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REVIEWS

# Perinatal Brain Damage: Mechanisms and Neuroprotective Strategies

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**Abstract |** Injury to the perinatal brain is a leading cause of childhood mortality and lifelong disability. Cerebral palsy and cognitive impairment are usually related to periventricular white matter damage, which is seen chiefly in babies born before 32 weeks' gestational age, and to cortico-subcortical lesions, which occur mainly in full term infants. Perinatal brain lesions remain largely unpreventable and untreatable despite recent improvements in neonatal care. However, partial neuroprotective efficacy has been proven using magnesium sulfate in preterm newborns and hypothermia in term newborns. Improved understanding of the pathophysiological mechanisms involved in perinatal brain lesions helps to identify potential targets for neuroprotective interventions, as discussed in this review.

Injury to the perinatal brain is a leading cause of death and disability in children. Despite major improvements in perinatal care, the incidence of neurological disabilities related to perinatal brain damage has not decreased significantly in Western countries over the last decades. Cerebral palsy and cognitive impairment are usually related to periventricular white matter damage (PWMD), which is seen chiefly in babies born before 32 weeks' gestational age, or to cortico-subcortical lesions, which occur mainly in full term infants. The formidable ethical, technical, and financial obstacles raised by research into perinatal brain damage have largely prevented the pharmaceutical industry from developing effective medications. To date, there are no effective treatments for perinatal brain lesions. Nevertheless, epidemiological and experimental data have allowed researchers to identify a number of potential targets for neuroprotective strategies. New animal models have led to the elucidation of biochemical events involved in neurodegeneration and neuroprotection.

The etiology is complex and multifactorial but hypoxia-ischemia (HI), infection/inflammation and excitotoxicity (see below) are considered important causes or precipitating insults of preventable /treatable forms of perinatal brain injury. In this review we will focus on mechanisms of brain injury in response to acute sterile insults in the developing brain that occur in term (e.g. neonatal encephalopathy) or preterm infants.

Genetic background, development age, sex and brain maturity affect vulnerability and the mechanisms of brain injury.[1,2] Furthermore, antecedents like infection/inflammation, intrauterine growth restriction or preexposures to hypoxia can modulate brain vulnerability in response to acute insults.[3–5] Brain injury evolves over time and different mechanisms are critical during the primary, secondary and tertiary phases (Fig. 1). Indeed, recent experimental data suggests that interventions can be effective if administered hours, days or even weeks after the primary insult.[6,7]

The aim of the present review is to describe the critical mechanisms of brain injury during the secondary and tertiary phases[8] after an acute insult and to present some therapeutic strategies that may have potential for clinical translation.

## 1 Concept of Secondary Brain Injury

In experimental studies, cerebral HI of sufficient severity to deplete tissue energy reserves (primary insult) is often followed by transient but complete restoration of glucose utilization,

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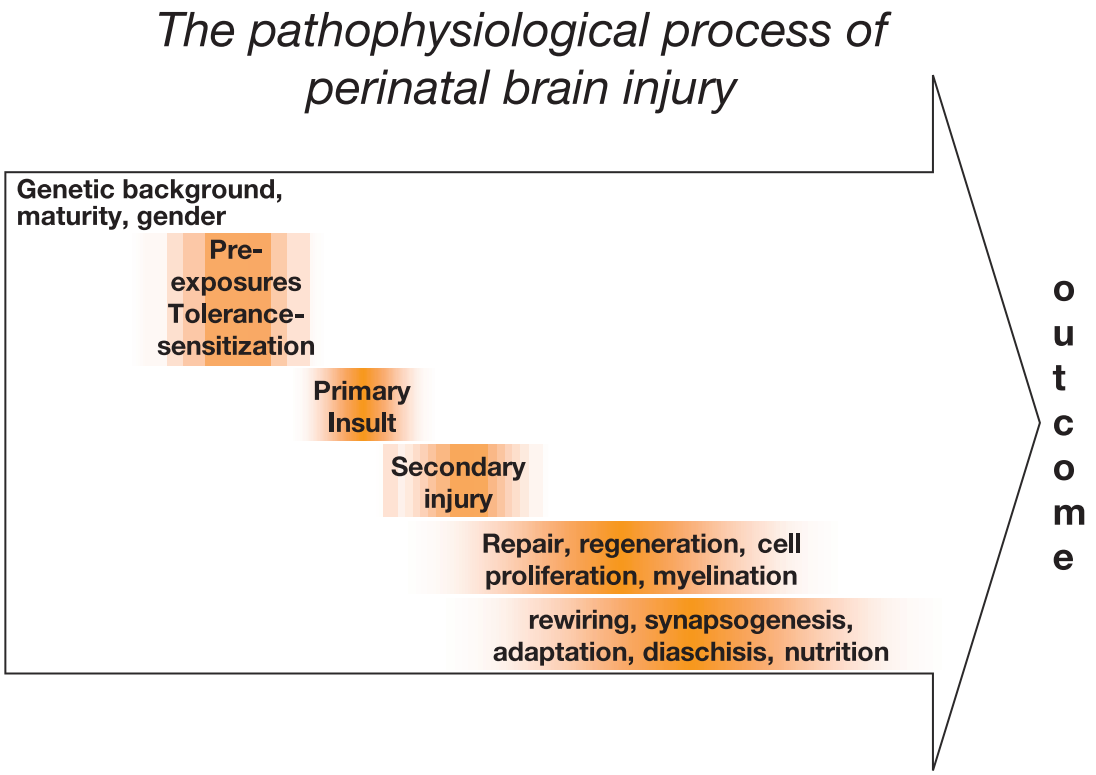


***Figure 1:*** Multiple factors occurring alone or in combination, prior, during and after the acute perinatal insult determine the outcome. Injurious and reparative processes are operative both during the secondary phase shortly after well as during the tertiary phase day-weeks after exposure.

ATP, and phosphocreatine upon reperfusion/reoxygenation.[9–11] Thereafter a secondary decrease of high energy phosphates occurs in parallel with a decrease in tissue glucose metabolism and development of cell injury.[9–11] Similarly, infants with neonatal encepalopathy show characteristic abnormalities in cerebral energy metabolism, which is often normal soon after birth, but shows a progressive decline in PCr]/Pi] some hours later.[12] Infants displaying this phenomenon develop severe neurodevelopmental impairment or die, and there is a close relationship between the magnitude of the late decline in PCr]/Pi], reduced brain growth and the severity of neurodevelopmental impairment four years later.[13]

These findings suggest that most of the injury after HI evolves over time *after* rather than during the insult. There are many examples of successful post-treatment after HI in animals suggesting a therapeutic window following HI prior to the secondary phase of tissue deterioration. Hypothermia following HI reduces secondary energy failure and brain injury in experimental studies[14] and was later proven effective as neuroprotective treatment in newborns with neonatal encephalopathy.[15] However, the mechanisms involved in secondary brain injury are incompletely understood and such information is critical for development of the next generation of therapies for preterm infants or to be combined with hypothermia in severely asphyxiated infants at term, hopefully, to further reduce serious disability in children and adults.

### 1.1 *Mechanisms of secondary brain injury*

The deficit in high-energy phosphates induced by HI leads to a primary failure to maintain transmembrane ionic gradients, release of neuroactive compounds into the extracellular compartments, accumulation of intracellular $Ca^{2+}$ and the activation of a series of mechanisms that if sustained will lead to immediate cell death. If the individual is resuscitated, these acute alterations are completely or partly reversed but the complex process has been started in which multiple interrelated factors may produce secondary brain injury.

The precise mechanisms of damage are incompletely understood but some components of the process have been elucidated. Excitatory amino acids (EAAs), mitochondrial impairment, intracellular calcium regulation, generation of reactive oxygen species (ROS) including nitric oxide (NO), apoptotic/necrotic mechanisms, changes in the availability of trophic factors and the

immuno-inflammatory system are all implicated in the process (Fig. 2).

**1.1.1 Excitotoxicity:** Glutamate and aspartate are the main excitatory transmitters in the brain, but they are known to exert toxic effects (excitotoxicity) if applied in excess to the nervous system. Both N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) /kainate receptors are expressed on neurons and oligodendroglial precursors (preferentially on somata) in vulnerable areas of grey and white matter.[16–18] The expression of EAA receptors is up regulated in the immature human brain which reflects the critical role of these receptors for brain development. Hence, the immature brain is also more vulnerable to excitotoxicity (especially NMDA) than the adult[19] affecting both white and grey matter.[20]

There is considerable evidence for a role of EAAs in the process leading to HI brain injury. EAA receptors are expressed by all types of neural cells. In particular, neurons[17] and oligodendrocyte precursor cells (OPCs)[21] express NMDA and AMPA receptors, while microglia[22] have been recently shown to express NMDA receptors (Fig. 3). NMDA receptors have also been described on endothelial cells.[23] Excess activation of neuronal NMDA or AMPA receptors leads to neuronal cell death. Excess activation of OPC AMPA receptors leads to OPC cell death while OPC NMDA receptor activation seems to lead to blockade of differentiation rather than cell death. Activation of microglial NMDA receptors leads to microglial activation and release of factors potentially toxic for neighboring neural cells. Extracellular concentrations of EAAs, and to some extent glycine, increase extracellularly during neonatal HI in mixed grey and white matter of fetal sheep[24] and is followed by a secondary increase during reflow.[25,26] EAAs also increase markedly in the CSF of newborns with neonatal encephalopathy and the levels are associated with the degree of encephalopathy and short-term outcome.[27]

Blocking NMDA receptors before or after HI reduces subsequent neuronal damage[28] and during "in vitro ischemia", NMDA receptor activation results in $Ca^{2+}$-dependent injury of oligodendroglial processes.[18] AMPA blockade reduces grey and white matter damage when given after HI or excitotoxic

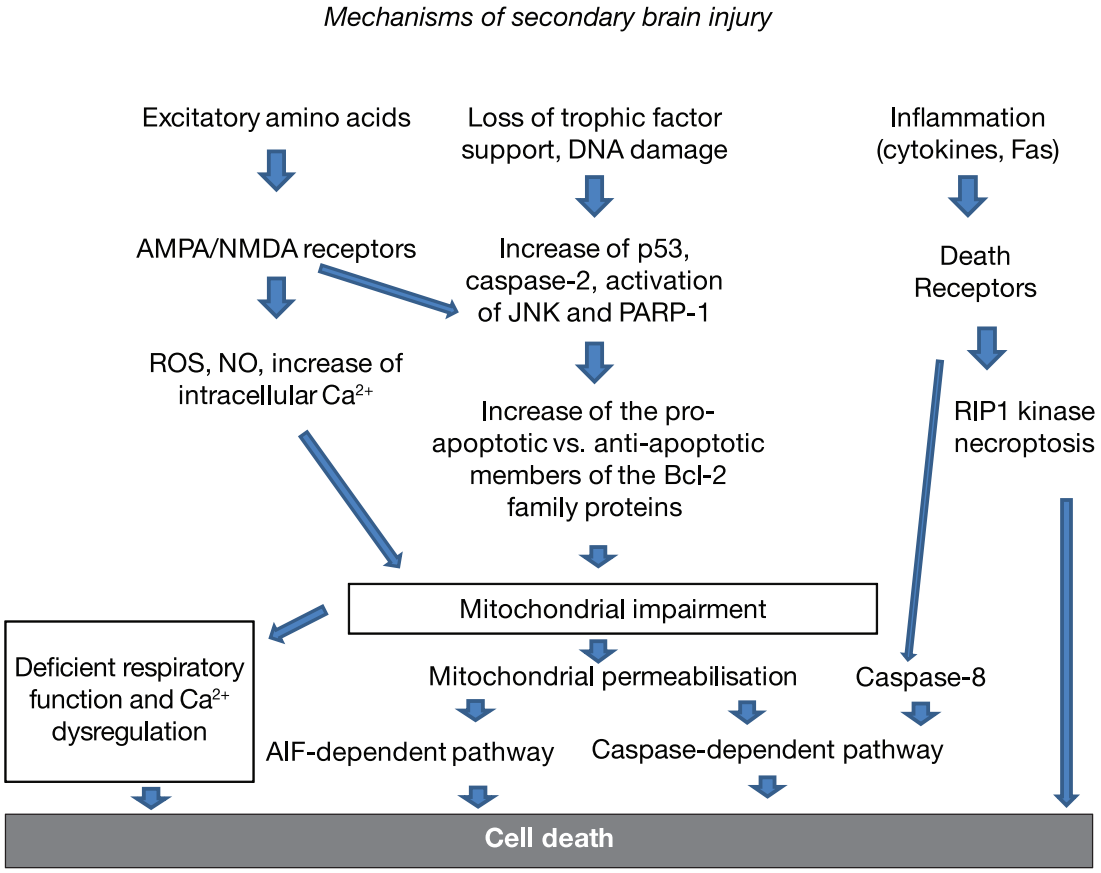


***Figure 2:*** Summary of injury mechanisms during the secondary phase.

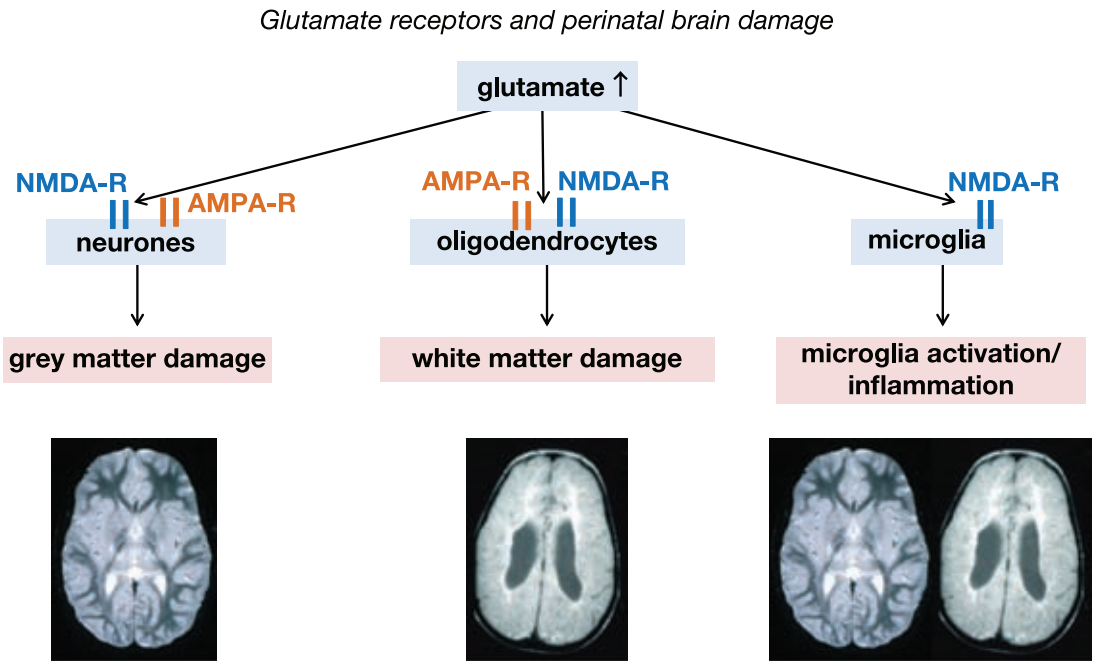


***Figure 3:*** Schematic representation of the expression of glutamate receptors on neural cells in the perinatal brain.

insult.[16, 28–29] The mechanism of excitotoxicity in response to HI probably involves perturbation of $Ca^{2+}$ homeostasis, triggering of NO and ROS production with subsequent mitochondrial impairment and activation of lipases and proteases (Fig. 2). Some NMDA receptor antagonists (eg. dizocilpine, Xenon) can, however, induce apoptosis in the immature brain,[30–31] which may complicate the use of such drugs for cerebroprotection. There are, however, other NMDA receptor antagonists like memantine that do not induce apoptosis in the immature brain in neuroprotective dosages.[32]

In addition to blocking directly glutamate receptors, alternate strategies targeting other neurotransmitter systems are capable of counteracting the deleterious effects of over stimulation of these receptors in the perinatal brain. For example, alpha-2-adrenoreceptor agonists such as dexmedetomidine are highly neuroprotective in a model of neonatal excitotoxicity induced by an NMDA agonist.[33,34] Accordingly, dexmedetomidine is also neuroprotective against a neonatal HI insult.[35] On the other hand, endocannabinoids protect the newborn brain against an AMPA-kainate receptor-mediated neonatal excitotoxic injury.[36]

**1.1.2 Calcium regulation:** Calcium ($Ca^{2+}$) is an intracellular second messenger, acting as key regulator of numerous cellular functions.[37] In order to allow efficient $Ca^{2+}$ dependent signaling, the intracellular $Ca^{2+}$ concentration ($Ca^{2+}$ic) is strictly regulated at a low level of 100 nM, i.e. 10,000 times lower than the extracellular concentration.[37] The large electrochemical gradient is being upheld through ATP requiring processes at the level of the cell membrane ($Na^+/Ca^{2+}$ exchange and $Na^+/K^+$ ATPase, $Ca^{2+}$ ATPase) mitochondria and endoplasmic reticulum.[37] In the adult brain, transmembrane ionic gradients cannot be maintained and a rapid depolarization occurs after a few minutes of complete anoxia or ischemia with a concomitant rise of $Ca^{2+}$ic. A marked rise of $Ca^{2+}$ic may trigger a number of toxic processes like activation of calpains, ROS, apoptosis, phospholipases, endonucleases and NO production.[38]

Intracellular calcium regulation is thus considered to play an important role in the cellular response to injury. However, its significance in immature brain injury is less clear. In studies *in vitro*, the rise of $Ca^{2+}$ic tends to be slower and less pronounced in immature neurons.[39] However, $Ca^{2+}$ accumulates to some extent in the brain tissue during HI[40] and calcium-dependent enzymes like calpains and phospholipase C are activated[41] which offers some indirect information in support of an increase of $Ca^{2+}$ic in the immature brain during HI.

It is equally unsettled exactly what happens to $Ca^{2+}$ic after delayed injury. Five to seventy-two hours following a period of HI there is a delayed accumulation of calcium in regions with brain injury.[40] In stroke models in adult rodents,

there are waves of NMDA-receptor dependent depolarization (spreading depression) in border-zone areas accompanied by neuronal uptake of $Ca^{2+}$. In the immature brain, spreading depression occurs at a slower rate and the degree of $Ca^{2+}$ influx appears to be lower than in the adult counterpart.[42] There is also a pronounced accumulation of calcium in axons and neuronal mitochondria detected by pyroantimonate technique/electron microscopy.[43] The pathophysiological role of these $Ca^{2+}$ elevations is yet unknown but could theoretically contribute to NMDA receptor dependent excitoxicity during the reperfusion phase.

**1.1.3 Mitochondrial impairment:** There seems to be a shift towards a more juxta-nuclear mitochondrial localization[43,44] and a progressive accumulation of calcium in the mitochondrial matrix of neurons 0.5 h–3 h after HI.[43] Some mitochondria developed a considerable degree of swelling reaching a diameter of several micrometers at 3 h of reflow, whereas the majority of mitochondria appeared moderately affected. Chromatin condensation was observed in the nuclei of many cells with severely swollen mitochondria with calcium deposits.

During early recovery after HI, high energy phosphates in the cerebral cortex are restored as previously mentioned.[11,45] During this phase, the 2-deoxyglucose (2-DG) utilization is increased, which correlates with increased levels of tissue lactate and a depression of mitochondrial respiration.[45] Post-HI administration of an NMDA receptor antagonist normalized 2-DG utilization, lactate levels, improved mitochondrial respiration and attenuated cortical brain injury.[11,45] These data suggest that NMDA-receptor activation in the early recovery phase suppresses mitochondrial respiration with a compensatory increase of anaerobic glucose cycling to lactate, which precedes development of cortical brain injury. Interestingly, a similar pattern of increased glucose use occurred in the CNS of asphyxiated infants, particularly in brain regions that were subsequently injured.[46] Such an increase in glucose utilization occurred in parallel with marked elevations of glutamate in the cerebrospinal fluid,[27] implying that HI brain injury also in post-asphyxiated infants is preceded by a phase of mitochondrial impairment related to activation of EAA receptors.

Mitochondrial impairment could, if persistent, have severe consequences on recovery of calcium regulation[37] and mitochondrial ATP production after HI. Furthermore, mitochondrial swelling and/or outer mitochondrial membrane permeabilisation lead to release of killer proteins that may lead to apoptotic-like execution of cell death (below).

**1.1.4 Apoptotic mechanisms**

***Apoptosis in the immature brain:*** Cell death is often classified as apoptotic or necrotic based on biochemical or morphological criteria.[47] Necrotic cell death is triggered by an acute insult resulting in severe primary energy failure, complete loss of membrane integrity and leaking of cytoplasmic contents into the extracellular matrix leading to an inflammatory response. Apoptotic cells do not lose membrane integrity and the organelles remain largely intact until the final stages when cell fragments bud off as apoptotic bodies that are subsequently phagocytized by microglia or healthy neighboring cells. In morphological terms mixed apoptotic-necrotic phenotypes predominate in HI, and recent data suggest apoptotic, necroptotic and necrotic pathways are all important.[47]

Apoptosis was initially recognized for its role in development. In some brain regions, half of the neurons die by apoptosis during normal brain development. Therefore it is entirely appropriate that many apoptosis-related factors are up regulated in the immature brain, such as caspase-3, Apaf-1, Bcl-2, and Bax.[48,49] Multiple apoptotic pathways converge on caspase-3, so this protease is critical in the execution of neuronal apoptosis both during brain development and after acute injury.[50] Caspase-3 appears to be particularly important in the brain, because mice devoid of caspase-3 through genetic targeting displayed a hyperplastic, disorganized brain, whereas other organs appeared normal.[50] The constitutive levels of caspase-3 and the activation after injury are several-fold more pronounced in the immature brain.[51,52] In summary, due to ongoing apoptotic processes during brain development, the apoptotic biochemical machinery is highly upregulated in the immature brain, which may confer heightened vulnerability.

***Caspase-dependent cell death and apoptosis-inducing factor (AIF) in HI:*** Studies suggest that mitochondria regulate apoptotic cell death through their capacity to undergo mitochondrial membrane permeabilization (MP) and release of proapoptotic proteins.[53] Cytochrome C (Cyt C), and other apoptogenic proteins, such as AIF, endonuclease G, SMAC/Diablo, and HtrA2/Omi, are released from the mitochondrial intermembrane space. Bax, Bad, Bid, and other members of the Bcl-2 family are involved in the regulation of mitochondrial release of proapoptotic

proteins. Cyt C interacts with APAF-1, ADP, and pro-caspase-9 to form the heptameric apoptosome, leading to activation of caspase-9, which in turn cleaves and activates pro-caspase-3.[53] AIF, on the other hand, promotes apoptosis in a caspase-independent manner.[53] In addition, the down-stream activation of executioner caspases like caspase-3 can be triggered through Fas receptor-mediated activation of caspase-8 without involvement of mitochondria, the so called extrinsic pathway.

Apoptosis is found in the brains of infants who die after intrauterine insults or perinatal HI.[54] and ample evidence support the concept that apoptotic mechanisms are critically involved.[55]

Caspase-3 is markedly activated after HI in the immature brain.[51,56] and cells with the cleaved active form of caspase-3 colocalize with markers of DNA fragmentation in injured brain regions.[56] Caspase-3 inhibitors[51] as well as transgenic overexpression of X-linked inhibitor of apoptosis (XIAP)[57] attenuate caspase-3 activation and provide a considerable degree of neuroprotection in the neonatal setting in some[51] but not in all studies.[58]

There are data to suggest that the extrinsic pathway is activated in response to HI[59] and Fas receptor deficiency seems to confer some degree of protection in neonatal HI.[60] Most data, however, suggest that activation of the intrinsic pathway is the key event in the immature brain. Assembly of the apoptosome is easily induced in homogenates from the immature brain,[61] Cyt C is released to the cytosol in response to HI,[44] caspase-9 is activated[62] and inhibition of APAF-1 reduces HI brain injury.[63] In addition, other proapoptotic proteins like AIF,[64] SMAC/Diablo,[57] and HtrA2/Omi[57] translocate from the mitochondria to a nuclear localization, suggesting that proapoptotic proteins are indeed released during the early recovery phase after HI. Cells with immunohistochemical translocation of Cyt C and AIF often exhibit signs of DNA fragmentation and nuclear condensation, and these cells are preferentially localized in regions with early loss of the neuronal marker MAP-2.[56]

Development of brain injury in immature mice after HI depends on AIF.[64] The distribution of AIF translocation matches the accumulation of poly(ADP-ribose) (PAR) suggesting that activation of poly(ADP-ribose) polymerase (PARP) might trigger AIF release from mitochondria.[65] AIF is released to the cytosol, binds to another protein, cyclophilin A (CyA)[66] and the AIF-CyA complex translocates to the nucleus and triggers DNA degradation. The release of pro-apoptotic proteins from mitochondria depends on induction of MP. In the mature brain, MP is mediated by cyclophilin D dependent opening of the mitochondrial permeability transition pore. However, in the immature brain MP depends on Bax/Bak that most likely forms a pore in the outer mitochondrial membrane.[67,68] Bax gene deficiency or Bax inhibitory peptide confers substantial neuroprotection after HI.[68–69] These data suggest that Bax dependent MP and subsequent activation of caspase- and AIF-dependent processes are critical in HI injury in the immature brain. The mechanisms of triggering of MP is partly unknown but a shift in the pro- vs. anti-apoptotic bcl-2 family balance is probably important.

***Bcl-2 family proteins in HI:*** Transgenic mice overexpressing human Bcl-xL postnatally were resistant to neonatal HI.[70] Using a site-specific antibody for phosphorylation of Bcl-2 at serine-24 (PS24-Bcl-2), the number of cells positive for PS24-Bcl-2 increased during 3–24 h of reperfusion in all investigated brain areas after neonatal HI.[62] Phosphorylation of Bcl-2 coincided with Cyt C translocation and co-localized with, but preceded, caspase-3 activation. In summary, Bcl-2 is phosphorylated, (and probably inactivated) and translocated to the nucleus, concomitant with increased mitochondrial Bax immunoreactivity, Cyt C release, and activation of caspase-3. Furthermore, Following HI, mice deficient in the BH3 only proteins Bad or Bim exhibited reduced activated caspase-3 and decreased parenchymal loss suggesting that these BH3 only proteins are also involved.[71]

The protein p53 is a tumor suppressor that triggers apoptosis via multiple pathways: by causing cell cycle arrest, inhibiting or stimulating autophagy[72] through transactivating pro-apoptotic and repressing antiapoptotic genes. P53 also has cytoplasmic actions at the mitochondrial level through interaction with bcl-2 family proteins and can promote Bax-dependent MP.[72] Recently it was shown that p53 translocates to mitochondria after HI and a peptide (pifithrin-μ) that selectively blocks the p53 interaction with mitochondria, reduces Cyt C release, caspase-3 activation and attenuates HI injury, whereas inhibition of p53 transactivation was less protective.[73]

***C-Jun N-terminal kinase 3 (JNK3) in neonatal HI:*** JNK3 is a member of the stress-activated group of mitogen-activated protein kinases (MAPK). The JNK isoform 3 (JNK3) is specifically expressed in the CNS, and stress-induced JNK3 contributes to brain injury, hence JNK3

deficiency renders adult mice resistant to excitotoxicity[74] and attenuates HI damage in newborn mice.[75] JNK3 deletion decreased caspase-3 cleavage and Bim/PUMA expression, coupled with an up-regulation of AKT/FOXO3a levels,[75] suggesting that the primary mode of JNK3 action is to promote apoptosis and that JNK3 is acting upstream of mitochondria (Figure 2).

***Calpains:*** Calpains are cysteine proteases involved in signal transduction cascades that differ from caspases as activity is calcium dependent and the proteolysis does not require an aspartic acid residue.[76] A high concentration of calcium can contribute to uncontrolled activation of calpains. They have been implicated in both apoptosis and necrosis, axonal degeneration and cytoskeletal disruption.[77]

Calpain activity is high in the developing brain especially in the white matter.[76] HI activates and relocates calpain to the membrane fraction[41] and inactivates the endogenous calpain inhibitor calpastatin in vulnerable brain regions.[41,48] Calpain cleavage products accumulate during delayed cerebral injury, especially in white matter.[41] Furthermore, synergistic activation of caspase-3 and m-calpains suggests a link between the two death signaling systems following HI.[48] The involvement of calpains is further supported by the neuroprotective efficacy of the calpain inhibitor MDL28170.[78]

**1.1.5 Necroptosis and autophagy:** Necroptosis is a cellular mechanism of necrotic cell death that can be induced in the absence of intracellular apoptotic signaling. It is triggered by death receptor engagement by TNF-α (or other ligands) leading to the activation of death domain receptor-associated adaptor kinase RIP1.[79] Allosteric Inhibition of RIP1 with necrostatin-1 attenuates delayed mouse ischemic brain injury in the adult.[79] Recently, it was shown that necrostatin-1 inhibits brain injury also in the immature brain subjected to HI, specifically in male mice.[80]

Autophagy is a process involving degradation of cytoplasmic macromolecules and organelles in mammalian cells via the lysosomal system, and can be induced during starvation and normal growth control to maintain cellular homeostasis and survival.[81,82] It has also been suggested that autophagy can trigger a type of cell death distinct from apoptosis.

Autophagy, as judged by the autophagosome-related marker LC-3 II, was induced after HI in both immature and adult brain[52] and was not different in male and female animals.[83] In order to investigate the importance of autophagy in neonatal HI, Atg7 gene deficient mice (*Atg7flox/flox*; nestin-*Cre*) were used. Atg7 is critical in autophagy as it together with Atg3, conjugates phosphatidyl ethanolamine to the C-terminal glycine residue of LC3-I, forming the membrane-bound form, LC3-II. Indeed, both caspase- dependent and caspase non-dependent cell death in hippocampus was substantially reduced in Atg7 deleted mice[84] suggesting that autophagy is a detrimental response. However, Atg7 may also take part in apoptosis which complicates the interpretation.[85] In another study, beclin 1, a Bcl-2-interacting protein required for autophagy, was shown to increase in neurons after neonatal HI. Further enhancement of beclin1 by pharmacological means reduced injury implicating that autophagy is protective.[86] Such a beneficial role of autophagy is further supported by its potential role in hypoxic or ischemic preconditioning.[86,87]

Additional studies are needed to further understand the importance of necroptosis and autophagy in perinatal brain injury, and how these processes interact with apoptotic mechanisms.

**1.1.6 Reactive Oxygen species (ROS):** ROS are molecules that contain one or more unpaired electrons,[88] which make the free radicals highly reactive and able to disrupt the molecular structure of lipids and proteins with devastating consequences for cellular function.[88] Depending on the cellular energy balance ROS can induce both necrosis and apoptosis by mechanisms that involve mitochondrial alterations following perinatal brain damage.

There are several pathways whereby ROS are produced in the brain.[88] The superoxide radicals ($O_2$.-) may be produced by: 1) electron leakage from the electron transport chain in mitochondria; 2) oxidation of hypoxanthine to xanthine and urate by xanthine oxidase (mainly in endothelial cells); 3) degradation of free fatty acids by phospholipase A2 into arachidonic acid and subsequent oxidation of arachidonic acid by cyclooxygenase and lipooxygenase; or 4) NADPH oxidase activity in macrophages, neutrophils and microglia.

The $O_2$.- radical has a relatively low reactivity and does not easily cross cell membranes. However $O_2$.- can react with $Fe^{2+}$ ions and form hydroxyl radicals (.OH) which reacts with almost every molecule in the presence of transition metals such as $Fe^{2+}$ ions and exerts toxic effects on DNA, activating poly(ADP-ribose)polymerase and depleting cellular $NAD^+$ and ATP. The .OH radical initiates lipid peroxidation in a self-perpetuating reaction that disrupts membrane function.

Thiol-groups on enzymes and structural proteins are oxidized with loss of enzyme function and cytoskeletal disruption.[88]

There are several defence systems in the brain to reduce the formation of OFRs and several pathways for their inactivation. The $O_2^{\cdot-}$ adduct is dismutated by superoxide dismutase (SOD) into hydrogen peroxide ($H_2O_2$), which is converted to water and oxygen by either of the two enzymes catalase or glutathione peroxidase. Compounds like vitamin E (α-tocopherol) act as lipid soluble scavengers, which inhibit lipid peroxidation. Chelation of transition metals such as iron is another endogenous protective mechanism against excessive formation of ROS. Intracellular concentrations of glutathione may be particularly important, and immature oligodendrocytes are especially prone to ROS-induced death because of limited glutathione stores.[89]

There is evidence for increased hypoxanthine levels, free radical formation and lipid peroxidation during reperfusion after HI in neonatal mice, newborn piglets, immature rats and fetal sheep.[55]

The neuroprotective strategy of use of free radical inhibitors and scavengers has been evaluated experimentally. Treatment with the 21-aminosteroid tirilazad mesylate, a lipid peroxidation inhibitor, after hypoxia-ischemia in 7-day-old rats reduces brain damage.[90] Allopurinol and its metabolite oxypurinol, being inhibitors of xanthine oxidase and ROS scavengers in high concentrations, reduce brain damage when administered before or after HI.[91] Furthermore, the iron chelator deferoxamine attenuates hypoxic-ischemic brain damage.[92] Recently several antioxidants, such as ascorbic acid, pyrrolydine dithiocarbamate, tanshionine and melatonin have been shown to be neuroprotective.[93–97] Other experimental data support the concept that ROS production has an important impact on the HI responses mediated by hypoxia-inducible factor-1 (HIF-1) and cyclooxygenase-2 (COX-2).[98,99]

In adult ischemia neutrophils are a major source of free radical production following ischemia and the major site of action for some neuroprotective free radical scavengers appears to be at the blood-brain barrier.[100] The role of neutrophils and NADPH expressed mainly in neutrophils appear to be less prominent in the immature.[101,102]

**1.1.7 Nitric oxide (NO):** As a second messenger NO is involved in distinct biological process such as maintenance of blood pressure, defense against microorganisms and cancer and neurotransmission. On the other hand, NO is involved in brain injury as the inhibition of NO synthesis attenuated NMDA neurotoxicity.[103] Production of NO, first identified as the endothelium-derived relaxing factor, occurs through conversion of arginine to citrulline by three different nitric oxide synthases (NOS): neuronal NOS (nNOS), endothelial NOS (eNOS) and macrophage or inducible NOS (iNOS).[104] Both eNOS and nNOS are expressed constitutively but all types of NOS can be induced in response to a variety of stimuli. Both eNOS and nNOS are dependent upon $Ca^{2+}$ binding for activation and nNOS is activated by NMDA receptor stimulation. The activity of iNOS is mainly expressed in inflammatory cells and produces large amounts of NO and its activity is $Ca^{2+}$ independent.[105] NO. and $O_2^{\cdot-}$ react very quickly to form peroxynitrite ($ONOO^-$) which is freely diffusible and oxidizes thiol groups, induces protein nitrosylation and mitochondrial impairment, thus contributing to brain damage (Fig. 2).[106]

Investigations on the role of NO in ischemic brain injury have yielded conflicting results, and the effects of different subtypes of NOS have to be considered separately.[105,107] In many studies eNOS confers protection through a beneficial vasodilator effect improving perfusion, while nNOS and iNOS enhance injury in response to focal ischemia.[107] Recent data also suggest that immature brain behaves differently from adult tissue. As in the adult, NO is produced in increasing amount during reperfusion,[25–26] and some data support a role for NO and NOS in injury to the developing brain: selective lesion of cells with NOS activity prior to an HI insult decreased brain injury;[108] neonatal mice lacking the gene for nNOS develops smaller brain injury than wild-type mice following hypoxia-ischemia;[109] and non-specific NOS inhibitors provide neuroprotection;[110] recently a neuronal NOS inhibitor prevented development of cerebral palsy-like motor deficits in a preterm model of injury in newborn rabbits was reported.[111] However, tissue concentrations of iNOS are very low in immature rat brain and do not appear to be induced within 36 hours of HI, and NOS inhibition was unable to prevent secondary energy failure after HI in immature mice.[112] Equally, intracerebral injection of the NO donor nitroprusside at doses that inflict damage in the adult brain is not toxic to the neonatal brain[113] suggesting that the immature brain may be more resistant to NO toxicity. In addition, NO released from nipradilol, an NO donor, exerts a neuroprotective effect on neonatal neurons.[114] In addition, it was recently shown that inhaled NO plays a key role in the myelination of the developing brain

and is neuroprotective in a model of neonatal excitotoxic brain damage.[115]

**1.1.8 Inflammation in neonatal brain injury:** Non-infectious exposure to excitotoxicity,[116] HI[117] or stroke[118] induces inflammation in the immature brain. Immuno-inflammatory cells, predominantly microglia/macrophages,[119,22] but also polymorphonuclear cells,[101] lymphocytes, NK-cells, and mast[120] and astroglia[101] are activated. The cellular changes are accompanied by altered expression of TLRs, cytokines, chemokines and reactive oxygen species.[121–125] The initiation of the microglial response is well studied in the infectious setting where microbial products bind to receptors (including toll-like receptors, TLRs) on the cell surface and activates the transcription factor NF-κB. The initial signals after sterile tissue damage are less well characterized, but once elicited the microglial response can be aggravated by pro-inflammatory cytokines including IL-1.[126] Microglia/macrophages may contribute to secondary brain injury through the production of pro-inflammatory cytokines, proteases, complement factors and excitotoxic amino acids.[127] In addition, microglial cells can induce oxidative injury through the production of reactive oxygen species (ROS) and nitric oxide. There are data suggesting that the early pro-inflammatory phase aggravates injury after HI as inhibition of platelet activating factor (PAF),[128] the pro-inflammatory cytokines IL-1β[122] and IL-18,[124] caspase-1 (activating IL-1 and IL-18)[129] and the complement C1q[130] all worsen HI injury. Furthermore, IL-1β, IL-6, IL-9, or TNF-α all enhance ibotenate excitotoxicity[4] whereas the anti-inflammatory cytokine IL-10 is protective.[131] Part of the mechanisms underlying the sensitizing effect of pro-inflammatory cytokines could be linked to an unbalance between pro- and anti-inflammatory response in the brain.[132] Inflammatory cells and mediators are highly context and time dependent, e.g. both beneficial and detrimental effects have been reported for microglia/macrophages[133] and components of the complement system.[134–135] We lack yet information as to the mechanisms of resolution of the pro-inflammatory phase and how repair and regain of CNS functions are achieved. Contradictory results have been obtained with regard to ischemia-reperfusion as both inhibition of microglial activation as well as addition of exogenous activated microglia have been shown to reduce injury.[136] Similarly, minocycline, which is known to reduce microglial activation, has yielded conflicting results in models of HI or excitotoxic perinatal brain damage.[116,137,138]

Another important issue is to what extent peripheral myeloid cells invade into the brain parenchyma and contribute to the population of activated microglia/macrophages after ischemia. A recent study suggest a small transfer of CD45high/Cd11b+ leukocytes (<10%) at 24h after neonatal stroke and a slightly higher contribution (30%) at 48h, indicating that fewer macrophages are coming from the blood in the immature compared to the adult brain after stroke.[135,139] Similar results were obtained in a model of neonatal excitotoxicity.[116] The possibility remains that the phenotypic expression of cells coming from the periphery changes rapidly when these cells settle in the brain parenchyma and/or that a subpopulation of microglia residing in the brain prior to the insult are expressing the CD45high/Cd11b+ phenotypic markers giving the false impression of cell invasion.

## 2 Delayed Interventions

One key issue for protecting the perinatal brain is the available window for intervention. From a clinical point of view, the longer this window, the better the chance for implementation of the treatment. For example, hypothermia has to be initiated within the first 6 hours of life to be protective in term infants with neonatal encephalopathy.[140] Such a short window does not allow applying this treatment to all neonates who might benefit from it.

In a schematic way, one could distinguish between strategies aiming at extending the window of opportunity from the acute phase to the sub-acute phase and strategies targeting more long-term events such as chronic inflammation or post-lesional plasticity. Although this is still a field in its youth, strategies for implementing delayed interventions (beyond the acute phase) are being tested in experimental models and can have different rationale and/or targets (Fig. 1).

### 2.1 *Delaying the acute phase*

As mentioned above, experimental and human data strongly support the fact that hypothermia is neuroprotective in term infants if introduced within the first 6 hours after birth. Although different groups are currently testing, in animal models and/or in clinical trials, the combination of early hypothermia and drugs such as Xenon or melatonin[141,142] in an add-on strategy to enhance the efficacy of hypothermia, some groups have been trying to extend the window of intervention of hypothermia by giving first an anti-epileptic drug prior to delayed hypothermia. Using the classical Rice-Vannucci P7 rat model, Liu et al.[143] have

shown that a combination of low dose topiramate administered 15 minutes after the hypoxic-ischemic insult and 3-hour hypothermia initiated 3 hours after the insult was neuroprotective while topiramate alone or hypothermia alone had no significant effect. More recently, the same group showed that early Phenobarbital also enhanced the efficacy of delayed hypothermia.[144]

An alternative strategy would be to use early but short-term hypothermia to enhance the window of opportunity for a protective drug. This strategy could allow reducing the duration of hypothermia. Accordingly, it was shown that fructose-1,6-biphosphate (FBP) was neuroprotective against neonatal excitotoxic cortical damage.[145] However, the drug had to be given the first 8 hours to be neuroprotective. Interestingly, a moderate but transient (4 hours) cooling immediately after the insult extended the therapeutic window for FBP, as FBP administered 24 h after the excitotoxic insult was still significantly neuroprotective in these pups.

### 2.2 *Stimulating/favouring M2 microglia*

Activated microglia have been shown to be detrimental for the production of hippocampal neurons but microglia and macrophages can also be beneficial and support neurogenesis, progenitor proliferation, survival, migration, and differentiation in other brain regions. Recent studies suggest that the phenotypic expression of macrophages can vary depending on the situation and pro-inflammatory macrophages (M1) can undergo transition into an anti-inflammatory-reparative (M2) phenotype. More recently, three activation states of microglia in CNS have been proposed: classical activation (tissue defence, pro-inflammatory), alternative activation (repair, anti-inflammatory, fibrosis, extracellular matrix reconstruction) and acquired deactivation (immunosuppression, phagocytosis of apoptotic cells).[136,146]

Strategies aiming at activating microglia when it has reached the M2 phase could be beneficial for facilitating repair and plasticity. Since the early phases of microglial activation are mostly deleterious (M1 type of activation) for the brain, such interventions should be delayed. Alternatively, or in parallel, strategies aiming at accelerating the M1-M2 switch could also be of major interest. At this point it is not known if modulation of the activation state of microglia/macrophages can be used for development of novel therapeutic strategies in the developing brain, but a recent report suggest that M2 (alternative activation/acquired deactivation) macrophage cell therapy indeed can provide protective effects in an animal model of multiple sclerosis.[136]

### 2.3 *Targeting the long lasting inflammation*

A recent and intriguing study performed in preterm infants with cerebral palsy[147] suggest that, at least in some patients with perinatal brain damage, there could be a long lasting inflammation as measured by increased TNF-$\alpha$ levels in the plasma and the supernatants of peripheral blood mononuclear cells after lipopolysaccharide stimulation. This long lasting altered inflammatory response could have deleterious effects on the progression of disease and/or on the clinical symptoms. If such a pathophysiological event was confirmed, recognizing and blocking such a persistent inflammation could be of therapeutic value.

Additional studies are necessary to confirm these new hypotheses and to determine whether or not there is a long lasting CNS inflammatory process. Techniques such as PET with markers of microglia or MRI using ferromagnetic particles taken up by activated microglia could be instrumental in this perspective if concerns about safety and ethics can be solved.

### 2.4 *Promoting positive post-lesional brain plasticity with pharmacological agents*

Fostering positive post-lesional plasticity appears as a very promising strategy for delayed interventions aiming at improving long-term neurological and cognitive function. However there is still limited knowledge about the cellular and molecular mechanisms underlying post-lesional brain plasticity.

Different growth factors, such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), insulin-like growth factor-1 (IGF-1), erythropoietin (EPO), or vasoactive intestinal peptide (VIP) have been shown to reduce delayed neuronal death in various animal models of perinatal brain damage.[148–152] As for hypothermia, the window for intervention, when tested, was rather restricted to the first hours after the insult. Beyond their potential capability to prevent on neuronal cell death, growth factors appear as good candidates to target mechanisms involved in plasticity such as proliferation of neuronal precursors, axonal growth and sprouting, or synaptogenesis and synaptic stabilization.

Accordingly, BDNF and VIP have been shown to promote axonal sprouting following excitotoxic

injury of the periventricular white matter in newborn mice.[151,152] Although growth factors like BDNF are big molecules unlikely to cross easily the intact blood-brain barrier, ampakines, which are allosteric positive modulators of glutamatergic AMPA receptors, are small and diffusible molecules able to induce BDNF production in the brain when administered systemically. Interestingly, ampakines have been shown to mimic BDNF effects on axonal sprouting in the mouse model of excitotoxic white matter injury.[153]

Similarly, melatonin was shown to promote plasticity using the same model of neonatal excitotoxic white matter damage.[93] Although melatonin did not prevent the initial appearance of white matter damage, it promoted repair of secondary lesion with axonal regrowth and/or sprouting. Recent data have shown that the window for intervention is at least 24 hours after the insult (Gressens P, personal communication). Behavioral studies support the hypothesis that melatonin-induced white matter histological repair is accompanied by improved learning capabilities. Neuroprotective properties of melatonin have been confirmed in several animal models of perinatal brain damage, including in fetal sheep.[154] Melatonin is a safe compound, including in newborns,[155] and it crosses the blood-brain barrier as well as the placenta. Based on these data, a clinical trial testing the neuroprotective effects of melatonin has been initiated in preterm infants at high risk to develop brain damage and neurological handicap.

Although this study needs to be replicated, an intriguing clinical study has recently shown that EPO, when given on an average of 24 hours after birth, had a very significant neuroprotective effects in human term infants with neonatal encephalopathy.[156] Evidently, the precise mechanism for this neuroprotection is unknown, but the timing of intervention argues in favour of an effect of EPO on post-lesional plasticity although a direct effect on delayed neuronal cell death cannot be excluded.

### 2.5 *Promoting positive post-lesional brain plasticity with exogenous stem cells*

The development of an adequate protocol for stem cell culturing and application has permitted to envisage the use of these cells for the reparation of perinatal cerebral lesions. Some studies have shown a positive effect of neural or mesenchymal stem cell therapy on the lesion extent and/or cognitive or motor outcome following perinatal brain lesions.[7,157] Interestingly, in some of these studies, positive effects were observed when stem cells were injected several days (up to 10 days) after the insult.

For example, the therapeutic potential of neural stem cells in acute neonatal brain injuries has been evaluated in rodent excitotoxic model.[157] Early (4-hour) and late (72-hour) neural stem cells implantation significantly reduced brain lesion size in this neonatal model. The implanted cells, modified *in vitro* prior to transplantation toward the oligodendrocytic lineage, were capable of migrating toward the lesion site even when implanted contralaterally to the lesion. At the lesion site, the neural stem cells underwent transient differentiation into neurons and oligodendrocytes but not astrocytes, suggesting that fate specification was achieved by the culture conditions. In parallel with the reduction in lesion size, the injured mice displayed a persistent and marked improvement in temporal and spatial memory at 3 and 6 weeks of age compared to littermates given intracerebroventricular injections of saline or fibroblasts.

Similarly, it was recently showed that two administrations of bone marrow-derived mesenchymal stem cells to neonatal mice 3 and 10 days after unilateral right carotid artery occlusion on P9 produced a 46% improvement in sensorimotor function as observed in the cylinder rearing test and a 60% decrease in neuronal loss, compared to vehicle-treated animals.[7] Moreover, cellular proliferation and differentiation of the proliferated cells into cells expressing neuronal, oligodendroglial and astrocyte markers was observed. Interestingly, remodeling of the corticospinal tract correlated with sensorimotor improvement.

However, it is not clear yet whether the stem cells themselves or factors secreted by stem cells mediate the positive effect. In case stem cells integration is important for their effect, these cells need to proliferate, find the site of lesion and differentiate into an adequate cell type (e.g. neuron, oligodendrocyte) and integrate into the tissue to be functional. The ethical problem associated with the use of human stem cells is less evident in mesenchymal stem cells or stem cells derived from cord blood. Such cells permit an autologous transplant and do not entail the problem of immune tolerance of the transplanted cells. A clinical study is currently being performed using stem cells in children with neonatal encephalopathy at the Duke University.

A further intriguing alternative to treatment with stem cells is to stimulate the production of endogenous neuronal stem cells. It has already

been shown that stem cells accumulate in the subventricular zone following an acute brain lesion. These results open a new perspective: the stimulation of this stem cell population to support the physiological reparation processes of a lesion. A variant of this strategy would be to redirect new cell production from astroglia to oligodendrocytes and neurons.[158] In any case, these newly produced cells will need to integrate and function correctly in the damaged tissue. Moreover, stimulation of stem cell proliferation bears the theoretical risk of cancer induction.

### 2.6 *Targeting epigenetic marks*

Prenatal factors like inflammation or maternal stress have been shown to make the developing brain more sensitive (sensitization process) to a second insult taking place around birth. This generally leads to destructive brain lesions that can be identified by brain imaging. There is also emerging evidence suggesting that the same prenatal factors, in the absence of a secondary insult, can lead to long-term disturbances of brain development. Although this latter phenomenon does not lead to clastic brain damage, it can be accompanied by long lasting cognitive, motor and/or behavioural impairments.[159]

The underlying mechanisms could involve myelin deficit linked to blockade of oligodendrocyte maturation, impaired neuronal migration, increased neuronal cell death, impaired axonal growth, or altered synaptogenesis.[159,160] At the molecular level, little is known but recent data suggest that modified epigenetic marks could be one of the underlying mechanisms. There is a rapidly growing knowledge about the epigenetic mechanisms and drugs specifically targeting epigenetic mechanisms are being developed and tested, raising hope for the future design of innovative treatments that could be implemented way beyond the perinatal insult.[8,161,162]

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He holds many awards and positions, which are young scientist award of 1990 at European Society for Neurochemistry (ESN), a recipient of The Eric Fernstrom Price, a Fullbright Scholarship, and the Lennander Award. He is an elected member of scientific peer review committees and scientific committees, a reviewer of LUA grant applications in Goteborg, a reviewer for the National Brain Foundation, a reviewer for the National Network in Neuroscience (NNN), a member of the Research Committee of the Medical Faculty, Goteborg University, Sweden, a member of the committee for research education a member of the Nominating Committee, Sahlgrenska Academy, a reviewer for Swedish Research Council in 2011. Also an External advisor for the neuroprotection drug development at Cerebrovascular Disorders, Hoechst AG, 1987–1995 and a member of the international advisory board at Neurophyxia (development of brain protective drugs for newborns) from October 2003 to present.