with normal brain development differ in their extent and quality depending on the nature, the timing, and the extent of the insult.[9] In the past decades, studies in rodents have provided substantial information on brain development.[9-14] Although there are variations in the rates of brain growth among mammals, a comparison of brain development between species is possible.[14,15] The developmental ages of human and rat embryos or fetuses are comparable when anatomical features and histological landmarks are similar in appearance in the two species, even though their exact chronological ages are different.[14] In the CNS, structures are created by cell proliferation, migration, and differentiation as well as the establishment of intercellular connections, i.e. the formation of networks.[9] Normal function requires a specific number of cells with the proper characteristics in the correct location at a specific time.[9] The cycle of neurogenesis of individual neuronal populations has been determined through autoradiography in rodent brain, and extrapolations have been made to the human brain.[9,14] These studies clearly indicate that different brain areas develop at different times during gestation, and within a single brain region, subpopulations of neurons develop at different rates and times. Cerebellar Purkinje cells, for example, develop early (embryonic days 13–15 in the rat, corresponding to gestational weeks 5–7 in humans), whereas granule cells are generated much later (postnatal days 4–19 in the rat, corresponding to gestational weeks 24–40 in humans).[14] Many agents, such as irradiation by x-rays, cause brain damage by interference with cell proliferation, and if the insult occurs during the stage of formation of a certain neuronal subpopulation, the involved cells may not develop.

Neurogenesis produces about twice as many neurons in a given structure as the number of neurons that survive in the adult organism. This initial excess of neurons is short lasting and followed by a process known as apoptosis or programmed cell death.[16] Apoptosis is regulated by growth factors and cytokines as well as by neurotransmitters and is executed by a number of intracellular proteins.[4,5,17,18] Any compound that interferes with these

processes may trigger apoptotic degeneration of neurons that would not have otherwise been deleted from the developing brain, or may, in contrast, promote survival of unnecessary cells.[19] The developing brain is especially vulnerable during a transient period when the brain is growing most rapidly, the so-called brain growth spurt.[11] This period occurs in the first two postnatal weeks in the rat and in the third trimester of gestation and first two years of life in humans.[12]

Cell migration, by which neurons reach their final location, is another important process during brain development. It is indispensable for the construction of complex circuits, and any interference with cell migration can have deleterious effects on the developing brain.[10] During development, migration occurs in waves associated with different cell types.[20] Neurons must subsequently form connections during the process of synaptogenesis. This developmental period is critical for the formation of the basic circuitry of the nervous system, although neurons are able to form new synapses throughout life.[20] Furthermore, evidence exists that neurotransmitters can modulate proliferation of neural stem cells, neuroblasts, and glioblasts, regulate migration, and induce differentiation.[21-24] Thus, pharmacological agents that interfere with neurotransmission during development may cause permanent defects in the central nervous system.

Pruning, defined as a loss of synapses, also occurs physiologically in the developing brain.[19] Such trimming of connections is a more extensive process than cell death and occurs late in childhood and adolescence.[19] Any interference with this process would be expected to affect the number of synaptic connections. Most of the developmental processes discussed so far have focused on neurons. However, it is well established that glial cells (astrocytes, oligodendrocytes, microglia) play a relevant role in brain function as well as in brain development. The vulnerability of the developing brain can thus also be attributed to these cells.

1.2 Causes of perinatal and infant brain damage

Causes of perinatal brain damage differ between term and preterm infants.[8,25,26] In term infants, perinatal asphyxia resulting in hypoxic-ischemic cerebral injury is the leading cause of infant mortality and neurologic morbidity such as cerebral palsy, mental retardation, visual impairment, and epilepsy.[1] In contrast, survivors of premature birth at 23-32 weeks of gestation have a predilection for perinatal brain injury especially to their

periventricular cerebral white matter.[8,27] Periventricular white matter injury can be caused by a wide spectrum of impacts including perinatal asphyxia (hypoxia-ischemia), hyperoxia, hemorrhage, trauma, infections, metabolic diseases, and intoxications.[28] Moreover, iatrogenic factors such as drug therapy can lead to infant brain damage. In the following, important causes of early childhood brain damage that are also the topic of the presented experimental work towards habilitation will be described.

1.2.1 Preterm infants and oxygen

The rate of preterm birth has risen in most industrialized coutries to 5-9% in Europe and up to 12-13% in the United States.[29] Although most preterm infants now survive the newborn period, they still have an increased risk of neurodevelopmental impairment accounting for about 50% of all children with early childhood brain damage.[29-31] There is often neither an obvious clinical explanation nor a clearly correlating finding in conventional ultrasound imaging studies for the neuropsychologic morbidity of formerly preterm infants.[32,33] In several cases, magnetic resonance imaging (MRI) modalities applied early in postnatal life of preterm infants revealed diffuse, multifocal lesions that did not develop into cystic periventricular leukomalacia later on.[34] Moreover, reduced brain volumes and delayed postnatal development of brain structures were found.[32,34-44] This developmental delay may be attributable to environmental pertubations associated with preterm birth.

Due to lung and brain stem immaturity, the preterm infant is exposed to fluctuating oxygen concentrations in the course of the early extrauterine development. These newborns exhibit a developmental immaturity of their free radical defences, and they are also inevitably exposed to relative hyperoxia compared to intrauterine hypoxic conditions (with an oxygen partial pressure of 25 mm Hg) and more likely encounter further situations of increased oxidative stress such as oxygen supplementation or systemic infections. Hypoxic situations as well as relative hyperoxia play a significant role in the genesis of brain damage.[45-49] Moreover, hypoxic insults often lead to cerebral hemorrhage in preterms, which significantly worsens the prognosis for these children.[1]

1.2.2 Mechanical trauma to the brain

Traumatic brain injury (TBI) is a major cause of long-term morbidity and mortality in the industrialized world.[50-54] The overall incidence of TBI is as high as 1.5 million affected per year in the United States, and 6% of the patients suffer from long-term disability.[50-54] In

Germany, approximately 100,000 children received in-patient care following head trauma in 2005 according to the annual report of the "Statistisches Bundesamt Deutschland". Children under the age of 6 years not only sustain TBI more frequently than any other age group,[55] but those under the age of 4 years also show the worst neurologic outcomes.[55-58] Even mild injuries may result in long-term morbidity in preschool children.[59,60] While mechanical brain injuries in the course of birth, often associated with asphyxia, and child abuse are frequent causes of TBI in newborns and infants, accidents (especially traffic accidents) are the number one cause for TBI in toddlers and young school children.[1,61-64]

1.2.3 Antiepileptic and sedative medications

Antiepileptic drugs (AEDs) or sedative medication taken by mothers during pregnancy are among the most common causes of fetal malformations.[65-69] Major malformations in the offsprings of mothers treated with AEDs during early pregnancy occur in about 3.5% of live births in comparison with 0.8% of live births in untreated cohorts.[69,70] These include neural tube defects, orofacial clefts and digital anomalies, growth retardation, developmental delay, and microcephaly.[65-67,71-73] Teratogenic effects have been associated with the use of phenytoin, carbamazepine, valproate, and phenobarbital. High maternal blood levels and combinations of several AEDs impose an increased risk of harm to human infants.[73] Moreover, it has been shown that AEDs may have adverse effects on the human intellect when given to treat seizures in pregnant women, infants, and toddlers. Long-lasting neurobehavioral effects, such as impaired cognitive development[74] and lower intelligence quotient (IQ) scores,[75] have been reported in humans following *in utero* exposure to **phenobarbital**. Despite the developmental neurotoxicity of phenobarbital, its embryotoxicity and teratogenic effects appear to be less than that of other anticonvulsants in animal models.[67] **Valproic acid**, on the other hand, is a clear human and animal teratogen.[76] Neural tube defects, specifically spina bifida, occur at a high rate upon *in utero* exposure to this compound.[77] Similar to valproic acid, *in utero* exposure to **carbamazepine** has been associated with an increased risk of spina bifida.[66,78] The pattern of other carbamazepine-associated malformations (facial dysmorphic features, microcephaly, growth retardation) resembles that of other anticonvulsants.[66,79] There is evidence that **phenytoin** is a developmental toxicant in humans: the fetal hydantoin syndrome is characterized by facial dysmorphologies, growth retardation, and other anomalies.[80,81] Microcephaly, learning disabilities, and decreased IQ scores have also been reported in this context.[82-84] Other AEDs or sedative drugs have also been found to be

teratogenic in humans.[85] For some of the newer drugs, adequate studies of their effects on the developing brain do not exist.[85]

The majority of studies, in which the effects of prenatal antiepileptic drug exposure were investigated, were performed in children under the age of 5 years. In many studies a trend towards lower developmental scores was reported; however, in a few studies no adverse effects could be found. In a longitudinal study, children exposed to carbamazepine and phenytoin had lower developmental and language scores compared to controls. In children receiving carbamazepine, a deficit in language development was not evident until an age beyond 3 years, suggesting that specific cognitive deficits may become apparent only when the child is older.[86]

Inconsistent results have also been obtained in studies that have investigated the long-term effects of *in utero* AED exposure on children older than 5 years.[84,87-90] Some studies have reported specific cognitive deficits in visuo-spatial functioning,[74,91] spelling as well as linguistic abilities.[86,92,93] Intrauterine exposure to phenytoin, phenobarbital, valproate, and carbamazepine is associated with lower intellectual functioning.[92-98] Carbamazepine appears to be the least developmentally neurotoxic compound among the major AEDs. Although in one study mild mental retardation has been reported in children exposed *in utero* to carbamazepine,[99] no neurologic or IQ differences were found by other investigators.[74,92] In humans, *in utero* exposure to valproic acid has been associated with developmental delay, mental retardation, cognitive impairment, and other behavioral deficits.[100,101] In a recent retrospective study exploring neuropsychological effects of exposure to anticonvulsant medication *in utero*, Vinten and coworkers reported that an *in utero* exposure to valproate was harmful to the later neuropsychological development. Children exposed to valproate had a significantly lower IQ when compared to children exposed to other AEDs or those not treated at all. The same children were more likely to have an IQ below 69 and more likely to have memory impairment when compared to other groups.[102]

In addition, postnatal exposure to AEDs during the first years of life may be harmful for cognitive development. In several studies it was shown that therapy with barbiturates during the first three years of life may cause cognitive impairment that persists into adulthood.[75,103-105] Adverse effects of AEDs have also been demonstrated in animal studies; reviewed in Kaindl *et al.* 2006.[85]

1.2.4 Drug abuse of pregnant women

The abuse of drugs such as alcohol, marihuana, cocain, ketamine ("special K") and phencyclidine ("angel dust") during pregnancy is a common cause of brain damage in the offspring.

Alcohol is the most widely abused drug in the world.[106] In two studies published in 2003, 4% and 14.8%, respectively, of all women interviewed confessed to alcohol abuse during pregnancy.[107,108] Transplacental exposure of the immature brain to ethanol can cause craniofacial anomalies, microcephaly, mental retardation, and neurobehavioral disturbances ranging from hyperactivity/attention deficit and learning disabilities to depression and psychosis.[109-112] While the distinctive clinical picture originally described by Jones and Smith (1973, 1975) is a neurotoxic syndrome referred to as the fetal alcohol syndrome (FAS), the fetotoxic effects of ethanol can also manifest themselves as a partial syndrome comprised largely of neurobehavioral disturbances unaccompanied by craniofacial malformations. [109,110,113,114] The latter are referred to as fetal alcohol effects (FAE) or alcohol related neurodevelopmental disorder (ARND).[109,110,113,114] A new term currently emerging to represent all clinicopathological manifestations of ethanol's fetotoxic effects is fetal alcohol spectrum disorder (FASD).[115] The prevalence of FASD is estimated to be approximately 1.5 per 1000 live births and that of FAE around 9 per 1,000 live births.[106,116]

„Angel dust", „special K", and marihuana can cause fetal developmental defects that can still be detected in juvenile age.[117-119] In the United States, 2.8% of pregnant women reported that they used illicit drugs such as marihuana, and 54% of these additionally consumed alcohol.[108] Adverse effects of these drugs have been demonstrated in animal studies; reviewed in Kaindl and Ikonomidou 2007.[120]

1.3 Model systems of perinatal brain damage

Multiple experimental and clinical approaches exist which allow for a study of the pathomechanisms underlying damage to the developing brain. In neuroscience, experimental models are used predominantly, since the possibilities to address clinical topics is naturally limited in children based on our ethic values. In the past several decades, studies in rodents have provided substantial information on brain development and have become indispensable for research groups focussing on developmental brain injury. [9-14,121] This has been enabled through extensive descriptions and comparisons of brain development between species.[14,15,121] The developmental ages of human and rat embryos or

fetuses are comparable when anatomical features and histological landmarks are similar in appearance in the two species, even though their exact chronological ages are different.[14]

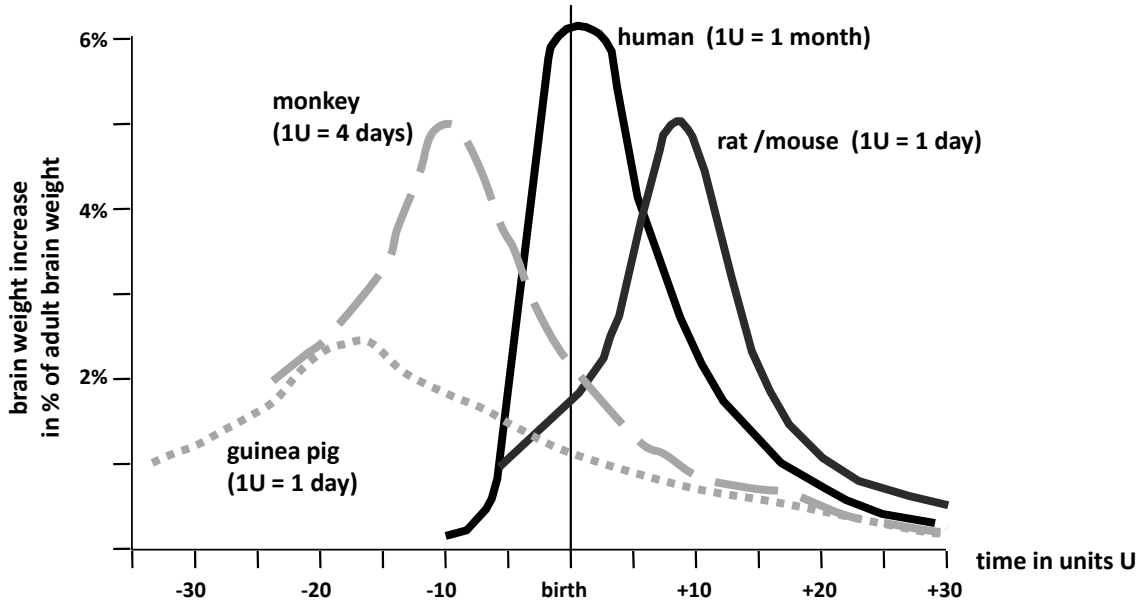

Figure 1. Brain growth spurt phase in various mammalian species. *The developing brain experiences a period of rapid growth (growth spurt phase) during which various otherwise innocious environmental factors cause widespread apoptotic neuronal death. The brain growth spurt starts at about midpregnancy in humans and extends well into the third postnatal year. In mice and rats, this developmental period occurs within the first three postnatal weeks. In contrast, monkeys, and guinea pigs are predominantly prenatal brain developers. Modified figure from Dobbing and Sands, 1973.*[11]

The developing mammalian brain undergoes a period of rapid growth, the so-called brain growth spurt period (Figure 1).[11] During this period, trillions of synaptic connections are formed while unsuccessful neurons are eliminated by instigation of regulated (or programmed) cellular events that lead to the demise and removal of these immature neurons in a very coordinated event, a process designated apoptosis. Different species run through this phase at different time periods. In the rat, the brain growth spurt period takes place within the first three postnatal weeks of life. The comparable period in humans starts in the sixth month of pregnancy, peaks around birth, and ends at the beginning of the third

year of life. Thus, several phenomena that take place in the developing human brain during the brain growth spurt period can be modeled by studying the developing rat brain during the first three postnatal weeks of life. In the following research work, we studied perinatal brain damage inflicted upon 7-day-old (P7) rats and mice, which are a good model for human infants when considering cross-species extrapolation with regard to the timing of the main growth spurt period. For a detailed description of rodent models for (i) hyperoxia, (ii) traumatic brain injury (TBI), and (iii) alteration of N-methyl-D-aspartic acid (NMDA) and/or gamma-aminobutyric acid type A ($GABA_A$) receptor neurotransmission, the reader is referred to the material and methods sections of individual research papers presented in chapter 3.

1.4 Mechanisms that lead to perinatal brain damage

In recent years, we have learned about silent triggers of cell death in the developing brain. Oxygen has been identified as a neurotoxic agent.[7,47-49] Moreover, it has been reported that compounds used as sedatives,[4,5] anesthetics,[122] or anticonvulsants[123] in neonatal intensive care units, which alter physiologic synaptic activity, such as antagonists at NMDA receptors (ketamine, nitric oxide), agonists at $GABA_A$ receptors (barbiturates, benzodiazepines, anesthetics), and sodium channel blockers (phenytoin, valproate), can cause massive apoptotic neurodegeneration in infant rats and mice. Similarly, traumatic brain injury, which affects the immature brain in a much more drastic way than the mature brain, induces not only excitotoxic but also massive apoptotic neurodegeneration. In rodents, these neurotoxic effects following various impacts are strictly confined to a developmental period characterized by rapid brain growth and are associated with an alteration of neurotrophins and an inflammatory response. Thus, human infants may be susceptible to and may sustain iatrogenic brain damage from treatments that are considered safe in older patients. Such mechanisms can potentially silently lead to diffuse brain injury in infancy and result in cognitive and/or motor impairment that can become evident later in life.

1.4.1 Hyperoxia

The exposure of infant rodents to high inspiratory concentrations of oxygen (80%) for several hours during the first postnatal weeks causes apoptotic neurodegeneration in their developing brains.[46,47] This cell death is disseminated in the brains and affects cortical areas, the basal ganglia, hypothalamus, hippocampus, and white matter tracts.[47] Vulnerability to oxygen-induced cell death is age dependent and peaks during the first

week of life in the rat.[47] Similarly, hyperoxia has been shown to induce maturation-dependent apoptosis of oligodendrocytes *in vitro*.[124,125] Several mechanisms that are responsible for hyperoxia-induced apoptotic death in the developing rodent brain have been revealed. Hyperoxia-induced cell death is associated with production of reactive oxygen species, an inflammatory response, reduced neurotrophin levels, and decreased activation of neurotrophin-activated pathways *in vivo*.[47,48]

The impairment of survival promoting signals (neurotrophins) and an imbalance between neuroprotective and neurodestructive mechanisms in the brain is likely to promote apoptotic cell death during a developmental period of ongoing physiological elimination of brain cells. Protein levels of active phosphorylated forms of extracellular signal-regulated kinase (ERK1/2) and protein kinase B (AKT) were reduced in the developing rodent brain following hyperoxia.[47] ERK1/2 and AKT are key players in two major survival promoting pathways, the mitogen-activated protein kinase ERK1/2 (MAPK-ERK1/2) and the phosphatidylinositol-3 kinase- (PI3K) AKT pathways, which are activated by tyrosine kinase receptors upon binding of growth factors.[126,127] Rat sarcoma (Ras) activation results from binding of growth factors to the respective receptors and initiates signaling via the MAPK and PI3K pathways. The synRas-transgenic mice, which postnatally overexpress activated Ras in neurons (that counteracts hyperoxia-induced reduction in Ras activity) and display higher levels of phosphorylated ERK1/2 in the cortex, were less susceptible to hyperoxia-induced apoptosis in the brain.[47] Since protection from hyperoxia-induced apoptosis was not complete in synRas-transgenic mice,[47] it is likely that mechanisms other than reduced transcription of growth factors and impairment of the ERK1/2 pathway contribute to hyperoxia-induced apoptotic cell death.

Hyperoxia-triggered apoptosis in the immature rodent brain is further associated with an increase of caspase-1 (interleukin (IL)-1β converting enzyme) and its substrates, the proinflammatory interleukins IL-1β and IL-18 in areas with pronounced apoptotic cell death.[48] These molecular changes are pathogenetically linked to cell death, as inhibition of IL-18 by IL-18 binding protein (IL-18BP) and disruption of the intracellular signaling cascade activated by IL-1β and IL-18 in interleukin-1 receptor-associated kinase (*IRAK-4*) knockout mice confer protection (IRAK-4 is a kinase most proximal to the IL-18 receptor and pivotal for signal transduction of IL-1ß and IL-18).[48] The mechanisms by which IL-1ß and IL-18 are capable of promoting tissue injury are not completely understood. Since both cytokines have been shown to activate c-*jun* terminal kinase (JNK) and p38,[128,129] which

can lead to apoptosis,[130,131] it is conceivable that these two signaling cascades become active and contribute to hyperoxia-induced cell death. Moreover, clinical studies have reported an association between high levels of IL-1β and IL-18 in cord blood of preterm infants and the occurrence of cerebral palsy.[132,133]

The injury caused by exposure to high levels of oxygen in the infant rat and mouse brain does not resemble the "classic large cystic lesions" of other animal models of periventricular leukomalacia; white matter injury is more diffuse.[134] It thereby resembles the lesions detected in premature infants through early-life MRI application.[34] Thus, the clinical data in combination with the experimental evidence presented suggest that apoptotic neurodegeneration triggered by a non-physiological, high oxygen environment (relative or absolute hyperoxia) during a critical stage of development may partly account for cognitive and also motor impairment of premature infants.

1.4.2 Traumatic brain injury

In traumatic brain injury (TBI), the primary damage may result in diffuse axonal injury, intraparenchymal contusions and/or intracranial hematomas.[53] These events are often followed by a secondary cascade of biochemical, cellular and molecular derangements as well as extra-cerebral complications which generate further damage.[7,53] Although similarities in the pathomechanisms triggered by TBI to pediatric and adult brains exist, an injury to a developing brain poses a unique challenge due to the often diffuse pattern of injury, the increased vulnerability of the brain, and ongoing developmental processes.

TBI can trigger two types of neurodegeneration in the developing brain, excitotoxic and apoptotic cell death. Whithin the area of impact, excitotoxic cell death occurs and expands rapidly within approximately 4 hours in rodent brains.[6,7,53,135] Approximately six hours after TBI, this local excitotoxic response is followed by a delayed, but much more extensive disseminated apoptosis in many brain regions ipsi- and contralateral to the trauma site hours after the excitotoxic degeneration has run its course.[6,7,53,135] Trauma-induced cell death is associated with oxidative stress, an activation of the intrinsic and the extrinsic apoptotic pathways as well as an increase in the transcription of neurotrophins.[7,136-141] The severity of apoptotic neurodegeneration following TBI is age-dependent; the magnitude of apoptotic response was highest in 3- and 7-day-old animals and subsequently followed by a rapid decline.[6,7,135-139] The timing of greatest vulnerability to TBI coincides with the peak of the brain growth spurt.[6,7,135-139]

1.4.3 Alteration of NMDA and/or GABAA receptor neurotransmission

Antiepileptic and sedative drugs as well as drugs abused by pregnant mothers interact with ion channels, metabolic enzymes, and neurotransmitter transporters in the brain.[142] In this chapter, the focus will be on mechanisms of developmental brain damage due to an alteration of the $GABA_A$ receptor ($GABA_AR$) and/or the NMDA receptor (NMDAR) neurotransmission, since this was also the subject of the presented research work. $GABA_AR$ agonists include benzodiazepines, barbiturates such as phenobarbital (which also act on calcium and sodium channels), propofol, and ethanol (which is also an NMDAR antagonist).[143-146] Antagonists at NMDARs include felbamate, ketamine, nitrous oxide, and ethanol (also a $GABA_AR$ agonist).[147]

Several mechanisms that explain the neurodevelopmental deficits following pre- and/or postnatal exposure to such drugs have been described. NMDAR antagonists and/or $GABA_AR$ agonists trigger widespread apoptotic neurodegeneration throughout the immature developing brain when administered to rodents during the period of the brain growth spurt between P0 and P14.[4,5,18,123] Drug-induced apoptotic neurodegeneration is associated with reduced levels of neurotrophins and subsequently reduced activation of neurotrophin-activated pathways.[148] SynRas transgenic mice were less susceptible to the proapoptotic effect of the NMDAR antagonist MK801 or the $GABA_AR$ agonist phenobarbital.[127] Such changes, which have also been described in *in vitro* systems following blockade of NMDA-receptors,[149] reflect an imbalance between neuroprotective and neurodestructive mechanisms in the brain of treated mice.

NMDAR antagonists and $GABA_AR$ agonists can not only cause wide-spread apoptotic neurodegeneration in the developing brain, but also influence other developmental processes such as cell proliferation and differentiation, synaptogenesis and synaptic plasticity, neuronal migration, and axonal arborisation and myelination.[85] A disruption of these developmental processes may potentially account for neurological deficits seen in humans exposed to such drugs pre- or postnatally.[85] Unfortunately, the effects of NMDAR antagonists and/or $GABA_AR$ agonists on these processes in the developing brain have not been analyzed systematically.[85]

Cell proliferation and differentiation. Glutamate and GABA neurotransmitter systems are implicated in neuronal proliferation and migration during CNS development. The application of a single dose of diazepam (5 mg/kg) at P11 induced a significant reduction of mitotic activity in rodent cerebral cortex and anterior pituitary gland.[150] Reactive

astrogliosis has previously been observed, along with microglial activation, in rats that were given 250 mg/kg/day vigabatrin, an irreversible inhibitor of the GABA-degrading enzyme GABA transaminase, for a period of eight weeks beginning at age P28.[151]

Synaptogenesis and synaptic plasticity. Concerns have been expressed that neurotransmission modulating drugs may disrupt synaptogenesis and synapse remodelling due to inhibition of excitatory neurotransmission.[152,153] Results of published studies are, however, somewhat contradictory. NMDARs are involved in the refinement of synaptic circuitry and the pruning of synaptic connections during brain development.[154] Pharmacological inhibition of NMDAR activity in 5- or 15-day-old rats for two weeks through intracranial application of 2-amino-5-phosphonovaleric acid (APV; selective, competitive NMDAR antagonist) or phencyclidine (PCP) has been associated with decreased brain weight, cortex layer depth, and total number of synapses.[155] Withdrawal from these NMDAR antagonists led to similar results initially, but was later (P36) displaced by a transitory rebound with increased molecular layer depth and total number of synapses. Chronic exposure of cultured mouse spinal cord neurons to phenobarbital led to reduced cell survival and decreased length and number of dendrite branches.[156,157] The application of NMDAR blockers to hippocampal slice cultures prepared from 6-day-old rats induced a change of spine appearance.[158] Intracerebral infusion of the NMDAR antagonist APV led to a disruption of experience-dependent synaptic modifications in the cortex of immature kittens,[159] and blocking of glutamatergic transmission decreased dendritic filopodial motility *in vitro*.[152] In contrast to these findings, Lüthi *et al*. demonstrated a substantial increase in synapse number in slice cultures prepared from newborn rats, a more complex dendritic arborisation, and an increased density of presynaptic buttons of CA1 and CA3 (CA = Cornu ammonis) pyramidal cells *in vitro* upon pharmacological blockage of NMDAR for 14 days.[154] Similarly, infusion of APV to 14-day-old ferrets led to an increase in the number of branch points and in the density of dendritic spines of the lateral geniculate nuclei compared with control animals.[160]

Neuronal migration and axon arborization. The relationship between neurotransmitter receptor activity and neuronal morphology has been studied in cultured dentate granule neurons from embryonic rat hippocampus. Here, the NMDAR antagonist MK801 blocked branching of neuronal processes.[161] A temporary block of NMDA and non-NMDA glutamate receptors in immature rats also disrupted the topographic refinement of thalamocortical connectivity and columnar organization, i.e. the topographic organization

of synaptic connections.[162]

Myelination. Based on results from animal studies, concerns were raised that prolonged vigabatrin administration may induce intramyelinic edema (IME).[163,164] Treatment of rodents with 30 mg/kg/day vigabatrin was associated with IME changes one year following the initiation of treatment, whereas a dose of 100-300 mg/kg/day triggered IME already within six months.[163] However, a progression of IME-lesions to demyelination has not been reported, and microvacuolation has been described as reversible after cessation of treatment.[163] Monkeys treated with high doses of vigabatrin (300 mg/kg/day) for up to 16 months demonstrated only occasional mild microvacuolation; monkeys treated with 50-100 mg/kg/day for six years showed no pathology.[163] Moreover, the phenomenon of IME has not been documented in humans. Other groups have reported a reduction in myelination in immature rat brains at P20 and P40 following subcutaneous vigabatrin injections of 40 mg/kg/day from P12 to P16 and 25 mg/kg/day from P12 to P26, respectively.[164,165] Similarly, long-lasting myelin abnormalities have been reported following pre- or postnatal administration of phenobarbital to immature mice.[166,167]

1.5 Neuroprotective strategies

Neuroprotection of the developing brain is a healthcare priority, since injury of the immature brain is a leading cause of mortality and long-term morbidity in children.[168] Moreover, adjunctive neuroprotective therapy in preterms is a promising approach, since secondary evolving brain injuries can be substantially larger than the damage caused by the primary insult, and - in an intensive care setting - certain potentially also harmful therapy regimens such as oxygen supplementation and/or an application of neurotransmission-affecting drugs are often inevitable. Neuroprotective strategies include hypothermia, i.e., a reduction in core body temperature to 34 °C.[169] The growth factors erythropoietin and granulocyte-colony stimulating factor have tissue-protective effects in several organs, which are unrelated to their hematopoietic functions.[170] Their recombinant forms are approved for clinical hematopoietic uses in neonatal populations, and long-term prospective clinical trials to analyze their neuroprotective effectiveness in humans have been initiated. Other strategies have been evaluated experimentally in specific models of perinatal brain damage, but have not been replicated in a systematic way in the human

neonate: (i) antioxidant drugs, like xanthine oxidase inhibitors (allopurinol, oxypurinol), inhibitors of hydroxyl radical formation from free iron (deferoxamine); (ii) anti-inflammatory agents, like steroids; (iii) growth factors such as brain derived neurotrophic factor and melatonin; (iv) magnesium sulphate, (v) blockers of apoptotic pathways; (vi) preconditioning; and (vii) stem cell therapy.[47,168,169,171]

1.6 Proteomic strategies to decipher neurodevelopmental diseases

The continous interplay of gene expression products (proteins) with each other and with the environment shapes the ongoing activity and development of the nervous system.[172] To understand why in acquired neurological disease a divergence from the normal developmental sequence occurs, changes of proteins both throughout physiologic CNS development as well as through interaction with the environment need to be analyzed. The availability of powerful instruments of genome, transcriptome, and proteome analysis has enabled new ways of formulating and addressing biological questions. The traditional approach of studying one gene, one mRNA, or protein at a time in hypothesis-driven research projects is now synergistically complemented by a more integrative approach, allowing the study of many genes, mRNAs, and/or proteins simultaneously. Moreover, novel techniques have revolutionized the type of experiments conducted currently, and the concept of protein networks has consolidated.

Investigating proteomes of healthy and diseased tissues enables the identification of molecular changes that potentially underlie disease pathogenesis and reparation processes.[173] The proteome, a term initially shaped by Marc Wilkins and Keith Williams in 1994, refers to the entire protein complement encoded in the genome of a subcellular compartment, a cell, a tissue, or an organism.[174] It is a dynamic system that is constantly subject to change. Protein compositions change from cell to cell type, within subcellular compartments, between different stages of development, and metabolic states. In this respect, they represent the functional status of a biological compartment. Proteome research (proteomics) can be defined as the large-scale characterization of proteins expressed by the genome. Unlike the study of a single protein or pathway, proteomic methods enable a systematic overview of expressed protein profiles. An advantage of proteomics over genomics and transcriptomics is the ability to study co- and post-translational modifications. The knowledge of protein modifications as well as of protein translocation and activity is not simply encoded in gene sequences and cannot be derived from mRNA expression, because of a lack of correlation between transcriptional profiles

and actual protein levels in cells.[175-177] For example, there is limited value in measuring signal transduction processes at the mRNA levels if they are characterized by protein phosphorylation or acetylation. Moreover, there are several genes with little correlation between RNA and protein expression levels.[175-177]

Proteomics employs techniques such as protein electrophoresis, mass spectrometry, and microarrays for the detection, identification, and characterization of proteins. These proteomic tools have their own individual advantages and limitations affecting their ability to assess the protein profile. Since the beginning of proteome research, the technology of two-dimensional polyacrylamide gel electrophoresis (2-DE) has been improved considerably and was supplemented by high sensitive protein detection techniques, image analysis software, mass spectrometry (MS) methods, and database search engines. Moreover, several gel-free high-throughput screening technologies for protein analysis such as multidimensional protein identification technology,[178] yeast two-hybrid and reverse two-hybrid assays,[179] protein microarrays,[180,181] phage-display antibody libraries,[182] and HysTag reagent[183] have been developed. MS techniques have matured rapidly in recent years, due to the invention of two ionization techniques: electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). Protein arrays are being developed involving up to a few hundred antibodies or based on surface-enhanced laser desorption/ionization (SELDI) for a wider coverage of the proteome.

Despite all drawbacks, 2-DE coupled with MS remains the central tool in proteome analysis and the most powerful tool to separate complex protein mixtures and thereby reveal simultaneously thousands of proteins and their co- and posttranslationally modified isoforms (Figure 2). 2-DE combines isoelectric focussing (IEF) in a polyacrylamide gel that has a pH gradient in the first dimension with a separation on an SDS polyacrylamide gel (SDS-PAGE) in the second dimension. The principle of 2-DE is to separate proteins according to two different characteristic parameters, in the first dimension according to the isoelectric point (pI) and in the second dimension according to the molecular weight (Mw). Protein spot patterns are obtained following protein staining procedures, and the proteins giving rise to a certain protein spot can be identified through mass spectrometry and subsequent protein database searches. On a 2-DE gel, a protein can be represented by one spot or constitute a pattern of multiple spots, referred to as isospots that are caused by co- and/or post-translational modifications of the primary protein product or by protein processing. Such modifications can result in alterations of the pI, the Mw, and/or the

conformation of a protein, causing in this way a shift in the position of a spot on a 2-DE gel (mobility variant) and thus a change of a spot intensity, i.e., a decrease in the relative concentration of an unmodified protein (presence/absence or amount variants). Protein isospots that are within close proximity to each other and emerge from one protein are referred to as spot complexes. Spots can also be considered in terms of spot families, which comprise the total number of isospots within a 2-DE protein pattern that were created from one primary protein.

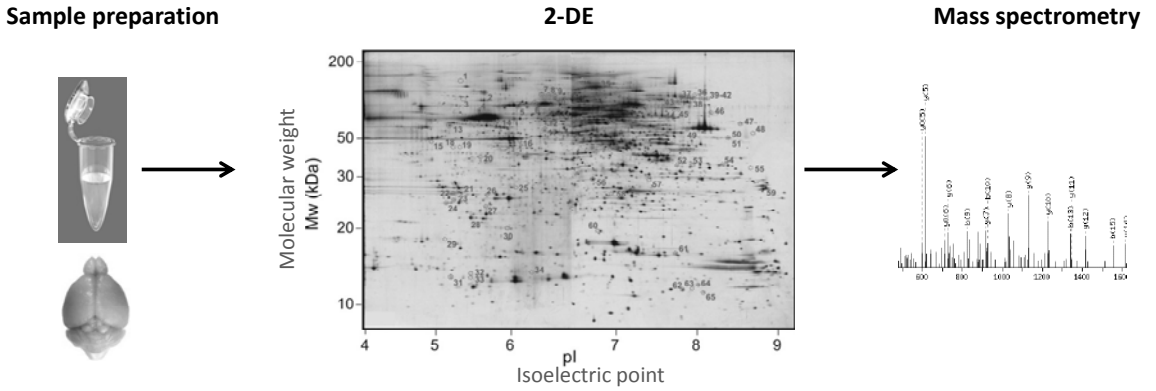

Figure 2. Typical proteome analysis setup applied in the presented research work. *Following sample preparation, total protein extracts were separated by two-dimensional gel electrophoresis (2-DE) according to their isoeletric point (pI) in the first dimension and their molecular weight (Mw) in the second dimension. After silver staining, up to 10,000 discrete protein spots per sample can be detected. Subtractive analyses comparing disease protein patterns with those of controls enable the detection of disease-associated protein spots, which can be identified through mass spectrometry.*

The power of 2-DE lies in its high resolution allowing for the distinction of up to 10,000 proteins per sample and its ability to detect simultaneously vast amounts of proteins and to visualize co- and post-translational modifications. Thereby, e.g., disease-associated proteins can be elucidated through subtractive analyses comparing disease protein patterns with control patterns. At the stage of subtractive analysis, the approach has the potential to unravel complex networks of protein interactions.

2 Topics of the presented research work

Various insults in critical phases of development can lead to a damage of the immature brain and subsequent neurological deficits. While the importance of research on acquired developmental disorders of the brain should rank high in healthcare priorities, based on morality and economic standpoints, research in this field has been accelerating only within the past few years. Several causes and mechanisms of brain damage acquired perinatally or in early childhood have been identified. Common pathomechanisms include an imbalance between neuroprotective and neurodestructive mechanisms, which promote apoptotic cell death in the developing brain. In many instances, however, it remains unclear in what way such impacts affect developmental processes acutely and disrupt the physiologic developmental program in the longrun. Moreover, targets for adjunctive neuroprotective therapy need to be identified. Such points can be addressed by comparative proteome analysis studies.

The aim of the presented research work was to explore acute and long-term changes in the brain proteome following different modes of injury to the immature brain and thereby gain insight into disrupted developmental events and identify novel proteins potentially involved in repair processes. Our results point towards a number of mechanisms that can potentially influence normal development and/or participate in reorganization events.

Since potentially harmful therapy regimens, such as oxygen supplementation, are sometimes inevitable in an intensive care setting, the effect of recombinant erythropoietin (rEpo) on hyperoxia-induced brain damage was analyzed. Our results demonstrate a protective effect of rEpo and suggest mechanisms involved in this process.

Finally, the finding of alterations of the same protein in different disease entities was addressed. In consideration of the protein network concept, the presence of nodal point proteins diseases of the central nervous system was explored. Such proteins may integrate different pathways into one pathway, but also diverge one pathway into several pathways. This finding offers a possible explanation for the clinical heterogeneity and phenotype overlap of distinct disorders. Moreover, changes in proteins at central network positions may generate an unspecific dysregulation of disease-unrelated proteins. This could explain the difficulties that arise in attempts to elucidate the fundamental mechanism that underlie a specific disorder when investigating diseases that are specific for a developmental stage.

3 Results in five selected original reports

3.1 Proteome changes of the immature brain following hyperoxia

Kaindl AM, Sifringer M, Zabel C, Nebrich G, Wacker M, Felderhoff-Mueser U, Endesfelder S, von der Hagen M, Stefovska V, Klose J, Ikonomidou C. Acute and long-term proteome changes induced by oxidative stress in the developing brain. *Cell Death Differ* 2006;13:1097-1109.

Premature infants not only exhibit developmental immaturity of their free radical defences, but they are also inevitably exposed to relative hyperoxia compared to intrauterine hypoxic conditions and are more likely to encounter further situations of increased oxidative stress, such as oxygen supplementation or systemic infections. Hyperoxia is a powerful trigger for widespread apoptotic cell death in the developing rodent brain. The aim of the presented research work was to explore acute and long-term changes in the brain proteome following hyperoxia in infancy, thereby gain insight into disrupted developmental events, and identify novel proteins potentially involved in repair processes. Our results point towards a number of mechanisms that can potentially influence normal development and/or participate in reorganization events.

3.2 Proteome changes of the immature brain following trauma

Kaindl AM, Zabel C, Stefovska V, Lehnert R, Sifringer M, Klose J, Ikonomidou C. Subacute proteome changes following traumatic injury of the developing brain: Implications for a dysregulation of neuronal migration and neurite arborization. *Proteomics - Clinical Application* 2007;1(7):640-649.

Traumatic brain injury (TBI) is a major cause of morbidity and mortality among children and adolescents in the industrialized world, and neurologic outcomes are especially unfavorable in very young patients. A mechanical injury to the immature rodent brain causes acute and rapidly expanding excitotoxic cell death within the area of impact and a delayed but much more extensive disseminated apoptosis in many brain regions ipsi- and contralateral to the trauma site. Neuronal loss following TBI is thus partly responsible for the described neurologic morbidity. The aim of the present study was to explore subacute changes in the brain proteome following TBI ipsi- and contralateral to the trauma site and thereby gain insight into developmental events which are disrupted by trauma as well as identify novel proteins potentially involved in reparative processes. Our results point towards a number of mechanisms that can potentially influence normal development and/or participate in reorganization events.

3.3 Proteome changes of the immature brain following alteration of NMDAR- or $GABA_AR$-mediated neurotransmission

Kaindl AM, Koppelstaetter A, Nebrich G, Stuwe J, Sifringer M, Zabel C, Klose J, Ikonomidou C. Brief alteration of NMDA or $GABA_A$ receptor mediated neurotransmission has long-term effects on the developing cerebral cortex. *Mol Cell Proteomics* 2008. - in press -

Sedative and anticonvulsant agents that reduce neuronal excitability via antagonism at N-methyl-D-aspartate receptors (NMDAR) and/or agonism at γ-amino-butyric acid subtype A receptors ($GABA_AR$) are applied frequently in obstetric and pediatric medicine. We demonstrate that a one-day treatment of infant mice at postnatal day 6 (P6) with the NMDAR antagonist dizocilpine or the $GABA_AR$ agonist phenobarbital not only has acute but also long-term effects on the cerebral cortex. Our results point towards several pathways modulated by a reduction of neuronal excitability that might interfere with critical developmental events and thus affirm concerns about the impact of NMDAR and/or $GABA_AR$ modulating drugs on human brain development.

3.4 Neuroprotection through recombinant erythropoietin

Kaindl AM, Sifringer M, Koppelstaetter A, Genz K, Loeber R, Boerner C, Stuwe J, Klose J, Felderhoff-Mueser U. Erythropoietin protects the developing brain from hyperoxia-induced cell death and proteome changes. *Ann Neurol* 2008. – in press -

Oxygen toxicity is a risk factor for neurocognitive morbidity in survivors of preterm birth and induces disseminated apoptotic neurodegeneration in infant rodent brains. In the present study, we demonstrate that a systemic treatment with recombinant erythropoietin (rEpo), widely used in neonatal medicine for its hematopoietic effect, significantly reduces hyperoxia-induced apoptosis in infant rodent brains. Moreover, rEpo inhibited most brain proteome changes observed when hyperoxia was applied exclusively. Our results suggest that rEpo generates its protective effect against oxygen toxicity through mechanisms such as a reduction of oxidative stress and a restoration of hyperoxia-induced increased levels of caspases and decreased levels of neurotrophins. Our findings are highly relevant from a clinical perspective since oxygen administration to neonates is often inevitable, and rEpo may be applied systemically as an adjunctive neuroprotective therapy.

3.5 Nodal point proteins

Zabel C*, Sagi D*, **Kaindl AM*** Steireif N, Kläre Y, Mao L, Peters H, Wacker MA, Kleene R, Klose J. Comparative Proteomics in Neurodegenerative and Non-neurodegenerative Diseases Suggest Nodal Point Proteins in Regulatory Networking. *J Proteome Res* 2006;5(8):1948-58.

In this research work, we address the occurrence of alterations of the same protein in different disease entities. Such proteins may be nodal point proteins within protein networks and thereby integrate different pathways into one pathway, but also diverge one pathway into several pathways. This finding offers a possible explanation for the clinical heterogeneity and phenotype overlap of distinct disorders. Moreover, changes in proteins at central network positions may generate an unspecific dysregulation of disease-unrelated proteins. This could explain the difficulties that arise in attempts to elucidate the fundamental mechanism that underlie a specific disorder when investigating diseases that are specific for a developmental stage.

4 Discussion

Various insults to the brain, when they occur in critical phases of development, result in brain damage and thereby contribute considerably to the mortality and long-term neurologic morbidity of children. Despite recent progress in this field, the underlying mechanisms are in many cases still unknown. In the presented research work, we reported for the first time on acute, subacute, and long-term proteome changes of the rodent brain subjected to hyperoxia/oxidative stress (Kaindl *et al.* 2006),[49] traumatic brain injury (Kaindl *et al.* 2007),[184] or substances influencing NMDAR and/or $GABA_AR$ neurotransmission (antiepileptic drugs, sedatives, abusive drugs; Kaindl *et al.* 2008)[185] during infancy, on postnatal day 6 (P6). Our results indicate that such insults lead to reproducible proteome alterations not only (sub)acutely on P7/P14, but, most remarkably, even four weeks later, on P35, when the animals have almost reached adulthood. The finding of proteome changes on P35 is in line with the long-term effects seen in humans following insults to the immature brain. We have disclosed mechanisms implemented in brain damage and repair following exogenous insults and following treatment with neuroprotective drugs such as erythropoietin (Kaindl *et al.* 2008).[186] We have further analyzed the overlap of brain proteome changes in several neurodegenerative diseases and discuss the presence of nodal point proteins (Zabel,* Sagi,* Kaindl* *et al.* 2006).[187]

4.1 Physiologic changes of the brain proteome throughout development

An analysis of the murine cerebral cortex proteome at three different stages during postnatal brain development (P7, P14, P35) and in adult mice (P56) disclosed a physiologic change of silver stained 2-DE protein patterns from the early postnatal age P7 to adult age of P56 (Figure 3). Between P7 and P56, 30 to 40% of protein spots in 2-DE gels of whole brain samples are differentially regulated,[188] and the actual percentage may be even higher than can be detected by quantitative measurements of silver stained 2-DE gels. The observed physiologic protein changes throughout brain development can be assigned to one of four groups: (i) *early expression group (EG)*: proteins that can be visualized during early brain development but diminish or disappear in the course of development, (ii) *late EG*: proteins that cannot be visualized or are only present at small amounts during early brain development and appear later at high concentrations, (iii) *transient EG*: proteins that appear only at a specific age, (iv) *stable EG*: proteins that can be detected largely unchanged at all ages evaluated (Figure 4).

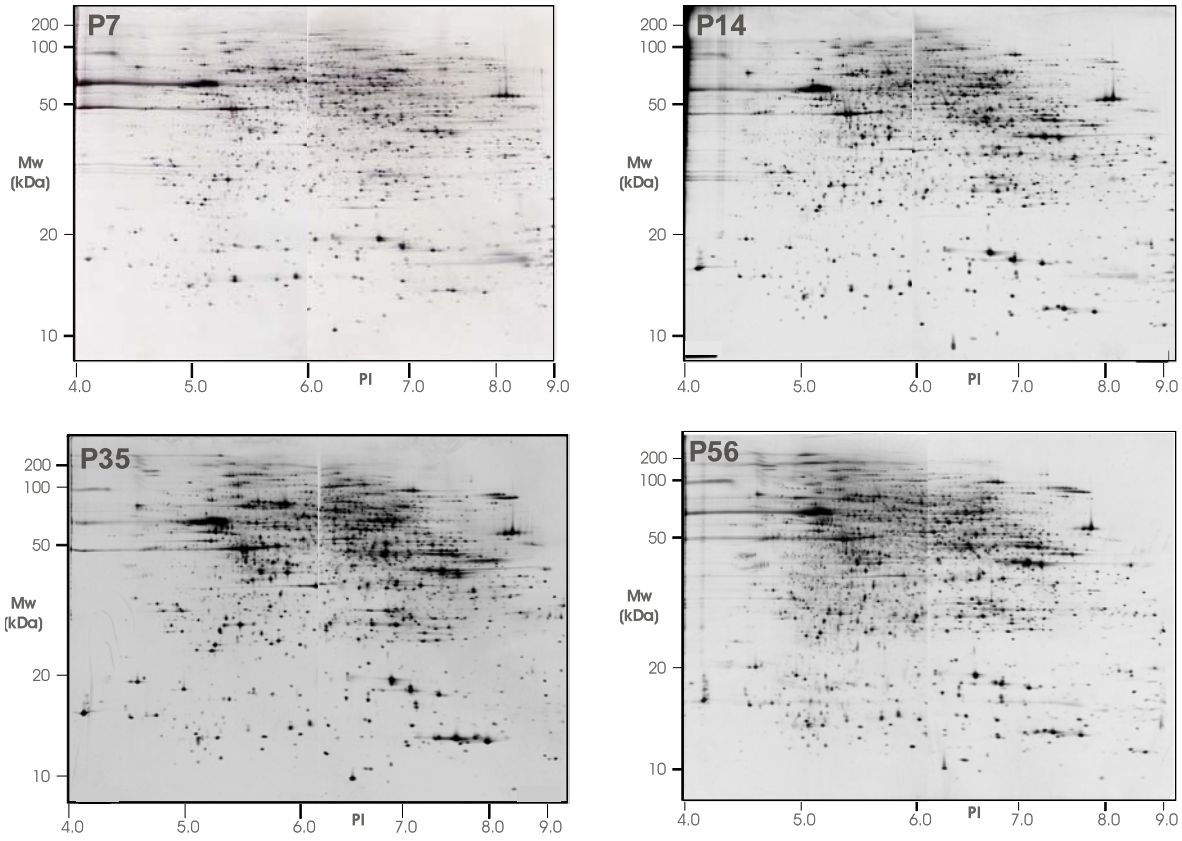

Figure 3. Cerebral cortex protein changes throughout development. *Cerebral cortex protein patterns in representative 2-DE gels from untreated male C57BL6 mice change from early postnatal to the adult age (P7, P14, P35, P56). Proteins were resolved by 2-DE according to isoelectric point (pI) in the first and molecular weight (Mw) in the second dimension and subsequently revealed by silver staining. Figure from Kaindl et al. 2008.*[185]

A classification of proteins into one of these groups may vary as further ages are introduced into the analysis, i.e., a protein assumed to be present at all ages may not be synthesized at earlier or much later ages. Also, various isospots (isoproteins) of one protein may fall into different groups. The change of phenotype (concentration, isospot number) throughout development is often in line with the associated protein function.

These dynamics of protein levels throughout physiologic brain development and in adulthood need to be considered when examining differences in brain proteomes following pharmacologic treatment at various ages. The detection of a protein change at a certain age upon drug treatment through proteome techniques applied in this study requires that a specific isoprotein is present and that the isoprotein level is high enough for detection in the physiologic or pathologic state. The finding that protein changes occur at a specific

time following drug treatment, i.e. acutely (P7), subacutely (P14), or as a long-term effect (P35), but not at all time points analyzed may reflect that (i) a protein change occurs truly only at a specific time, (ii) a (iso)protein does not exist yet or not anymore at a specific age, (iii) a (iso)protein quantity is below detection level due to a reduced gene expression, reduced protein synthesis, change in co-/post-translational modification and/or increased turnover at specific ages, or (iv) the dynamics of protein changes may differ between ages and thus be present but not significant at certain ages. Protein isoforms may possess different functions at various developmental periods and/or be part of a different pathway at various ages.

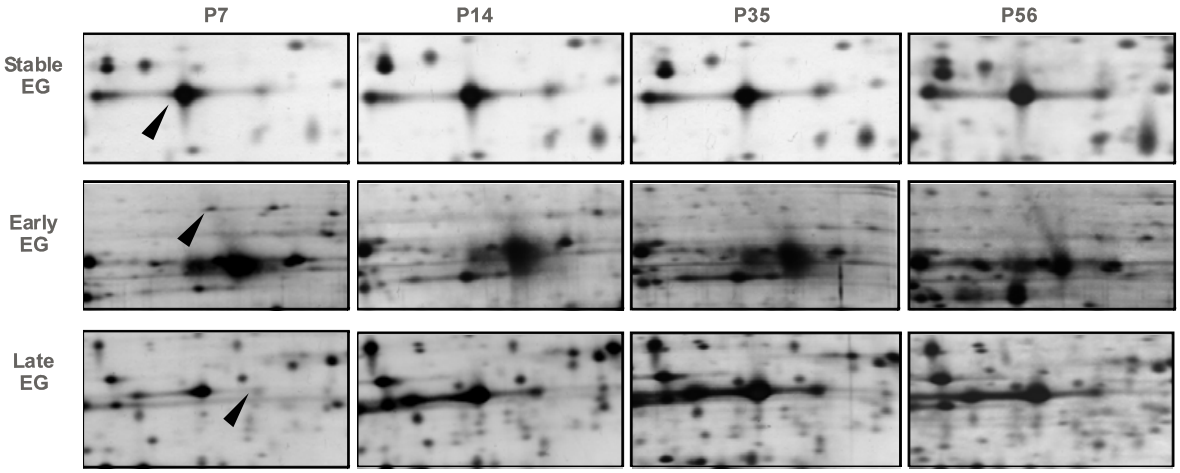

Figure 4. Protein expression groups throughout brain development and in adult age. *Protein spots can be classified in expression groups (EG): (i) stable EG: protein spot abundance is largely unchanged at all evaluated ages; (ii) early EG: protein spots can be visualized early in development but diminish or disappear with age; (iii) late EG: proteins appear at later ages; (iv) transient EG: protein spots can be visualized only within a specific period (not illustrated). Figure from Kaindl et al. 2008.*[185]

4.2 Brain proteome changes following damage of the immature brain

Through a comparison of brain proteomes in 2-DE protein patterns, we detected reproducible qualitative and quantitative differences in proteins of mice exposed to hyperoxia/oxidative stress, traumatic brain injury, or treated with the NMDAR antagonist dizocilpine or the $GABA_AR$ agonist phenobarbital in infancy as compared to untreated

littermates at P7, P14, and P35. Most of these isoproteins can be assigned to the stable expression group. Protein dysregulation occurred primarily in the acute phase after treatment and resolved within one (P14) to four weeks (P35). While acute protein alterations can be explained as direct effects of treatment on cell metabolism (e.g. apoptosis), protein changes that persist or even occur later in development indicate long-term disruption of brain function/morphology and/or ongoing reorganization processes. The above named injuries cause acute functional and morphological changes in the developing brain.[4,5,18,47-49,85,120,123,138-140] In addition, acute functional and morphological changes may induce further functional and morphological changes in the period of rapid brain growth and thereby result in an irreversibly damaged adult brain.

4.3 Mechanisms of brain damage

Acute and long-term modifications of brain proteins detected in the presented studies indicate that hyperoxia/oxidative stress, traumatic brain injury or a brief alteration of NMDAR- or $GABA_AR$-mediated neurotransmission in infant rodents not only result acutely in an increased rate of apoptosis, oxidative stress, and inflammation in their brains, but may also elicit long-term alterations in cell maintenance/proliferation and neuronal circuit formation.[49,184,185] Most alterations observed in infant mice did not occur in adult mice exposed to similar insults and thus appear to be specific for the infant brain.

4.3.1 Apoptosis, oxidative stress, and inflammation

We identified acute and long-term changes in brain proteins that can be linked to increased rates of apoptosis and oxidative stress levels in the developing brain following hyperoxia/ oxidative stress, traumatic brain injury, or a brief alteration of NMDAR- or $GABA_AR$-mediated neurotransmission. Consistent with the results of the proteome analysis, we demonstrated previously that the above named injuries significantly increase the rate of apoptotic cell death in the brain.[4-6,47,123,140] This vulnerability is significantly increased in neonatal rodents and subsides in the course of the growth spurt period by P14 in rodents. [4-6,47,123,140] Apoptotic cell death following hyperoxia, traumatic brain injury, or treatment with NMDAR-antagonists or $GABA_AR$-agonists may be associated with oxidative stress as suggested by observed protein changes. The dysregulation of free radical defenses/ scavengers such as peroxiredoxin (Prdx) isoforms, dysregulated in all of the animal models studied likely reflects a physiologic activation of endogenous antioxidant defense mechanisms to prevent oxidative damage or a consumption of these proteins as a result of overwhelming oxidative stress. Prdx2 was downregulated in brains of neonatal mice

following hyperoxia, Prdx1 and 6 were upregulated following traumatic brain injury, and Prdx1 was upregulated in the cortex of neonatal mice treated with the NMDAR antagonist MK801 or the $GABA_AR$ agonist phenobarbital on P7. The role of peroxiredoxins in coping with oxidative stress has long been established. A neuroprotective role of peroxiredoxins against oxidative damage has been demonstrated in the neonate in a model of NMDAR-mediated brain lesions and in oxygen-mediated injury of the lung in neonatal baboons.[189,190] Moreover, a dysregulation of Prdx1 has been reported in adult mouse models of neurodegenerative and non-neurodegenerative diseases,[187] and PRDX6 was upregulated in the frontal cortex of patients with Parkinson's disease.[191]

4.3.2 Cell proliferation

Acute and long-term changes of proteins that indicate a modulation of cell maintenance and proliferation were detected following hyperoxia, traumatic brain injury, or a brief alteration of NMDAR- or $GABA_AR$-mediated neurotransmission. Oxidative stress has been linked to reduced cell proliferation in the brain.[192] Correspondingly, our recent results indicate that both NMDAR antagonists and $GABA_AR$ agonists markedly suppress cell proliferation within the brain when administered to infant rats during the second postnatal week of life.[193] In line with our finding, an activation of the NMDAR has been reported to increase proliferation and differentiation of hippocampal neuronal progenitor cells in the developing brain[194] and to decrease the diameter of neurospheres in the embryonic rat brain.[195] Glutamate has also been reported to enhance proliferation and neurogenesis in human neuronal progenitor cell cultures derived from the fetal cortex[196] and to act as a positive regulator of neurogenesis.[197] Our findings are in line with these reports on the role of NMDAR in the developing brain which is a stimulatory effect on cell proliferation. GABA constitutes a developmental signal during stages of embryonic neurogenesis, progenitor proliferation, neuronal migration, and neurite outgrowth.[198] The effect of traumatic brain injury on cell proliferation in the developing brain has not been studied. In the adult, traumatic brain injury stimulates cell proliferation and neurogenesis.[199,200]

4.3.3 Neuronal network formation

Neuronal network development depends not only on genetic determinants but also on exogenous factors. In our experimental models proteins that can play a role in neuronal migration and axonal arborisation were dysregulated following hyperoxia/oxidative stress, traumatic brain injury, and drug-induced modulation of neuronal excitability in infancy but not in adulthood. Quantitative and qualitative differences of proteins involved in growth

cone guidance were detected in the above named animal models in response to treatment. These include Rab and Rho GDP dissociation inhibitor (RabGDI, RhoGDI) isoproteins as well as isoproteins of downstream effectors such as collapsin response mediator proteins (Crmp).

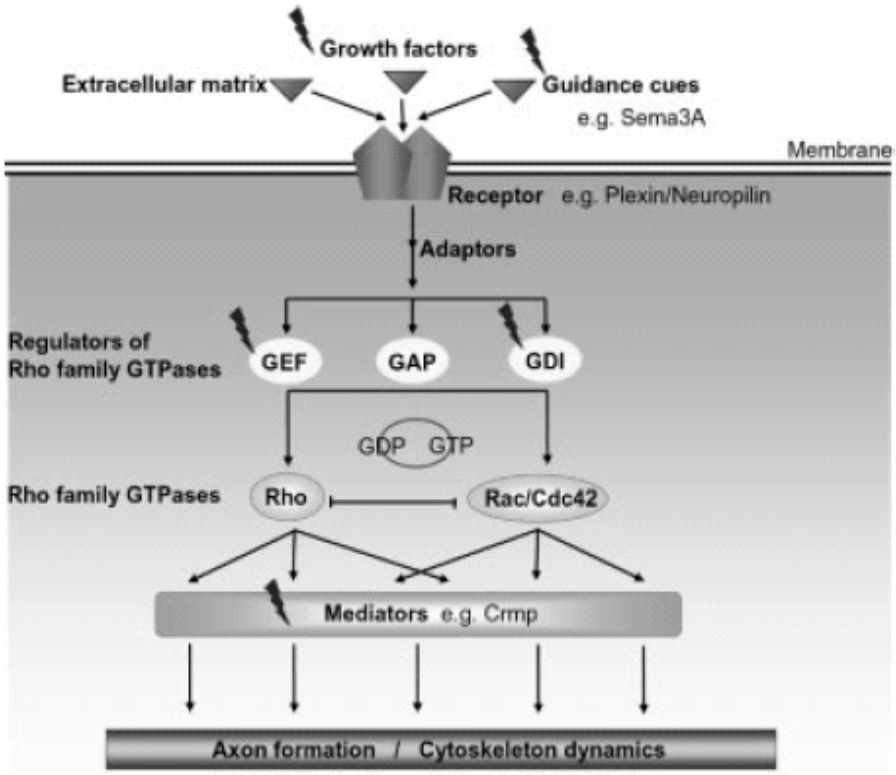

Figure 5. Dysregulation of axon growth and guidance-associated proteins through hyperoxia/oxidative stress, traumatic brain injury, and drug-induced modulation of neuronal excitability. *In this simplified scheme of prospective signalling through Rho family small GTPases, proteins/protein groups dysregulated by the insults mentioned above are indicated by thunderbolts symbols. Extracellular signals (extracellular matrix, growth factors, guidance cues) directly or indirectly control cytoskeleton dynamics by modulating the function of Rho family small GTPase regulators such as guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs). GTP-bound Rho family GTPases modulate cytoskeletal morphology through a wide variety of effector molecules including collapsin response mediator protein (Crmp) isoforms. Figure from Kaindl et al. 2007.*[184]

Growth cone guidance may be influenced by the acute and long-term modulation of Rho family small GTPase regulators RabGDI1 and RhoGDI1; both proteins bind to the

GDP form of Rho GTPases, slow the rate of GDP dissociation from Rho GTPases and thereby decrease the activity of these proteins. GTP-bound Rho family GTPases modulate cytoskeletal morphology through a wide variety of effector molecules (Figure 5).[201] Molecular guidance cues directly or indirectly control cytoskeleton dynamics by modulating the function of Rho family small GTPase regulators such as guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs).[201] Moreover, GTP-bound Rho family GTPases modulate cytoskeletal morphology through a wide variety of effector molecules including Crmps.[201] The function of Crmps, proteins initially identified as mediators of semaphorin3A/ collapsin-induced growth-cone collaps, is crucial for axonal growth and branching, determination of axon-dendrite fate, establishment of neuronal polarity, and kinesin I-dependent transport of proteins to growth cones.[202-204] In line with data from other research groups, we have found that total Crmp levels are high at early stages of development and then diminish massively following the period of rapid brain growth within the first postnatal weeks.[49,185]

The observed Crmp isoform dysregulation as well as the upregulation of total Crmp2 and 4 mRNA and protein levels following hyperoxia, traumatic brain injury, and dizocilpine or phenobarbital-induced neurodegeneration may reflect neuronal regeneration or detrimental CNS damage through the formation of aberrant axons and/or a loss of neuronal polarity as demonstrated in the context of other models *in vitro*.[204-206] This hypothesis is based on the following observations: A *Crmp2* overexpression in primary hippocampal neuronal cultures disrupted neuronal polarity and induced the formation of supernumerary axons.[206] On the other hand, *Crmp2* overexpression in dorsal root ganglions accelerated the regeneration of injured neurons,[205] and a re-expression of *CRMP4* has been detected during axonal regrowth following axotomy.[204] Moreover, nematodes with a mutated Crmp-homolog *C. elegans* gene have coordination disturbances and display an axon guidance disorder with a premature termination of axon arborisation.[204] The hypothesis of a pathologic neurite formation following hyperoxia/oxidative stress is supported by the finding of Katoh and co-workers, who described a hyperoxia-induced formation of a differentiated neuronal phenotype in PC12 cells which is otherwise induced only by the supplementation of nerve growth factor.[207] In the adult brain, plasticity-associated events (increase in dendritic arborization, spine density, and synaptogenesis) have been described.[208,209] Moreover, NMDAR antagonists have been reported to affect

branching of neuronal processes in embryonic rat hippocampal neurons, and a temporary block of NMDA and non-NMDA glutamate receptors in immature rats also disrupted the topographic refinement of thalamocortical connectivity and columnar organization, i.e. the topographic organization of synaptic connections.[161,162] Similarly, agonists and antagonists of $GABA_AR$ have been associated with effects on neuronal migration and differentiation including neurite outgrowth.[198,210,211]

4.4 Neuroprotective effect of erythropoietin

Adjunctive neuroprotective therapy in infants is a promising approach, since secondary evolving brain injuries can be substantially larger than the damage caused by the primary insult, and certain potentially also harmful therapy regimens such as oxygen supplementation and/or an application of neurotransmission-affecting drugs can be inevitable. We reported on the neuroprotective effect of systemic treatment with recombinant erythropoietin (rEpo) in our hyperoxia-animal model and pointed out several mechanisms through which rEpo may entertain its tissue-protective effect against oxygen toxicity.[186]

Pursuing the question by what mechanisms rEpo ameliorates the apoptotic component of hyperoxia-induced brain damage, we compared brain proteomes of infant mice treated concomitantly with oxygen and rEpo to those of controls (normoxia, normal saline injections). Through a comparison of brain proteomes in 2-DE protein patterns and identification of protein spots by mass spectrometry, we detected reproducible qualitative and quantitative differences in 24 proteins in mice treated with hyperoxia and rEpo in infancy as compared to untreated littermates.

We demonstrated previously that apoptotic cell death following hyperoxia is associated with a dysregulation of brain proteins in infant mice that have been associated with oxidative stress, apoptosis, cell maintenance and growth, and the formation of neuronal connections.[49] Consistent with our histological results, systemic rEpo treatment prior to the initiation of hyperoxia decreased the range of infant murine brain protein changes detected by proteome analysis. Only myosin regulatory light chain was dysregulated following hyperoxia regardless of a co-treatment with rEpo.

4.4.1 Decrease of apoptotic cell death

An exposure of infant rodents at P6 to a high inspiratory oxygen concentration of 80% (hyperoxia) over a period of 24 hrs increased the rate of cell death in the developing rat

brain acutely. In previous studies, we demonstrated that hyperoxia-induced cell death displays morphological features of apoptosis and occurs in a disseminated fashion throughout the immature rat brain with the highest susceptibility towards oxygen in the first week of life.[46,47] A significant decrease of cell death was detected when rats were treated systemically with 10,000 or 20,000 IE/kg rEpo prior to an exposure to high oxygen levels (apoptotic score decreased by 70%). This effect indicates possible therapeutic implications of rEpo in neonatal medicine as a preventive neuroprotective agent.

A neuroprotective effect of rEpo has been reported in experimental rodent models of hypoxia-ischemia, excitotoxicity and neonatal stroke, as reviewed by Juul and Felderhoff-Mueser.[170] Moreover, an improvement of long-term neurological outcome of neonatal stroke/hypoxia-ischemia has been reported.[212,213] Still, tissue-protective properties of rEpo have been more extensively studied in adult models than in developing organisms, whilst the precise mechanisms of rEpo action in the immature CNS, which might differ from those in the adult CNS,[214] have not been fully defined. Potential effects of rEpo include an induction of anti-apoptotic signaling pathways,[215-217] a decrease of inflammation,[217] and a decrease of excitotoxicity.[218,219]

4.4.2 Decrease of effector caspase production

A systemic concomitant rEpo treatment blocked the dysregulation of apoptosis-associated brain proteins following hyperoxia. To determine whether the decrease in hyperoxia-induced apoptotic cell death following rEpo treatment is associated with a decreased activation of intrinsic and extrinsic apoptosis/caspase pathways, we analyzed activated caspase 2, 3, and 8 protein levels as well as caspase 2 and 8 enzyme activities in rats at P6 subjected to hyperoxia with and without rEpo treatment and in normoxic controls. Hyperoxia during infancy increased the production of caspase 2, 3, and 8 protein levels and enzyme activities, and this effect was reduced by a treatment with rEpo. In line with our data, rEpo has been demonstrated to prevent inflammatory cell demise through pathways that involve phosphatidylserine exposure, microglial activation, protein kinase B (Akt), and the regulation of caspases.[220]

4.4.3 Antioxidant defense

Proteins that can be linked to rEpo countersteering increased production of reactive oxygen species in the developing brain were modulated upon combined rEpo and hyperoxia as well as upon single rEpo treatment. We reported that levels of oxidized glutathione and protein carbonyls in the brains of infant rodents exposed to hyperoxia are elevated when

compared to those kept at room air, findings consistent with oxidative stress.[47,49] The protein carbonyl levels decreased through a concomitant systemic rEpo treatment. Our finding that rEpo decreases hyperoxia-induced oxidative stress is supported by reports of other groups that rEpo (i) reduces NO-mediated formation of free radicals or antagonizes their toxicity through, e.g., an increase in the activity of antioxidant enzymes in neurons,[221] (ii) decreases increased lipid peroxidation levels in fetal rat ischemia-reperfusion and hypoxic-ischemic (HI) brain injury models,[222,223] (iii) increases glutathione peroxidase enzymatic activity and decreases nitric oxide overproduction in a HI model,[222] (iv) stimulates astroglial glutathione peroxidase production in astrocyte cultures,[224] (v) protects microglia cells from cell death during oxygen and glucose deprivation-induced oxidative stress,[225] and (vi) improves the survivability of manganese-superoxide dismutase double knockout (*Sod2-/-*) mice astrocytes *in vitro*.[226] Such properties may be relevant in the therapeutic prevention of hyperoxia injury to the developing brain of premature infants, in whom antioxidant systems are immature.

Brain proteins altered upon systemic rEpo treatment indicate molecular mechanisms potentially involved in a reduction of oxidative stress. When rEpo was applied prior to hyperoxia-treatment, we detected a dysregulation of glyoxalase I (Glo1), heat shock protein 2 (Hsp2), and carbonyl reductase 1 (Cbr1) isoproteins. Systemic rEpo treatment similarly lead to a dysregulation of Glo1 and Prdx2, but also induced a change in the proteins latexin (Lxn), protein phosphatase 3 regulatory subunit B, alpha isoform (Pp3r1), ferritin light chain (Ftl1), and glia maturation factor beta (Gmfb).

Glo1 modulation may be part of rEpo antioxidant defense mechanism, as its expression is partially regulated by oxidative stress,[227] it is part of a glutathione-dependent glyoxylase enzyme system that protects cells from advanced glycation endproduct formation,[227-230] and it is over-expressed in several apoptosis-resistant tumor cells.[231] *Glo1* gene expression is also regulated by mitogen-activated protein kinases (MAPK) under stress conditions in *Saccharomyces cerevisiae*[232] and by tumor necrosis factor-α (TNF-α).[226] On the other hand, glycation end products can activate multiple signaling pathways including Erk1/2 MAPK, Rho GTPases, phosphoinositol-3 kinase, and the Jak/Stat pathway as well as downstream effectors.[228,230]

4.4.4 Neurotrophin rescue

A further potential mechanism of rEpo action in the amelioration of hyperoxia-induced damage of the immature brain is the recovery of decreased neurotrophic factors since

several proteins that were modulated by rEpo have been associated with cell maintenance and growth and may influence neurotrophins. Neurotrophins provide trophic support to developing neurons, the withdrawal of which may lead to neuronal death.[233] We detected a decreased synthesis of neurotrophic factors BDNF and GDNF as well as reduced levels of the active phosphorylated forms of ERK1/2 and Akt. ERK1/2 and Akt are key players in two major survival promoting pathways, the Mek-ERK1/2 and the PI3 kinase-Akt pathways, which are activated by tyrosine kinase receptors upon binding of growth factors. Ras activation results from binding of growth factors to the respective receptors and initiated signaling via the MEK and PI3 pathways. rEpo application partially counteracted the effects of hyperoxia on brain BDNF mRNA expression/protein levels and restored levels of phosphorylated ERK1/2 and Akt in the brain. These results indicate that rEpo-mediated signaling crosstalks with intracellular signaling mediated by neurotrophins. Such crosstalking of Epo-mediated signals with other second messengers may account for its neuroprotective action. Supportive observations exist in the literature. rEpo has been shown to exert cytoprotective effects in a variety of experimental models.[234-240] Thus, rEpo inhibits apoptosis of cultured cortical neurons deprived of growth factors or exposed to kainic acid and of endothelial cells by activating MEK-ERK1/2 and PI3-kinase-Akt pathways.[241] rEpo may also exert a neuroprotective effect by recruiting the transcriptional factor NF-κB, which upon phosphorylation translocates to the nucleus and activates expression of neuroprotective genes such as superoxide dismutase or inhibitors of apoptosis proteins.[242]

4.4.5 Potential deleterious effects on the developing brain

To assess the impact of erythropoietin on infant brain proteins and thus distinguish it from effects caused by a co-treatment of rEpo with hyperoxia, we assessed brain protein changes following a single systemic rEpo treatment of infant mice at P6. In response to systemic rEpo treatment, we detected reproducible qualitative and quantitative differences in 37 protein spots of mice treated with rEpo in infancy as compared to untreated littermates with 2-DE, and mass spectrometry enabled the identification of 36 discrete proteins which have been associated with processes such as oxidative stress, inflammation, cell maintenance and growth, and neuronal circuit formation. Though this protein modulation may be beneficial, our results also raise the question whether rEpo may also exert negative effects on brain development. rEpo does, e.g., increase the level of Crmp2 and does not ameliorate hyperoxia-induced modulation of this cell polarity and axon

outgrowth-associated protein. It remains to be elucidated if this is a beneficial (regeneration of injured neurons?) or a further detrimental (abnormal circuit development) effect.

This concern is supported by reports on neurotoxic effects of very high dose rEpo treatment *in vitro* in neuronal culture and brain-slice models of mild hypoxia, as well as *in vivo* in a neonatal rat model of mild brain injury (40,000 IE/kg/d i.p.).[241] Moreover, the concern that Epo may amplify diffuse axonal injury has been recently expressed.[234] Reassuringly, lower doses of 2500 or 5000 IE/kg did not show acute toxicity in neonatal rats or adverse long-term behavioral effects,[241,243] and single clinical studies in preterm babies report no negative long-term neurodevelopmental effects of erythropoietic doses of rEpo when compared to placebo.[244] Still, it needs to be questioned what impact the effect of a decrease of physiologic apoptosis, an important aspect of normal brain development, and the modulation of further developmental processes will have in the long-run. Thus, caution needs to be exercised as it may not only be beneficial but may also influence normal brain development.

4.5 Nodal point proteins

In different disease entities alterations of the same protein can often be detected. To further address this point, we compared the brain proteome changes found in mouse models of seven neurodegenerative and non-neurodegenerative disorders of the brain.[187] Thereby, we identified proteins which were altered in several neurodegenerative diseases (ND) but not in non-ND as well as proteins which were altered in both ND and non-ND. In addition, we investigated whether proteins altered in disease show changes in normal mice due to protein polymorphisms. In all diseases investigated, numerous brain proteome changes were found. Interestingly, however, up to 36% of abnormal protein changes occurred in multiple disease states. Seven proteins (11 protein isospots) were differentially regulated in at least three out of seven investigated neurological conditions. Protein expression changes that are not specific for a single disease may be explained by integration of proteins within networks. Widespread interaction between proteins in highly complex protein networks has been demonstrated for several organisms such as yeast,[245] drosophila,[246] and humans.[247,248] An alteration of one protein in a protein network may therefore cause

changes in many other proteins within the network by direct or indirect protein-protein interactions. Thus, while genetic defects, non-genetic disorders, and naturally occurring variations such as polymorphisms may primarily modulate only one or a few proteins specifically, many other proteins may become involved due to network effects. Proteins most frequently targeted may be those which occupy nodal points in the proteome network. Therefore, alterations in a tissue or cell induced by conditions that differ as much as ND, non-ND, and normal populations (polymorphisms) may, nevertheless, affect to some extent the same nodal point proteins, which will become obvious by quantitative changes in these proteins.

An interesting observation of our study was that all seven nodal point proteins detected were altered in their expression between different mouse strains as well. These differences were in some cases very similar to those observed under disease conditions. Expression polymorphisms of a nodal point protein may be caused by alterations of proteins interacting with it. Nodal point protein encoding genes are unlikely to carry mutations themselves since adaptive evolution suggests that this is least likely for genes encoding proteins that hold a high number of interactions.[249,250] Between mouse strains *C57BL/6* and *SPR*, a frequency of 15% protein polymorphisms has been observed, representing a high level of variation between mouse strains.[251] Therefore, proteins altered by polymorphisms may act on nodal point proteins and alter their expression which is seen as protein expression polymorphisms. In this way, protein polymorphisms may serve as disease associated modifiers, e.g., changing the onset of disease.

In conclusion, our investigation has shown that quantitative changes of proteins in various neurological diseases can be quite unspecific to the disease under consideration and may even reflect frequently normal variability of proteins due to polymorphisms. These proteins may occupy nodal points in the network of protein-protein interaction and regulation and be responsible for overlapping metabolic pathways and clinical symptoms of different genetic disorders. Thus, these proteins may play an important role in disease pathology.

5 Conclusion and outlook

The immature brain reacts differently from the mature brain when exposed to potentially damaging environmental factors or insults. Our knowledge on mechanisms underlying injuries to the developing brain has progressed throughout the past years, though most processes have been studied only in the mature brain. It is now known, that several insults have the potential to induce widespread apoptotic neurodegeneration in infant rodent brains during the brain growth spurt phase, a transient period when the brain is growing most rapidly. In what way other developmental processes are affected has not been analyzed in detail so far.

In the presented research work, we have disclosed mechanisms that can potentially influence normal development and/or participate in reorganization events following damage to the developing brain through hyperoxia, traumatic brain injury, or an exposure to substances influencing NMDAR and/or $GABA_AR$ neurotransmission. Depending upon their timing, lesions of the immature brain carry the potential of influencing developmental events in their natural sequence and redirecting subsequent development.

While acute brain protein alterations in response to the above named impacts within the period of rapid brain growth most likely represent direct effects on cell metabolism, sustained or newly evolved differences in brain protein phenotypes four weeks after an impact may constitute secondary changes such as irreversible morphological alterations (a possibly ongoing shift in normal developmental program) and/or reorganization events. In comparison to the adult brain, insults disturb a vulnerable and developing system in which the cellular phenotypes, protein concentrations, protein compositions, and interactions change rapidly according to a predetermined developmental program. So far, little is known about the effects of antiepileptic and sedative drugs on dynamic processes in the developing brain. Our results demonstrate that comparative analysis of the brain proteome facilitates insight into the nature of developmental events which may be disrupted by oxygen, traumatic brain injury, or drugs. Impairment beyond that of acute apoptotic neurodegeneration became evident. The described effects did not occur in mice at an age beyond the period of rapid brain growth. Our findings indicate that the above named injuries may cause oxidative stress, inhibit cell proliferation, and induce abnormal neuronal migration/arborization. Ongoing studies will need to explore functional and morphological aspects of these effects.

Detailed knowledge on pathways involved in a damage of the developing brain may

enable the identification of therapeutic targets which again may permit a selective inhibition of pathologic processes without disturbing intact cells/physiologic cell function. We have identified erythropoietin as a neuroprotective agent in hyperoxie-induced brain damage. This finding is highly relevant from a clinical perspective since oxygen administration to neonates is often inevitable and rEpo may be adopted as adjunctive neuroprotective therapy. The experimental evidence we present here and data of others, however, also call for caution with the unrestricted use of rEpo in neonatal medicine prior to further studies on the effect of this drug on dynamic processes of physiological brain development and subsequently randomized, controlled clinical trials. It remains to be elucidated if the protective effects of the drug provide a net benefit to the immature brain.

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7 References

1. Volpe, JJ, (2001) Perinatal brain injury: from pathogenesis to neuroprotection. Ment Retard Dev Disabil Res Rev 7: 56-64.
2. Berger, R, Garnier, Y and Jensen, A, (2002) Perinatal brain damage: underlying mechanisms and neuroprotective strategies. J Soc Gynecol Investig 9: 319-28.
3. Olney, JW, Farber, NB, Wozniak, DF, Jevtovic-Todorovic, V and Ikonomidou, C, (2000) Environmental agents that have the potential to trigger massive apoptotic neurodegeneration in the developing brain. Environ Health Perspect 108 Suppl 3: 383-8.
4. Ikonomidou, C, Bosch, F, Miksa, M, Bittigau, P, Vockler, J, Dikranian, K et al., (1999) Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. Science 283: 70-4.
5. Ikonomidou, C, Bittigau, P, Ishimaru, MJ, Wozniak, DF, Koch, C, Genz, K et al., (2000) Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. Science 287: 1056-60.
6. Bittigau, P, Sifringer, M, Pohl, D, Stadthaus, D, Ishimaru, M, Shimizu, H et al., (1999) Apoptotic neurodegeneration following trauma is markedly enhanced in the immature brain. Ann Neurol 45: 724-35.
7. Pohl, D, Bittigau, P, Ishimaru, MJ, Stadthaus, D, Hubner, C, Olney, JW et al., (1999) N-Methyl-D-aspartate antagonists and apoptotic cell death triggered by head trauma in developing rat brain. Proc Natl Acad Sci U S A 96: 2508-13.
8. McQuillen, PS and Ferriero, DM, (2004) Selective vulnerability in the developing central nervous system. Pediatr Neurol 30: 227-35.
9. Rodier, PM, (1980) Chronology of neuron development: animal studies and their clinical implications. Dev Med Child Neurol 22: 525-45.
10. Rodier, PM, (1994) Vulnerable periods and processes during central nervous system development. Environ Health Perspect 102 Suppl 2: 121-4.
11. Dobbing, J and Sands, J, (1973) Quantitative growth and development of human brain. Arch Dis Child 48: 757-67.
12. Dobbing, J, (1974) The later growth of the brain and its vulnerability. Pediatrics 53: 2-6.
13. Smart, JL, (1991) Critical periods in brain development. Ciba Found Symp 156: 109-24; discussion 124-8.
14. Bayer, SA, Altman, J, Russo, RJ and Zhang, X, (1993) Timetables of neurogenesis in the human brain based on experimentally determined patterns in the rat. Neurotoxicology 14: 83-144.
15. Passingham, RE, (1985) Rates of brain development in mammals including man. Brain Behav Evol 26: 167-75.
16. Johnson, EM, Jr. and Deckwerth, TL, (1993) Molecular mechanisms of developmental neuronal death. Annu Rev Neurosci 16: 31-46.
17. Henderson, CE, (1996) Programmed cell death in the developing nervous system. Neuron 17: 579-85.
18. Ikonomidou, C, Bittigau, P, Koch, C, Genz, K, Hoerster, F, Felderhoff-Mueser, U et al., (2001) Neurotransmitters and apoptosis in the developing brain. Biochem Pharmacol 62: 401-5.
19. Webb, SJ, Monk, CS and Nelson, CA, (2001) Mechanisms of postnatal neurobiological development: implications for human development. Dev Neuropsychol 19: 147-71.
20. Rodier, PM, (1995) Developing brain as a target of toxicity. Environ Health Perspect 103 Suppl 6: 73-6.
21. Emerit, MB, Riad, M and Hamon, M, (1992) Trophic effects of neurotransmitters during brain maturation. Biol Neonate 62: 193-201.

22. Retz, W, Kornhuber, J and Riederer, P, (1996) Neurotransmission and the ontogeny of human brain. J Neural Transm 103: 403-19.
23. Levitt, P, Harvey, JA, Friedman, E, Simansky, K and Murphy, EH, (1997) New evidence for neurotransmitter influences on brain development. Trends Neurosci 20: 269-74.
24. Nguyen, L, Rigo, JM, Rocher, V, Belachew, S, Malgrange, B, Rogister, B et al., (2001) Neurotransmitters as early signals for central nervous system development. Cell Tissue Res 305: 187-202.
25. Folkerth, RD, (2007) The neuropathology of acquired pre- and perinatal brain injuries. Semin Diagn Pathol 24: 48-57.
26. Vexler, ZS and Ferriero, DM, (2001) Molecular and biochemical mechanisms of perinatal brain injury. Semin Neonatol 6: 99-108.
27. Back, SA, Riddle, A and McClure, MM, (2007) Maturation-dependent vulnerability of perinatal white matter in premature birth. Stroke 38: 724-30.
28. Kirpalani, H and Asztalos, E, (2001) Neonatal brain injury. Curr Opin Pediatr 13: 227-33.
29. Goldenberg, RL, Culhane, JF, Iams, JD and Romero, R, (2008) Epidemiology and causes of preterm birth. Lancet 371: 75-84.
30. Berkowitz, GS and Papiernik, E, (1993) Epidemiology of preterm birth. Epidemiol Rev 15: 414-43.
31. Tommiska, V, Heinonen, K, Kero, P, Pokela, ML, Tammela, O, Jarvenpaa, AL et al., (2003) A national two year follow up study of extremely low birthweight infants born in 1996-1997. Arch Dis Child Fetal Neonatal Ed 88: F29-35.
32. Maalouf, EF, Duggan, PJ, Counsell, SJ, Rutherford, MA, Cowan, F, Azzopardi, D et al., (2001) Comparison of findings on cranial ultrasound and magnetic resonance imaging in preterm infants. Pediatrics 107: 719-27.
33. Huppi, PS, (2002) Advances in postnatal neuroimaging: relevance to pathogenesis and treatment of brain injury. Clin Perinatol 29: 827-56.
34. Maalouf, EF, Duggan, PJ, Rutherford, MA, Counsell, SJ, Fletcher, AM, Battin, M et al., (1999) Magnetic resonance imaging of the brain in a cohort of extremely preterm infants. J Pediatr 135: 351-7.
35. Huppi, PS, Schuknecht, B, Boesch, C, Bossi, E, Felblinger, J, Fusch, C et al., (1996) Structural and neurobehavioral delay in postnatal brain development of preterm infants. Pediatr Res 39: 895-901.
36. Inder, TE, Warfield, SK, Wang, H, Huppi, PS and Volpe, JJ, (2005) Abnormal cerebral structure is present at term in premature infants. Pediatrics 115: 286-94.
37. Inder, TE, Anderson, NJ, Spencer, C, Wells, S and Volpe, JJ, (2003) White matter injury in the premature infant: a comparison between serial cranial sonographic and MR findings at term. AJNR Am J Neuroradiol 24: 805-9.
38. Inder, TE, Wells, SJ, Mogridge, NB, Spencer, C and Volpe, JJ, (2003) Defining the nature of the cerebral abnormalities in the premature infant: a qualitative magnetic resonance imaging study. J Pediatr 143: 171-9.
39. Inder, TE, Huppi, PS, Warfield, S, Kikinis, R, Zientara, GP, Barnes, PD et al., (1999) Periventricular white matter injury in the premature infant is followed by reduced cerebral cortical gray matter volume at term. Ann Neurol 46: 755-60.
40. McKinstry, RC, Mathur, A, Miller, JH, Ozcan, A, Snyder, AZ, Schefft, GL et al., (2002) Radial organization of developing preterm human cerebral cortex revealed by non-invasive water diffusion anisotropy MRI. Cereb Cortex 12: 1237-43.
41. Peterson, BS, Anderson, AW, Ehrenkranz, R, Staib, LH, Tageldin, M, Colson, E et al., (2003) Regional brain volumes and their later neurodevelopmental correlates in term and preterm infants. Pediatrics 111: 939-48.

42. Battin, M, Maalouf, EF, Counsell, S, Herilhy, AH and Edwards, AD, (1997) Magnetic resonance imaging of the brain of premature infants. Lancet 349: 1741.
43. Ajayi-Obe, M, Saeed, N, Cowan, FM, Rutherford, MA and Edwards, AD, (2000) Reduced development of cerebral cortex in extremely preterm infants. Lancet 356: 1162-3.
44. Skranes, J, Vangberg, TR, Kulseng, S, Indredavik, MS, Evensen, KA, Martinussen, M et al., (2007) Clinical findings and white matter abnormalities seen on diffusion tensor imaging in adolescents with very low birth weight. Brain 130: 654-66.
45. Taglialatela, G, Perez-Polo, JR and Rassin, DK, (1998) Induction of apoptosis in the CNS during development by the combination of hyperoxia and inhibition of glutathione synthesis. Free Radic Biol Med 25: 936-42.
46. Hoehn, T, Felderhoff-Mueser, U, Maschewski, K, Stadelmann, C, Sifringer, M, Bittigau, P et al., (2003) Hyperoxia causes inducible nitric oxide synthase-mediated cellular damage to the immature rat brain. Pediatr Res 54: 179-84.
47. Felderhoff-Mueser, U, Bittigau, P, Sifringer, M, Jarosz, B, Korobowicz, E, Mahler, L et al., (2004) Oxygen causes cell death in the developing brain. Neurobiol Dis 17: 273-82.
48. Felderhoff-Mueser, U, Sifringer, M, Polley, O, Dzietko, M, Leineweber, B, Mahler, L et al., (2005) Caspase-1-processed interleukins in hyperoxia-induced cell death in the developing brain. Ann Neurol 57: 50-9.
49. Kaindl, AM, Sifringer, M, Zabel, C, Nebrich, G, Wacker, MA, Felderhoff-Mueser, U et al., (2006) Acute and long-term proteome changes induced by oxidative stress in the developing brain. Cell Death Differ 13: 1097-109.
50. Goldstein, M, (1990) Traumatic brain injury: a silent epidemic. Ann Neurol 27: 327.
51. Sosin, DM, Sniezek, JE and Waxweiler, RJ, (1995) Trends in death associated with traumatic brain injury, 1979 through 1992. Success and failure. Jama 273: 1778-80.
52. Thurman, D and Guerrero, J, (1999) Trends in hospitalization associated with traumatic brain injury. Jama 282: 954-7.
53. Potts, MB, Koh, SE, Whetstone, WD, Walker, BA, Yoneyama, T, Claus, CP et al., (2006) Traumatic injury to the immature brain: inflammation, oxidative injury, and iron-mediated damage as potential therapeutic targets. NeuroRx 3: 143-53.
54. Bruns, J, Jr. and Hauser, WA, (2003) The epidemiology of traumatic brain injury: a review. Epilepsia 44 Suppl 10: 2-10.
55. Adelson, PD and Kochanek, PM, (1998) Head injury in children. J Child Neurol 13: 2-15.
56. Koskiniemi, M, Kyykka, T, Nybo, T and Jarho, L, (1995) Long-term outcome after severe brain injury in preschoolers is worse than expected. Arch Pediatr Adolesc Med 149: 249-54.
57. Thakker, JC, Splaingard, M, Zhu, J, Babel, K, Bresnahan, J and Havens, PL, (1997) Survival and functional outcome of children requiring endotracheal intubation during therapy for severe traumatic brain injury. Crit Care Med 25: 1396-401.
58. Mahoney, WJ, D'Souza, BJ, Haller, JA, Rogers, MC, Epstein, MH and Freeman, JM, (1983) Long-term outcome of children with severe head trauma and prolonged coma. Pediatrics 71: 756-62.
59. McKinlay, A, Dalrymple-Alford, JC, Horwood, LJ and Fergusson, DM, (2002) Long term psychosocial outcomes after mild head injury in early childhood. J Neurol Neurosurg Psychiatry 73: 281-8.
60. Massagli, TL, Fann, JR, Burington, BE, Jaffe, KM, Katon, WJ and Thompson, RS, (2004) Psychiatric illness after mild traumatic brain injury in children. Arch Phys Med Rehabil 85: 1428-34.
61. Deputy, S, (2003) Shaking-impact syndrome of infancy. Semin Pediatr Neurol 10: 112-9.

62. Hawley, CA, Ward, AB, Magnay, AR and Long, J, (2002) Children's brain injury: a postal follow-up of 525 children from one health region in the UK. Brain Inj 16: 969-85.
63. Keenan, HT, Runyan, DK, Marshall, SW, Nocera, MA, Merten, DF and Sinal, SH, (2003) A population-based study of inflicted traumatic brain injury in young children. Jama 290: 621-6.
64. Ewing-Cobbs, L, Kramer, L, Prasad, M, Canales, DN, Louis, PT, Fletcher, JM et al., (1998) Neuroimaging, physical, and developmental findings after inflicted and noninflicted traumatic brain injury in young children. Pediatrics 102: 300-7.
65. Speidel, BD and Meadow, SR, (1972) Maternal epilepsy and abnormalities of the fetus and newborn. Lancet 2: 839-43.
66. Jones, KL, Lacro, RV, Johnson, KA and Adams, J, (1989) Pattern of malformations in the children of women treated with carbamazepine during pregnancy. N Engl J Med 320: 1661-6.
67. Holmes, LB, Harvey, EA, Coull, BA, Huntington, KB, Khoshbin, S, Hayes, AM et al., (2001) The teratogenicity of anticonvulsant drugs. N Engl J Med 344: 1132-8.
68. Dean, JC, Hailey, H, Moore, SJ, Lloyd, DJ, Turnpenny, PD and Little, J, (2002) Long term health and neurodevelopment in children exposed to antiepileptic drugs before birth. J Med Genet 39: 251-9.
69. Kaaja, E, Kaaja, R and Hiilesmaa, V, (2003) Major malformations in offspring of women with epilepsy. Neurology 60: 575-9.
70. Jick, SS and Terris, BZ, (1997) Anticonvulsants and congenital malformations. Pharmacotherapy 17: 561-4.
71. Strickler, SM, Dansky, LV, Miller, MA, Seni, MH, Andermann, E and Spielberg, SP, (1985) Genetic predisposition to phenytoin-induced birth defects. Lancet 2: 746-9.
72. Buehler, BA, Delimont, D, van Waes, M and Finnell, RH, (1990) Prenatal prediction of risk of the fetal hydantoin syndrome. N Engl J Med 322: 1567-72.
73. Zahn, C, (1998) Neurologic care of pregnant women with epilepsy. Epilepsia 39 Suppl 8: S26-31.
74. van der Pol, MC, Hadders-Algra, M, Huisjes, HJ and Touwen, BC, (1991) Antiepileptic medication in pregnancy: late effects on the children's central nervous system development. Am J Obstet Gynecol 164: 121-8.
75. Reinisch, JM, Sanders, SA, Mortensen, EL and Rubin, DB, (1995) In utero exposure to phenobarbital and intelligence deficits in adult men. Jama 274: 1518-25.
76. Wyszynski, DF, Nambisan, M, Surve, T, Alsdorf, RM, Smith, CR and Holmes, LB, (2005) Increased rate of major malformations in offspring exposed to valproate during pregnancy. Neurology 64: 961-5.
77. Lindhout, D and Schmidt, D, (1986) In-utero exposure to valproate and neural tube defects. Lancet 1: 1392-3.
78. Rosa, FW, (1991) Spina bifida in infants of women treated with carbamazepine during pregnancy. N Engl J Med 324: 674-7.
79. Matalon, S, Schechtman, S, Goldzweig, G and Ornoy, A, (2002) The teratogenic effect of carbamazepine: a meta-analysis of 1255 exposures. Reprod Toxicol 16: 9-17.
80. Hanson, JW, (1986) Teratogen update: fetal hydantoin effects. Teratology 33: 349-53.
81. Hansen, DK, (1991) The embryotoxicity of phenytoin: an update on possible mechanisms. Proc Soc Exp Biol Med 197: 361-8.
82. Adams, J, Vorhees, CV and Middaugh, LD, (1990) Developmental neurotoxicity of anticonvulsants: human and animal evidence on phenytoin. Neurotoxicol Teratol 12: 203-14.

83. Vanoverloop, D, Schnell, RR, Harvey, EA and Holmes, LB, (1992) The effects of prenatal exposure to phenytoin and other anticonvulsants on intellectual function at 4 to 8 years of age. Neurotoxicol Teratol 14: 329-35.
84. Dessens, AB, Cohen-Kettenis, PT, Mellenbergh, GJ, Koppe, JG, van De Poll, NE and Boer, K, (2000) Association of prenatal phenobarbital and phenytoin exposure with small head size at birth and with learning problems. Acta Paediatr 89: 533-41.
85. Kaindl, AM, Asimiadou, S, Manthey, D, Hagen, MV, Turski, L and Ikonomidou, C, (2006) Antiepileptic drugs and the developing brain. Cell Mol Life Sci 63: 399-413.
86. Adab, N, Jacoby, A, Smith, D and Chadwick, D, (2001) Additional educational needs in children born to mothers with epilepsy. J Neurol Neurosurg Psychiatry 70: 15-21.
87. Mawer, G, Clayton-Smith, J, Coyle, H and Kini, U, (2002) Outcome of pregnancy in women attending an outpatient epilepsy clinic: adverse features associated with higher doses of sodium valproate. Seizure 11: 512-8.
88. Gaily, E, Kantola-Sorsa, E and Granstrom, ML, (1988) Intelligence of children of epileptic mothers. J Pediatr 113: 677-84.
89. Gaily, E, Kantola-Sorsa, E and Granstrom, ML, (1990) Specific cognitive dysfunction in children with epileptic mothers. Dev Med Child Neurol 32: 403-14.
90. Hanson, JW, Myrianthopoulos, NC, Harvey, MA and Smith, DW, (1976) Risks to the offspring of women treated with hydantoin anticonvulsants, with emphasis on the fetal hydantoin syndrome. J Pediatr 89: 662-8.
91. Koch, S, Titze, K, Zimmermann, RB, Schroder, M, Lehmkuhl, U and Rauh, H, (1999) Long-term neuropsychological consequences of maternal epilepsy and anticonvulsant treatment during pregnancy for school-age children and adolescents. Epilepsia 40: 1237-43.
92. Scolnik, D, Nulman, I, Rovet, J, Gladstone, D, Czuchta, D, Gardner, HA et al., (1994) Neurodevelopment of children exposed in utero to phenytoin and carbamazepine monotherapy. Jama 271: 767-70.
93. Leavitt, AM, Yerby, MS, Robinson, N, Sells, CJ and Erickson, DM, (1992) Epilepsy in pregnancy: developmental outcome of offspring at 12 months. Neurology 42: 141-3.
94. Losche, G, Steinhausen, HC, Koch, S and Helge, H, (1994) The psychological development of children of epileptic parents. II. The differential impact of intrauterine exposure to anticonvulsant drugs and further influential factors. Acta Paediatr 83: 961-6.
95. Ornoy, A and Cohen, E, (1996) Outcome of children born to epileptic mothers treated with carbamazepine during pregnancy. Arch Dis Child 75: 517-20.
96. Moore, SJ, Turnpenny, P, Quinn, A, Glover, S, Lloyd, DJ, Montgomery, T et al., (2000) A clinical study of 57 children with fetal anticonvulsant syndromes. J Med Genet 37: 489-97.
97. Kozma, C, (2001) Valproic acid embryopathy: report of two siblings with further expansion of the phenotypic abnormalities and a review of the literature. Am J Med Genet 98: 168-75.
98. Vinten, J, Adab, N, Kini, U, Gorry, J, Gregg, J and Baker, GA, (2005) Neuropsychological effects of exposure to anticonvulsant medication in utero. Neurology 64: 949-54.
99. Farwell, JR, Lee, YJ, Hirtz, DG, Sulzbacher, SI, Ellenberg, JH and Nelson, KB, (1990) Phenobarbital for febrile seizures--effects on intelligence and on seizure recurrence. N Engl J Med 322: 364-9.
100. Sulzbacher, S, Farwell, JR, Temkin, N, Lu, AS and Hirtz, DG, (1999) Late cognitive effects of early treatment with phenobarbital. Clin Pediatr (Phila) 38: 387-94.

101. Thorp, JA, O'Connor, M, Jones, AM, Hoffman, EL and Belden, B, (1999) Does perinatal phenobarbital exposure affect developmental outcome at age 2? Am J Perinatol 16: 51-60.
102. Fishman, RH and Yanai, J, (1983) Long-lasting effects of early barbiturates on central nervous system and behavior. Neurosci Biobehav Rev 7: 19-28.
103. Diaz, J, Schain, RJ and Bailey, BG, (1977) Phenobarbital-induced brain growth retardation in artificially reared rat pups. Biol Neonate 32: 77-82.
104. Yanai, J, Rosselli-Austin, L and Tabakoff, B, (1979) Neuronal deficits in mice following prenatal exposure to phenobarbital. Exp Neurol 64: 237-44.
105. Schain, RJ and Watanabe, K, (1975) Effect of chronic phenobarbital administration upon brain growth of the infant rat. Exp Neurol 47: 509-15.
106. Abel, EL and Sokol, RJ, (1987) Incidence of fetal alcohol syndrome and economic impact of FAS-related anomalies. Drug Alcohol Depend 19: 51-70.
107. Burd, L, Martsolf, J, Klug, MG, O'Connor, E and Peterson, M, (2003) Prenatal alcohol exposure assessment: multiple embedded measures in a prenatal questionnaire. Neurotoxicol Teratol 25: 675-9.
108. Ebrahim, SH and Gfroerer, J, (2003) Pregnancy-related substance use in the United States during 1996-1998. Obstet Gynecol 101: 374-9.
109. Sulik, KK, Johnston, MC and Webb, MA, (1981) Fetal alcohol syndrome: embryogenesis in a mouse model. Science 214: 936-8.
110. Faingold, CL, N'Gouemo, P and Riaz, A, (1998) Ethanol and neurotransmitter interactions--from molecular to integrative effects. Prog Neurobiol 55: 509-35.
111. Streissguth, AP and O'Malley, K, (2000) Neuropsychiatric implications and long-term consequences of fetal alcohol spectrum disorders. Semin Clin Neuropsychiatry 5: 177-90.
112. Famy, C, Streissguth, AP and Unis, AS, (1998) Mental illness in adults with fetal alcohol syndrome or fetal alcohol effects. Am J Psychiatry 155: 552-4.
113. Jones, KL, Smith, DW, Ulleland, CN and Streissguth, P, (1973) Pattern of malformation in offspring of chronic alcoholic mothers. Lancet 1: 1267-71.
114. Jones, KL and Smith, DW, (1975) The fetal alcohol syndrome. Teratology 12: 1-10.
115. Barr, HM and Streissguth, AP, (2001) Identifying maternal self-reported alcohol use associated with fetal alcohol spectrum disorders. Alcohol Clin Exp Res 25: 283-7.
116. Sampson, PD, Streissguth, AP, Bookstein, FL, Little, RE, Clarren, SK, Dehaene, P et al., (1997) Incidence of fetal alcohol syndrome and prevalence of alcohol-related neurodevelopmental disorder. Teratology 56: 317-26.
117. Wachsman, L, Schuetz, S, Chan, LS and Wingert, WA, (1989) What happens to babies exposed to phencyclidine (PCP) in utero? Am J Drug Alcohol Abuse 15: 31-9.
118. Deutsch, SI, Mastropaolo, J and Rosse, RB, (1998) Neurodevelopmental consequences of early exposure to phencyclidine and related drugs. Clin Neuropharmacol 21: 320-32.
119. Fried, PA, Watkinson, B and Gray, R, (2003) Differential effects on cognitive functioning in 13- to 16-year-olds prenatally exposed to cigarettes and marihuana. Neurotoxicol Teratol 25: 427-36.
120. Kaindl, AM and Ikonomidou, C, (2007) Glutamate antagonists are neurotoxins for the developing brain. Neurotox Res 11: 203-18.
121. Hagberg, H, Ichord, R, Palmer, C, Yager, JY and Vannucci, SJ, (2002) Animal models of developmental brain injury: relevance to human disease. A summary of the panel discussion from the Third Hershey Conference on Developmental Cerebral Blood Flow and Metabolism. Dev Neurosci 24: 364-6.
122. Jevtovic-Todorovic, V, Hartman, RE, Izumi, Y, Benshoff, ND, Dikranian, K, Zorumski, CF et al., (2003) Early exposure to common anesthetic agents causes widespread

neurodegeneration in the developing rat brain and persistent learning deficits. J Neurosci 23: 876-82.
123. Bittigau, P, Sifringer, M, Genz, K, Reith, E, Pospischil, D, Govindarajalu, S et al., (2002) Antiepileptic drugs and apoptotic neurodegeneration in the developing brain. Proc Natl Acad Sci U S A 99: 15089-94.
124. Gerstner, B, Buhrer, C, Rheinlander, C, Polley, O, Schuller, A, Berns, M et al., (2006) Maturation-dependent oligodendrocyte apoptosis caused by hyperoxia. J Neurosci Res 84: 306-15.
125. Gerstner, B, DeSilva, TM, Genz, K, Armstrong, A, Brehmer, F, Neve, RL et al., (2008) Hyperoxia causes maturation-dependent cell death in the developing white matter. J Neurosci 28: 1236-45.
126. Finnell, RH, Waes, JG, Eudy, JD and Rosenquist, TH, (2002) Molecular basis of environmentally induced birth defects. Annu Rev Pharmacol Toxicol 42: 181-208.
127. Laeng, P, Pitts, RL, Lemire, AL, Drabik, CE, Weiner, A, Tang, H et al., (2004) The mood stabilizer valproic acid stimulates GABA neurogenesis from rat forebrain stem cells. J Neurochem 91: 238-51.
128. Lee, JK, Kim, SH, Lewis, EC, Azam, T, Reznikov, LL and Dinarello, CA, (2004) Differences in signaling pathways by IL-1beta and IL-18. Proc Natl Acad Sci U S A 101: 8815-20.
129. Wald, D, Commane, M, Stark, GR and Li, X, (2001) IRAK and TAK1 are required for IL-18-mediated signaling. Eur J Immunol 31: 3747-54.
130. Harris, C, Maroney, AC and Johnson, EM, Jr., (2002) Identification of JNK-dependent and -independent components of cerebellar granule neuron apoptosis. J Neurochem 83: 992-1001.
131. Takeda, K and Ichijo, H, (2002) Neuronal p38 MAPK signalling: an emerging regulator of cell fate and function in the nervous system. Genes Cells 7: 1099-111.
132. Nelson, KB, Dambrosia, JM, Grether, JK and Phillips, TM, (1998) Neonatal cytokines and coagulation factors in children with cerebral palsy. Ann Neurol 44: 665-75.
133. Minagawa, K, Tsuji, Y, Ueda, H, Koyama, K, Tanizawa, K, Okamura, H et al., (2002) Possible correlation between high levels of IL-18 in the cord blood of pre-term infants and neonatal development of periventricular leukomalacia and cerebral palsy. Cytokine 17: 164-70.
134. Redecker, C, Hagemann, G, Witte, OW, Marret, S, Evrard, P and Gressens, P, (1998) Long-term evolution of excitotoxic cortical dysgenesis induced in the developing rat brain. Brain Res Dev Brain Res 109: 109-13.
135. Ikonomidou, C, Qin, Y, Labruyere, J, Kirby, C and Olney, JW, (1996) Prevention of trauma-induced neurodegeneration in infant rat brain. Pediatr Res 39: 1020-7.
136. Felderhoff-Mueser, U and Ikonomidou, C, (2000) Mechanisms of neurodegeneration after paediatric brain injury. Curr Opin Neurol 13: 141-5.
137. Felderhoff-Mueser, U, Sifringer, M, Pesditschek, S, Kuckuck, H, Moysich, A, Bittigau, P et al., (2002) Pathways leading to apoptotic neurodegeneration following trauma to the developing rat brain. Neurobiol Dis 11: 231-45.
138. Bittigau, P, Pohl, D, Sifringer, M, Shimizu, H, Ikeda, M, Ishimaru, M et al., (1998) Modeling pediatric head trauma: mechanisms of degeneration and potential strategies for neuroprotection. Restor Neurol Neurosci 13: 11-23.
139. Bittigau, P, Sifringer, M, Felderhoff-Mueser, U, Hansen, HH and Ikonomidou, C, (2003) Neuropathological and biochemical features of traumatic injury in the developing brain. Neurotox Res 5: 475-90.

140. Bittigau, P, Sifringer, M, Felderhoff-Mueser, U and Ikonomidou, C, (2004) Apoptotic neurodegeneration in the context of traumatic injury to the developing brain. Exp Toxicol Pathol 56: 83-9.
141. Kochanek, PM, Clark, RS, Ruppel, RA, Adelson, PD, Bell, MJ, Whalen, MJ et al., (2000) Biochemical, cellular, and molecular mechanisms in the evolution of secondary damage after severe traumatic brain injury in infants and children: Lessons learned from the bedside. Pediatr Crit Care Med 1: 4-19.
142. Rogawski, MA and Loscher, W, (2004) The neurobiology of antiepileptic drugs for the treatment of nonepileptic conditions. Nat Med 10: 685-92.
143. Rudolph, U, Crestani, F, Benke, D, Brunig, I, Benson, JA, Fritschy, JM et al., (1999) Benzodiazepine actions mediated by specific gamma-aminobutyric acid(A) receptor subtypes. Nature 401: 796-800.
144. Crestani, F, Martin, JR, Mohler, H and Rudolph, U, (2000) Mechanism of action of the hypnotic zolpidem in vivo. Br J Pharmacol 131: 1251-4.
145. Macdonald, RL and Olsen, RW, (1994) GABAA receptor channels. Annu Rev Neurosci 17: 569-602.
146. ffrench-Mullen, JM, Barker, JL and Rogawski, MA, (1993) Calcium current block by (-)-pentobarbital, phenobarbital, and CHEB but not (+)-pentobarbital in acutely isolated hippocampal CA1 neurons: comparison with effects on GABA-activated Cl- current. J Neurosci 13: 3211-21.
147. Kleckner, NW, Glazewski, JC, Chen, CC and Moscrip, TD, (1999) Subtype-selective antagonism of N-methyl-D-aspartate receptors by felbamate: insights into the mechanism of action. J Pharmacol Exp Ther 289: 886-94.
148. Hansen, HH, Briem, T, Dzietko, M, Sifringer, M, Voss, A, Rzeski, W et al., (2004) Mechanisms leading to disseminated apoptosis following NMDA receptor blockade in the developing rat brain. Neurobiol Dis 16: 440-53.
149. Ogura, H, Yasuda, M, Nakamura, S, Yamashita, H, Mikoshiba, K and Ohmori, H, (2002) Neurotoxic damage of granule cells in the dentate gyrus and the cerebellum and cognitive deficit following neonatal administration of phenytoin in mice. J Neuropathol Exp Neurol 61: 956-67.
150. Pawlikowski, M, Stepien, H, Mroz-Wasilewska, Z and Pawlikowska, A, (1987) Effects of diazepam on cell proliferation in cerebral cortex, anterior pituitary and thymus of developing rats. Life Sci 40: 1131-5.
151. Jackson, GD, Williams, SR, Weller, RO, van Bruggen, N, Preece, NE, Williams, SC et al., (1994) Vigabatrin-induced lesions in the rat brain demonstrated by quantitative magnetic resonance imaging. Epilepsy Res 18: 57-66.
152. Wong, WT and Wong, RO, (2001) Changing specificity of neurotransmitter regulation of rapid dendritic remodeling during synaptogenesis. Nat Neurosci 4: 351-2.
153. Bashir, ZI, Bortolotto, ZA, Davies, CH, Berretta, N, Irving, AJ, Seal, AJ et al., (1993) Induction of LTP in the hippocampus needs synaptic activation of glutamate metabotropic receptors. Nature 363: 347-50.
154. Luthi, A, Schwyzer, L, Mateos, JM, Gahwiler, BH and McKinney, RA, (2001) NMDA receptor activation limits the number of synaptic connections during hippocampal development. Nat Neurosci 4: 1102-7.
155. Brooks, WJ, Petit, TL and LeBoutillier, JC, (1997) Effect of chronic administration of NMDA antagonists on synaptic development. Synapse 26: 104-13.
156. Serrano, EE, Kunis, DM and Ransom, BR, (1988) Effects of chronic phenobarbital exposure on cultured mouse spinal cord neurons. Ann Neurol 24: 429-38.

157. Bergey, GK, Swaiman, KF, Schrier, BK, Fitzgerald, S and Nelson, PG, (1981) Adverse effects of phenobarbital on morphological and biochemical development of fetal mouse spinal cord neurons in culture. Ann Neurol 9: 584-9.
158. McKinney, RA, Capogna, M, Durr, R, Gahwiler, BH and Thompson, SM, (1999) Miniature synaptic events maintain dendritic spines via AMPA receptor activation. Nat Neurosci 2: 44-9.
159. Bear, MF, Kleinschmidt, A, Gu, QA and Singer, W, (1990) Disruption of experience-dependent synaptic modifications in striate cortex by infusion of an NMDA receptor antagonist. J Neurosci 10: 909-25.
160. Rocha, M and Sur, M, (1995) Rapid acquisition of dendritic spines by visual thalamic neurons after blockade of N-methyl-D-aspartate receptors. Proc Natl Acad Sci U S A 92: 8026-30.
161. Brewer, GJ and Cotman, CW, (1989) NMDA receptor regulation of neuronal morphology in cultured hippocampal neurons. Neurosci Lett 99: 268-73.
162. Fox, K, Schlaggar, BL, Glazewski, S and O'Leary, DD, (1996) Glutamate receptor blockade at cortical synapses disrupts development of thalamocortical and columnar organization in somatosensory cortex. Proc Natl Acad Sci U S A 93: 5584-9.
163. Cohen, JA, Fisher, RS, Brigell, MG, Peyster, RG and Sze, G, (2000) The potential for vigabatrin-induced intramyelinic edema in humans. Epilepsia 41: 148-57.
164. Sidhu, RS, Del Bigio, MR, Tuor, UI and Seshia, SS, (1997) Low-dose vigabatrin (gamma-vinyl GABA)-induced damage in the immature rat brain. Exp Neurol 144: 400-5.
165. Qiao, M, Malisza, KL, Del Bigio, MR, Kozlowski, P, Seshia, SS and Tuor, UI, (2000) Effect of long-term vigabatrin administration on the immature rat brain. Epilepsia 41: 655-65.
166. Fishman, RH, Ornoy, A and Yanai, J, (1989) Correlated ultrastructural damage between cerebellum cells after early anticonvulsant treatment in mice. Int J Dev Neurosci 7: 15-26.
167. Patsalos, PN and Wiggins, RC, (1982) Brain maturation following administration of phenobarbital, phenytoin, and sodium valproate to developing rats or to their dams: effects on synthesis of brain myelin and other subcellular membrane proteins. J Neurochem 39: 915-23.
168. Saliba, E, Favrais, G and Gressens, P, (2007) Neuroprotection of the newborn: from bench to cribside. Semin Fetal Neonatal Med 12: 239-40.
169. Schulzke, SM, Rao, S and Patole, SK, (2007) A systematic review of cooling for neuroprotection in neonates with hypoxic ischemic encephalopathy - are we there yet? BMC Pediatr 7: 30.
170. Juul, S and Felderhoff-Mueser, U, (2007) Epo and other hematopoietic factors. Semin Fetal Neonatal Med 12: 250-8.
171. Sizonenko, SV, Bednarek, N and Gressens, P, (2007) Growth factors and plasticity. Semin Fetal Neonatal Med 12: 241-9.
172. Strohman, R, (1994) Epigenesis: the missing beat in biotechnology? Biotechnology (N Y) 12: 156-64.
173. Hanash, S, (2003) Disease proteomics. Nature 422: 226-32.
174. Wilkins, MR, Sanchez, JC, Gooley, AA, Appel, RD, Humphery-Smith, I, Hochstrasser, DF et al., (1996) Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it. Biotechnol Genet Eng Rev 13: 19-50.
175. Anderson, L and Seilhamer, J, (1997) A comparison of selected mRNA and protein abundances in human liver. Electrophoresis 18: 533-7.

176. Paulson, L, Martin, P, Persson, A, Nilsson, CL, Ljung, E, Westman-Brinkmalm, A et al., (2003) Comparative genome- and proteome analysis of cerebral cortex from MK-801-treated rats. J Neurosci Res 71: 526-33.
177. Gygi, SP, Rochon, Y, Franza, BR and Aebersold, R, (1999) Correlation between protein and mRNA abundance in yeast. Mol Cell Biol 19: 1720-30.
178. Washburn, MP, Wolters, D and Yates, JR, 3rd, (2001) Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nat Biotechnol 19: 242-7.
179. Vidal, M and Legrain, P, (1999) Yeast forward and reverse 'n'-hybrid systems. Nucleic Acids Res 27: 919-29.
180. Cutler, P, (2003) Protein arrays: the current state-of-the-art. Proteomics 3: 3-18.
181. Melton, L, (2004) Protein arrays: proteomics in multiplex. Nature 429: 101-7.
182. Sidhu, SS, (2000) Phage display in pharmaceutical biotechnology. Curr Opin Biotechnol 11: 610-6.
183. Olsen, JV, Andersen, JR, Nielsen, PA, Nielsen, ML, Figeys, D, Mann, M et al., (2004) HysTag--a novel proteomic quantification tool applied to differential display analysis of membrane proteins from distinct areas of mouse brain. Mol Cell Proteomics 3: 82-92.
184. Kaindl AM, SM, Zabel C, Lehnert R, Stefovska V, Klose J, Ikonomidou C, (2007) Subacute proteome changes following traumatic injury of the developing brain: Implications for a dysregulation of neuronal migration and neurite arborization. Proteomics - Clinical Application 1: 640-649.
185. Kaindl AM, KA, Nebrich G, Stuwe J, Sifringer M, Klose J, Ikonomidou C, (2008) Brief alteration of NMDA- or GABAA-receptor mediated neurotransmission has long-term effects on the developing cerebral cortex. Moll Cell Proteomics in press.
186. Kaindl AM, SM, Koppelstaetter A, Genz K, Stuwe J, Klose J, Felderhoff-Mueser U. , (2008) Erythropoietin attenuates hyperoxia-induced cell death and proteome changes in the developing brain. Ann Neurol in press.
187. Zabel, C, Sagi, D, Kaindl, AM, Steireif, N, Klare, Y, Mao, L et al., (2006) Comparative proteomics in neurodegenerative and non-neurodegenerative diseases suggest nodal point proteins in regulatory networking. J Proteome Res 5: 1948-58.
188. Seefeldt, I, Nebrich, G, Romer, I, Mao, L and Klose, J, (2006) Evaluation of 2-DE protein patterns from pre- and postnatal stages of the mouse brain. Proteomics 6: 4932-9.
189. Plaisant, F, Clippe, A, Vander Stricht, D, Knoops, B and Gressens, P, (2003) Recombinant peroxiredoxin 5 protects against excitotoxic brain lesions in newborn mice. Free Radic Biol Med 34: 862-72.
190. Das, KC, (2004) Thioredoxin system in premature and newborn biology. Antioxid Redox Signal 6: 177-84.
191. Krapfenbauer, K, Engidawork, E, Cairns, N, Fountoulakis, M and Lubec, G, (2003) Aberrant expression of peroxiredoxin subtypes in neurodegenerative disorders. Brain Res 967: 152-60.
192. Soerensen, J, Jakupoglu, C, Beck, H, Forster, H, Schmidt, J, Schmahl, W et al., (2008) The role of thioredoxin reductases in brain development. PLoS ONE 3: e1813.
193. Stefovska V, UO, Czuczwar M, Smitka M, Czuczwar P, Kis J, Kaindl AM, Turski L, Turski WA, Ikonomidou C, (2008) Sedative and anticonvulsant drugs suppress postnatal neurogenesis. Ann Neurol in press.
194. Joo, JY, Kim, BW, Lee, JS, Park, JY, Kim, S, Yun, YJ et al., (2007) Activation of NMDA receptors increases proliferation and differentiation of hippocampal neural progenitor cells. J Cell Sci 120: 1358-70.

195. Mochizuki, N, Takagi, N, Kurokawa, K, Kawai, T, Besshoh, S, Tanonaka, K et al., (2007) Effect of NMDA receptor antagonist on proliferation of neurospheres from embryonic brain. Neurosci Lett 417: 143-8.
196. Suzuki, M, Nelson, AD, Eickstaedt, JB, Wallace, K, Wright, LS and Svendsen, CN, (2006) Glutamate enhances proliferation and neurogenesis in human neural progenitor cell cultures derived from the fetal cortex. Eur J Neurosci 24: 645-53.
197. Schlett, K, (2006) Glutamate as a modulator of embryonic and adult neurogenesis. Curr Top Med Chem 6: 949-60.
198. Owens, DF and Kriegstein, AR, (2002) Is there more to GABA than synaptic inhibition? Nat Rev Neurosci 3: 715-27.
199. Xiong, Y, Mahmood, A, Lu, D, Qu, C, Goussev, A, Schallert, T et al., (2007) Role of gender in outcome after traumatic brain injury and therapeutic effect of erythropoietin in mice. Brain Res 1185: 301-12.
200. Urrea, C, Castellanos, DA, Sagen, J, Tsoulfas, P, Bramlett, HM and Dietrich, WD, (2007) Widespread cellular proliferation and focal neurogenesis after traumatic brain injury in the rat. Restor Neurol Neurosci 25: 65-76.
201. Kaibuchi, K, Kuroda, S and Amano, M, (1999) Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells. Annu Rev Biochem 68: 459-86.
202. Arimura, N, Menager, C, Fukata, Y and Kaibuchi, K, (2004) Role of CRMP-2 in neuronal polarity. J Neurobiol 58: 34-47.
203. Arimura, N, Inagaki, N, Chihara, K, Menager, C, Nakamura, N, Amano, M et al., (2000) Phosphorylation of collapsin response mediator protein-2 by Rho-kinase. Evidence for two separate signaling pathways for growth cone collapse. J Biol Chem 275: 23973-80.
204. Quinn, CC, Gray, GE and Hockfield, S, (1999) A family of proteins implicated in axon guidance and outgrowth. J Neurobiol 41: 158-64.
205. Suzuki, Y, Nakagomi, S, Namikawa, K, Kiryu-Seo, S, Inagaki, N, Kaibuchi, K et al., (2003) Collapsin response mediator protein-2 accelerates axon regeneration of nerve-injured motor neurons of rat. J Neurochem 86: 1042-50.
206. Inagaki, N, Chihara, K, Arimura, N, Menager, C, Kawano, Y, Matsuo, N et al., (2001) CRMP-2 induces axons in cultured hippocampal neurons. Nat Neurosci 4: 781-2.
207. Katoh, S, Mitsui, Y, Kitani, K and Suzuki, T, (1999) Hyperoxia induces the neuronal differentiated phenotype of PC12 cells via a sustained activity of mitogen-activated protein kinase induced by Bcl-2. Biochem J 338 (Pt 2): 465-70.
208. Keyvani, K and Schallert, T, (2002) Plasticity-associated molecular and structural events in the injured brain. J Neuropathol Exp Neurol 61: 831-40.
209. Pastrana, E, Moreno-Flores, MT, Gurzov, EN, Avila, J, Wandosell, F and Diaz-Nido, J, (2006) Genes associated with adult axon regeneration promoted by olfactory ensheathing cells: a new role for matrix metalloproteinase 2. J Neurosci 26: 5347-59.
210. Matsutani, S and Yamamoto, N, (1998) GABAergic neuron-to-astrocyte signaling regulates dendritic branching in coculture. J Neurobiol 37: 251-64.
211. Barbin, G, Pollard, H, Gaiarsa, JL and Ben-Ari, Y, (1993) Involvement of GABAA receptors in the outgrowth of cultured hippocampal neurons. Neurosci Lett 152: 150-4.
212. Chang, YS, Mu, D, Wendland, M, Sheldon, RA, Vexler, ZS, McQuillen, PS et al., (2005) Erythropoietin improves functional and histological outcome in neonatal stroke. Pediatr Res 58: 106-11.
213. Demers, EJ, McPherson, RJ and Juul, SE, (2005) Erythropoietin protects dopaminergic neurons and improves neurobehavioral outcomes in juvenile rats after neonatal hypoxia-ischemia. Pediatr Res 58: 297-301.

214. Sola, A, Wen, TC, Hamrick, SE and Ferriero, DM, (2005) Potential for protection and repair following injury to the developing brain: a role for erythropoietin? Pediatr Res 57: 110R-117R.
215. Digicaylioglu, M and Lipton, SA, (2001) Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-kappaB signalling cascades. Nature 412: 641-7.
216. Matsushita, H, Johnston, MV, Lange, MS and Wilson, MA, (2003) Protective effect of erythropoietin in neonatal hypoxic ischemia in mice. Neuroreport 14: 1757-61.
217. Sun, Y, Zhou, C, Polk, P, Nanda, A and Zhang, JH, (2004) Mechanisms of erythropoietin-induced brain protection in neonatal hypoxia-ischemia rat model. J Cereb Blood Flow Metab 24: 259-70.
218. Kawakami, M, Iwasaki, S, Sato, K and Takahashi, M, (2000) Erythropoietin inhibits calcium-induced neurotransmitter release from clonal neuronal cells. Biochem Biophys Res Commun 279: 293-7.
219. Keller, M, Yang, J, Griesmaier, E, Gorna, A, Sarkozy, G, Urbanek, M et al., (2006) Erythropoietin is neuroprotective against NMDA-receptor-mediated excitotoxic brain injury in newborn mice. Neurobiol Dis 24: 357-66.
220. Maiese, K, Li, F and Chong, ZZ, (2004) Erythropoietin in the brain: can the promise to protect be fulfilled? Trends Pharmacol Sci 25: 577-83.
221. Sakanaka, M, Wen, TC, Matsuda, S, Masuda, S, Morishita, E, Nagao, M et al., (1998) In vivo evidence that erythropoietin protects neurons from ischemic damage. Proc Natl Acad Sci U S A 95: 4635-40.
222. Kumral, A, Gonenc, S, Acikgoz, O, Sonmez, A, Genc, K, Yilmaz, O et al., (2005) Erythropoietin increases glutathione peroxidase enzyme activity and decreases lipid peroxidation levels in hypoxic-ischemic brain injury in neonatal rats. Biol Neonate 87: 15-8.
223. Solaroglu, I, Solaroglu, A, Kaptanoglu, E, Dede, S, Haberal, A, Beskonakli, E et al., (2003) Erythropoietin prevents ischemia-reperfusion from inducing oxidative damage in fetal rat brain. Childs Nerv Syst 19: 19-22.
224. Genc, S, Akhisaroglu, M, Kuralay, F and Genc, K, (2002) Erythropoietin restores glutathione peroxidase activity in 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine-induced neurotoxicity in C57BL mice and stimulates murine astroglial glutathione peroxidase production in vitro. Neurosci Lett 321: 73-6.
225. Li, F, Chong, ZZ and Maiese, K, (2006) Microglial integrity is maintained by erythropoietin through integration of Akt and its substrates of glycogen synthase kinase-3beta, beta-catenin, and nuclear factor-kappaB. Curr Neurovasc Res 3: 187-201.
226. Liu, Q, Yu, L, Gao, J, Fu, Q, Zhang, J, Zhang, P et al., (2000) Cloning, tissue expression pattern and genomic organization of latexin, a human homologue of rat carboxypeptidase A inhibitor. Mol Biol Rep 27: 241-6.
227. Thornalley, PJ, (2003) Glyoxalase I--structure, function and a critical role in the enzymatic defence against glycation. Biochem Soc Trans 31: 1343-8.
228. Basta, G, Lazzerini, G, Massaro, M, Simoncini, T, Tanganelli, P, Fu, C et al., (2002) Advanced glycation end products activate endothelium through signal-transduction receptor RAGE: a mechanism for amplification of inflammatory responses. Circulation 105: 816-22.
229. Thornalley, PJ, (1998) Glutathione-dependent detoxification of alpha-oxoaldehydes by the glyoxalase system: involvement in disease mechanisms and antiproliferative activity of glyoxalase I inhibitors. Chem Biol Interact 111-112: 137-51.
230. Yan, SF, Ramasamy, R, Naka, Y and Schmidt, AM, (2003) Glycation, inflammation, and RAGE: a scaffold for the macrovascular complications of diabetes and beyond. Circ Res 93: 1159-69.

231. Inoue, Y, Tsujimoto, Y and Kimura, A, (1998) Expression of the glyoxalase I gene of Saccharomyces cerevisiae is regulated by high osmolarity glycerol mitogen-activated protein kinase pathway in osmotic stress response. J Biol Chem 273: 2977-83.
232. Van Herreweghe, F, Mao, J, Chaplen, FW, Grooten, J, Gevaert, K, Vandekerckhove, J et al., (2002) Tumor necrosis factor-induced modulation of glyoxalase I activities through phosphorylation by PKA results in cell death and is accompanied by the formation of a specific methylglyoxal-derived AGE. Proc Natl Acad Sci U S A 99: 949-54.
233. Huang, EJ and Reichardt, LF, (2001) Neurotrophins: roles in neuronal development and function. Annu Rev Neurosci 24: 677-736.
234. Adembri, C, Massagrande, A, Tani, A, Miranda, M, Margheri, M, De Gaudio, R et al., (2008) Carbamylated erythropoietin derivative is neuroprotective in an experimental model of traumatic brain injury. Crit Care Med.
235. Dzietko, M, Felderhoff-Mueser, U, Sifringer, M, Krutz, B, Bittigau, P, Thor, F et al., (2004) Erythropoietin protects the developing brain against N-methyl-D-aspartate receptor antagonist neurotoxicity. Neurobiol Dis 15: 177-87.
236. Gorio, A, Gokmen, N, Erbayraktar, S, Yilmaz, O, Madaschi, L, Cichetti, C et al., (2002) Recombinant human erythropoietin counteracts secondary injury and markedly enhances neurological recovery from experimental spinal cord trauma. Proc Natl Acad Sci U S A 99: 9450-5.
237. Grimm, C, Wenzel, A, Groszer, M, Mayser, H, Seeliger, M, Samardzija, M et al., (2002) HIF-1-induced erythropoietin in the hypoxic retina protects against light-induced retinal degeneration. Nat Med 8: 718-24.
238. Junk, AK, Mammis, A, Savitz, SI, Singh, M, Roth, S, Malhotra, S et al., (2002) Erythropoietin administration protects retinal neurons from acute ischemia-reperfusion injury. Proc Natl Acad Sci U S A 99: 10659-64.
239. Kumral, A, Ozer, E, Yilmaz, O, Akhisaroglu, M, Gokmen, N, Duman, N et al., (2003) Neuroprotective effect of erythropoietin on hypoxic-ischemic brain injury in neonatal rats. Biol Neonate 83: 224-8.
240. Li, Y, Lu, ZY, Ogle, M and Wei, L, (2007) Erythropoietin prevents blood brain barrier damage induced by focal cerebral ischemia in mice. Neurochem Res 32: 2132-41.
241. Weber, A, Dzietko, M, Berns, M, Felderhoff-Mueser, U, Heinemann, U, Maier, RF et al., (2005) Neuronal damage after moderate hypoxia and erythropoietin. Neurobiol Dis 20: 594-600.
242. Tubbs, RS, Shoja, MM, Jamshidi, M and Shokouhi, G, (2007) Does the neuroprotective agent erythropoietin amplify diffuse axonal injury in its early stages? Med Hypotheses 69: 1385-6.
243. McPherson, RJ and Juul, SE, (2007) Recent trends in erythropoietin-mediated neuroprotection. Int J Dev Neurosci.
244. Ohls, RK, Ehrenkranz, RA, Das, A, Dusick, AM, Yolton, K, Romano, E et al., (2004) Neurodevelopmental outcome and growth at 18 to 22 months' corrected age in extremely low birth weight infants treated with early erythropoietin and iron. Pediatrics 114: 1287-91.
245. Schwikowski, B, Uetz, P and Fields, S, (2000) A network of protein-protein interactions in yeast. Nat Biotechnol 18: 1257-61.
246. Giot, L, Bader, JS, Brouwer, C, Chaudhuri, A, Kuang, B, Li, Y et al., (2003) A protein interaction map of Drosophila melanogaster. Science 302: 1727-36.
247. Goehler, H, Lalowski, M, Stelzl, U, Waelter, S, Stroedicke, M, Worm, U et al., (2004) A protein interaction network links GIT1, an enhancer of huntingtin aggregation, to Huntington's disease. Mol Cell 15: 853-65.

248. Stelzl, U, Worm, U, Lalowski, M, Haenig, C, Brembeck, FH, Goehler, H et al., (2005) A human protein-protein interaction network: a resource for annotating the proteome. Cell 122: 957-68.
249. Jain, R, Rivera, MC and Lake, JA, (1999) Horizontal gene transfer among genomes: the complexity hypothesis. Proc Natl Acad Sci U S A 96: 3801-6.
250. Aris-Brosou, S, (2005) Determinants of adaptive evolution at the molecular level: the extended complexity hypothesis. Mol Biol Evol 22: 200-9.
251. Klose, J, Nock, C, Herrmann, M, Stuhler, K, Marcus, K, Bluggel, M et al., (2002) Genetic analysis of the mouse brain proteome. Nat Genet 30: 385-93.

8 Declaration in lieu of oath

Eidesstattliche Versicherung

gemäß Habilitationsordnung der Charité – Universitätsmedizin Berlin

Hiermit erkläre ich, daß

- weder früher noch gleichzeitig ein Habilitationsverfahren durchgeführt oder angemeldet wurde;
- die vorgelegte Habilitationsschrift ohne fremde Hilfe verfaßt wurde, die beschriebenen Ergebnisse selbst gewonnen wurden und die verwendeten Hilfsmittel, die Zusammenarbeit mit anderen Wissenschaftlerinnen, oder Wissenschaftlern und technischen Hilfskräften sowie die Literatur vollständig angegeben sind;
- mir die geltende Habilitationsordnung der Chaité bekannt ist.

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