Tertiary mechanisms of brain damage: a new hope for treatment of cerebral palsy?

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Cerebral palsy is caused by injury or developmental disturbances to the immature brain and leads to substantial motor, cognitive, and learning deficits. In addition to developmental disruption associated with the initial insult to the immature brain, injury processes can persist for many months or years. We suggest that these tertiary mechanisms of damage might include persistent inflammation and epigenetic changes. We propose that these processes are implicit in prevention of endogenous repair and regeneration and predispose patients to development of future cognitive dysfunction and sensitisation to further injury. We suggest that treatment of tertiary mechanisms of damage might be possible by various means, including preventing the repressive effects of microglia and astrocyte over-activation, recapitulating developmentally permissive epigenetic conditions, and using cell therapies to stimulate repair and regeneration Recognition of tertiary mechanisms of damage might be the first step in a complex translational task to tailor safe and effective therapies that can be used to treat the already developmentally disrupted brain long after an insult.

Introduction

Cerebral palsy is caused by non-progressive malformations or lesions to the immature brain that lead to developmental disturbance (panel 1).[1] Although cerebral palsy characteristically presents with motor dysfunction, substantial impairments in sensation, behaviour, cognition, and communication are common comorbidities. Extensive multicentre trials have revealed that only about 10% of cases of cerebral palsy can be attributed to hypoxia-ischaemia around the time of birth, as defined by blood lactate, pH and base deficit, and Apgar score.[2,3] Inflammation, genetic factors, low birthweight, and prematurity are among the strongest risk factors for cerebral palsy;[4–6] however, in most cases the causal mechanism is unknown. A large proportion of, but not all, cases are caused by events in the perinatal period and we focus much of this Review on the effects of injury to the brain in this developmental period. Also, because the specific details of the perinatal events that lead to cerebral palsy are often unknown, we cannot derive a causal relation to later neurological dysfunction in human beings. As such, where there are limited data on the human neonatal disorder, we will discuss animal models and studies of adult injury, where cause and effect are more easily identified. However, we acknowledge that direct extrapolation of conclusions on the basis of findings from adult studies is not possible because of the substantial differences in damage and repair mechanisms between the neonatal and adult brains.

Findings from animal and clinical studies[7] suggest that in a subset of infants with cerebral palsy there are persistent active mechanisms that prevent regeneration or exacerbate brain damage, or both, and sensitise the patient's brain to further injury. We define these processes and their effects as tertiary brain damage (panel 2). In conjunction with and beyond the commonly outlined phases of acute and secondary cellular responses, such as cell death and metabolic disturbances (figure 1), this tertiary damage phase might persist for months or years after the initiating injury. Cerebral palsy is defined by its effect on development, and we cannot completely separate the effect of a disrupted developmental trajectory from the effects of persisting active mechanisms of tertiary injury. However, with additional research, these active ongoing processes might be targets for pharmacological treatment to improve long-term neurological outcome in patients with cerebral palsy.

Changes that persist in the brain after perinatal injury might include myelin deficits, reduced plasticity, and altered cell number. The tertiary mechanisms that govern these effects, we speculate, include persistent inflammation and epigenetic changes, which cause a blockade of oligodendrocyte maturation, impaired neurogenesis, impaired axonal growth, or altered synaptogenesis (figure 2).[8,9] Also, we suggest that, long after injury, tertiary brain damage might become apparent only in conjunction with a so-called second hit. In such a scenario, sensitisation to cognitive dysfunction, cell loss, and seizures during subsequent inflammation[10–12] would occur as a result of the tertiary processes of persistent glial priming leading to accumulation of excitatory neurotransmitters and aberrant proinflammatory signalling.

The processes of delayed cell death after hypoxia-ischaemia were only recognised in the 1980s, and soon after the notion of the window of therapeutic intervention was developed.[13–15] These were key steps in the development of therapeutic hypothermia—the only effective therapy for perinatal brain injury.[16] In a similar way, we suggest a change in the conceptualisation of injury to incorporate tertiary damage processes. We hope this concept will provide a new outlook on treatment options for perinatal brain damage, which will expand the treatment window and substantially increase the number of treatable patients.

Mechanisms of damage

Gliosis

Aberrant gliosis might cause tertiary brain damage after brain injury, even though coordinated and regulated

glial responses are crucial in repair and regeneration.[17] Evidence that glial activity is increased long after inflammation or injury has come from studies in several species. In mice, microglial gene expression for pro-inflammatory cytokines was still increased 7 months after irradiation-induced inflammation.[18] In non-human primates, prolonged microglial reactivity and increased cytokine expression have been noted 12 months after traumatic brain injury (TBI).[19] In human beings, PET imaging with a ligand against translocator protein (TSPO; panel 3)[20,21] revealed that activation of microglia persists in the human brain for more than 10 years after TBI and is associated with poor cognitive function.[22] Also, in a perinatal animal model of cerebral palsy, PET imaging for activated microglia showed that severity of motor deficit was associated with TSPO binding in the short term.[23] Advances in imaging have been crucial for identification of the persistence of injury processes, and imaging of activated microglia could be refined into a crucial diagnostic and monitoring aid in the future.

No long-term studies of microglial activation in animal models of neonatal hypoxia-ischaemia or inflammation or in human neonatal encephalopathy have been done. However, a study undertaken in 7-year-old children with cerebral palsy[24] who had been born prematurely revealed increased tumour necrosis factor α (TNFα) concentrations in the plasma and supernatants of peripheral blood mononuclear cells after lipopolysaccharide stimulation. Because increased peripheral cytokines are a marker for cerebral inflammation, this finding suggests that an altered inflammatory response persists for at least 7 years after brain damage in patients with cerebral palsy. This response could have deleterious effects on clinical symptoms or later disease progression, or both, and recognising and blocking such persistent inflammation could be of therapeutic value.

After focal injury, reactive astrocytes are the primary constituent cells of a glial scar. In the acute phase this scar is protective, in part by forming a barrier where the blood-brain barrier (BBB) has been damaged, and thereby preventing the uncontrolled access of blood proteins and red blood cells into the brain. This protective astrocyte response is initiated by activation of the TGF-β/SMAD pathway by albumin.[25] Although astrocytes are involved in regeneration and repair in the CNS, astrogliosis can persist for many years after injury and products of activated astrocytes inhibit axonal regrowth and remyelination.[17] Microglial signalling might exacerbate astrocyte cytotoxicity,[26] further suggesting the need for microglia-specific immunomodulatory treatment to enable long-term recovery and regeneration. Astrocytes also regulate the bioavailability of neurotransmitters, and glutamate transporter 1 expression is decreased at least 21 days after hypoxia-ischaemia in neonatal rats,[27] which might sensitise the brain to seizures[10] and suggests a further mechanism by which the effects of injury persist in the brain. Persistent increased reactivity might also occur in perivascular macrophages (PVM). Located within the neurovascular unit, PVM might influence BBB function, including lymphocyte transmigration or activation profile, or both.[28,29] Although the importance of BBB integrity and lymphocyte infiltration in acute-phase injury is known, further investigation into the subtle changes that persist in the long term is warranted.

Boys suffer more severe brain damage due to perinatal brain injury than girls[30] and also have poorer cognitive outcome 1 year after paediatric TBI, even if the severity of their injury is controlled.[31] In-vitro astrocytes from male neonatal mice that were stimulated with lipopolysaccharide expressed higher concentrations of interleukin-6, TNFα, and interleukin-1B than matched female microglia, suggesting that sex might influence the effects of persistent gliosis.[32] These differences might be relevant in vivo for several reasons. First, interleukin-6 and TNFα stimulate astrocyte proliferation and reduce neural precursor cell proliferation in rodents,[33,34] which might perpetuate the inhibitory effects of the gliotic scar and delay repair in male rodents. Second, interleukin-1B sensitises the brain to neurotoxicity[35] and inhibits oligodendrocyte maturation in mice,[9] increasing the severity of the acute and long-term effects of any second-hit insult during inflammation. Together with the immunomodulatory effects of sex hormones on outcome after damage,[36,37]

***Panel 1:* The definition of cerebral palsy from the executive classification committee[1]**

"Cerebral palsy describes a group of disorders of the development of movement and posture, causing activity limitation, that are attributed to non-progressive disturbances that occurred in the developing fetal or infant brain. The motor disorders of cerebral palsy are often accompanied by disturbances of sensation, cognition, communication, perception, and/or behaviour, and/or by a seizure disorder."

***Panel 2:* Tertiary brain damage**

Definition
Tertiary forms of brain damage are those that worsen outcome, predispose a patient to further injury, or prevent repair or regeneration after an initial insult to the brain

Cause
Mechanisms that facilitate this phase of damage include an altered epigenome and ongoing inflammation

Treatment
We suggest that these mechanisms are active processes for which treatments can be developed, including treatments that target microglia, innate immune processes, or astrocytes; recapitulate permissive epigenetic conditions; or promote plasticity

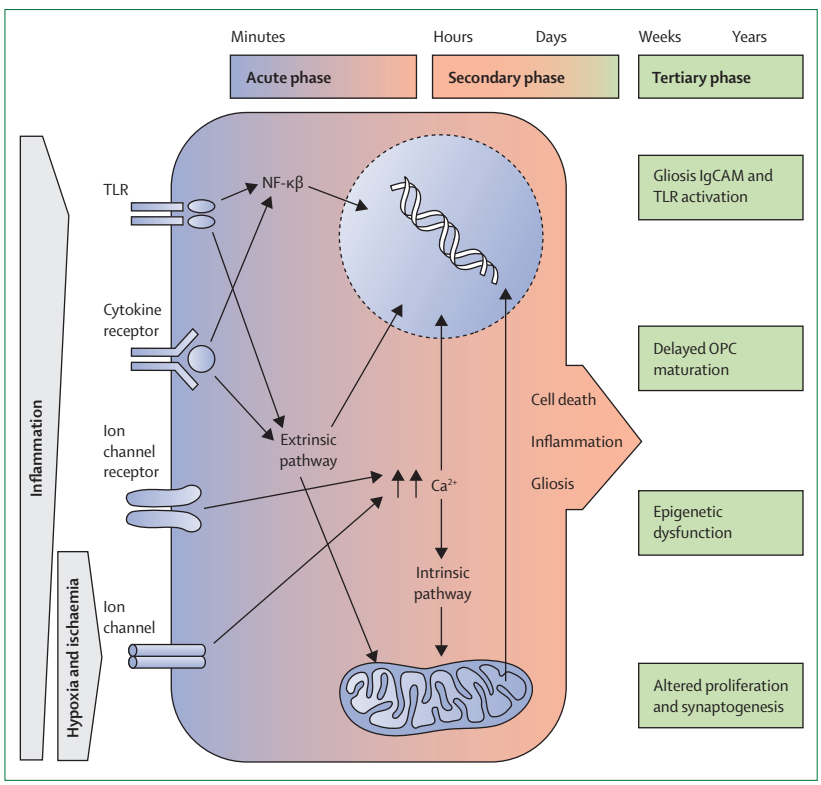


Figure 1: **Outline of the acute, secondary, and tertiary damage phases in cerebral palsy**
Activation of an array of receptors and ion channels leads to NF-κβ activation, intrinsic and extrinsic apoptotic cascades, and an increase in intracellular calcium, which in combination affect cell death, perpetuate inflammation, and induce gliosis. Selected tertiary-phase mechanisms are also shown, including persisting effects on glia, the epigenome, and neurogenesis. NF-κβ=nuclear factor-κβ. TLR=toll-like receptor. IgCAM=immunoglobulin cell adhesion molecule. OPC=oligodendrocyte precursor cell.

differences in the endogenous cell-based inflammatory response might influence tertiary brain damage.

Microglia contribute directly to the development of a mature level of spontaneous pathway activity and plasticity by eliminating synapses.[38] Microglia also participate in proliferation, causing phagocytosis of hundreds of new cells in the subgranular zone as part of the normal proliferative processes.[39] Inflammation increases this phagocytic capacity, and concentrations of activated microglia correlate with a reduction in hippocampal long-term potentiation in adult rats.[40] Microglial interleukin-1B plays an important part in memory acquisition, and after neonatal infection hippocampal learning is impaired in adult mice exposed to a second immune challenge.[12] Thus, we know that microglial activation can persist long after brain injury,[18,19,22] but we can only speculate that in the long term this activation also alters synaptic pruning, neurogenesis, pathway excitability, and memory—key features that predispose the brain to altered seizure susceptibility and learning impairments.

Inflammatory receptor activation

Immunoglobulin cell adhesion molecules (IgCAMs) have diverse cell-dependent and context-dependent roles in cell proliferation, survival, migration, neuritogenesis, synaptogenesis, and axonal regeneration.[41] Similarly, the chondroitin sulfate proteoglycans are crucial in neural development because they are involved in complex signalling with other extracellular matrix components.[42] Chondroitin sulfate proteoglycans and IgCAMs are glial scar components that inhibit regeneration of the injured mouse spinal cord. The glial scar can persist for years after injury, and expression of these adhesion molecules also remains raised for weeks[43] or months after injury.[44] As such, they might represent an active signalling mechanism that inhibits regeneration and repair processes long after the acute and secondary phases of injury.

Toll-like receptors (TLRs) are a family of pattern recognition receptors. TLRs initiate innate immune responses, including cytokine release, after activation by damage-associated or pathogen-associated molecular pattern molecules. Activation of TLRs in the brain causes inflammation, and cytokines produced because of peripheral TLR activation signal via endothelial cells of the neurovascular unit to induce central inflammation. Aberrant TLR activation during the perinatal period sensitises the brain to further experimental injury in the acute inflammatory phase.[45,46] Additionally, in rodent models perinatal TLR activation reprogrammes the adult neuroimmune response, sensitising it to adult brain inflammation and injury, accelerating age-related cognitive decline, and increasing susceptibility to seizures in adults.[11,47–50] Also, well into adulthood one exposure to the TLR-4 agonist lipopolysaccharide in the neonatal mouse disrupts fate determination in cells of the subgranular zone (Smith LP, Naylor AS, Gothenburg University, personal communication). Because neurogenic potential is linked to the capacity for memory and learning,[51] altered neurogenic potential probably represents a tertiary mechanism by which the ability of the brain to adapt and respond to later challenge is impaired.

Furthermore, arrest of oligodendrocyte maturation is caused by activation of TLR-2 by hyaluronan, a product of the glial scar.[52] As such, persistent glial reactivity after brain injury (see Gliosis section) might be a mechanism by which repair and regeneration are impaired. Additionally, accumulation of immature oligodendrocytes after perinatal injury might sensitise the brain to further injury.[53]

Epigenetic changes

Epigenetic modifications include enzymatic regulation of transcription by the modification of permissive tags (acetylation, methylation, ubiquitination, phosphorylation, and sumoylation) on histones or DNA or via microRNA-mediated regulation of translation. In human beings, acetylation regulates transcription of up to 5% of the genome, and every human gene can be regulated by at least one microRNA.[54–56] As such, epigenetic changes are, not surprisingly, crucial for every aspect of normal development and brain function. Epigenetic modifications are a major mechanism by

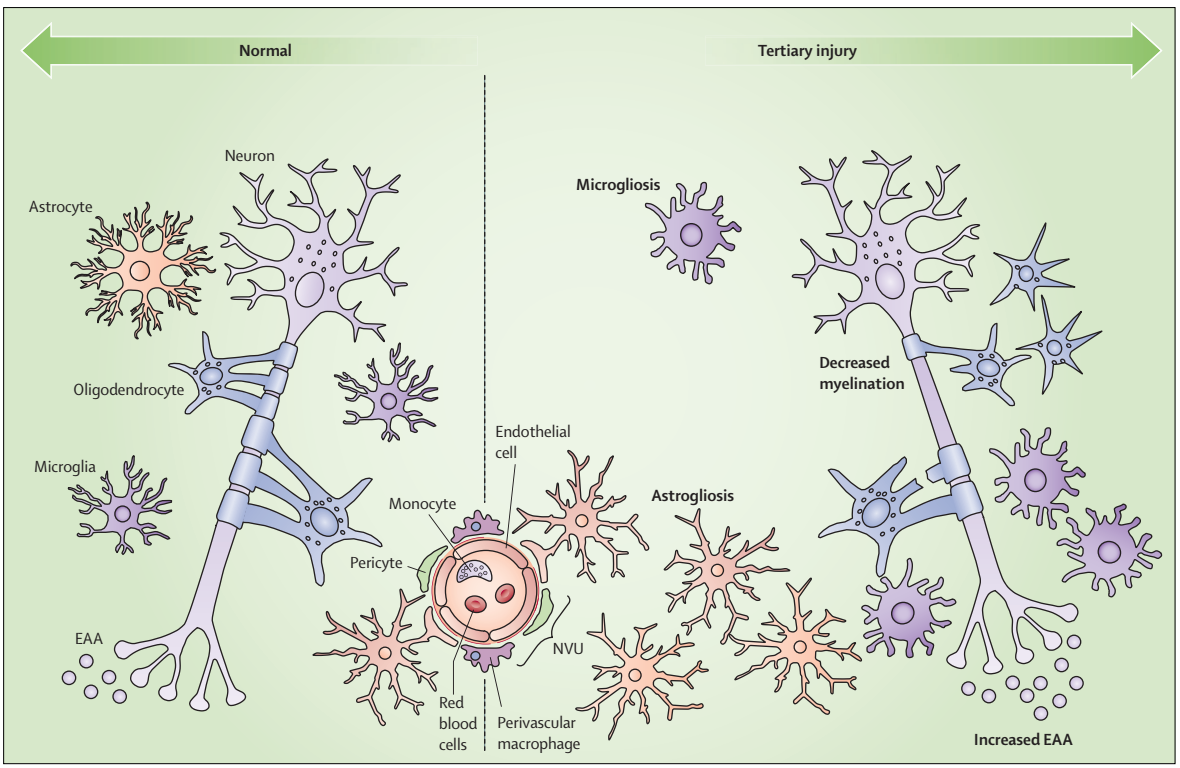


Figure 2: **Schematic representation of tertiary phase damage**
Changes in oligodendrocytes, maturational blockade, and the production of glial scar products leading to decreased myelination, changes to glia, astrogliosis and microgliosis, and possible changes in EAA bioavailability are shown. Also shown is the NVU, a possible target for novel therapeutics because of its ability to mediate transmigration of inflammatory mediators. EAA= excitatory aminoacid. NVU= neurovascular unit.

which injury and destructive prenatal environmental factors can lead to long-term disturbances of brain development, such as cognitive, motor, or behavioural impairments.[57]

During the acute and secondary phases of brain injury there is substantial loss of acetylation and methylation tags[58,59] and considerable variation in microRNA expression.[60,61] Reduced acetylation is associated with cognitive decline,[62,63] which is accelerated after brain injury.[64,65] Whether epigenetic changes persist after immature brain injury or in inflammatory models in the long term is unknown. However, prenatal stress increases methylation and acetylation well into adulthood in rodents and human beings, leading to substantial changes in stress reactivity.[66,67] Also, for at least 1 year after low-level radiation exposure in mice there is an increase in concentrations of microRNA-21,[68] an onco-miRNA that stimulates cell growth and proliferation. Another microRNA, miRNA-107 might contribute to the pathogenesis of Alzheimer's disease and is reduced after TBI, ischaemia, and hypoxia.[60] Together, these findings suggest that changes in epigenetic processes, which can disrupt neurodevelopment, might also persist and cause tertiary brain damage.

Changes to epigenetic processes might be particularly relevant for white matter injury and the persistent failure in further oligodendrocyte precursor cell (OPC) maturation. These changes are key features of brain damage in neonatal models and in preterm infants[9,53,69,70] and typically cause substantial lasting cognitive impairments. OPCs are sensitive to injury because they have a lower antioxidant capacity than other cells[71] and possibly also because of increased death receptor expression in these cells.[72] However, the persisting active process of delayed oligodendrocyte maturation might be linked to altered microRNA expression[73,74] and acetylation.[9,75] In particular, oligodendrocyte differentiation and maturation depend on a reduction in acetylation during the transition from embryonic stem

Panel 3: Imaging microglia in vivo

The mitochondrial network-located translocator protein (TSPO; 18 kDa) was previously known as the mitochondrial peripheral benzodiazepine receptor (PBR). TSPO is located at the contact sites between the outer and inner mitochondrial network membranes and is involved in cholesterol transport and steroid synthesis. Activated microglia have an increased expression of TSPO, and various PET ligands for TSPO have enabled visualisation of microglial activation during neurodegeneration and after injury.[20,21]

cell to OPC[75] and the transition from OPC to myelin-producing oligodendrocytes.[76] We have recently established a model of white matter injury in which chronic perinatal inflammation, caused by interleukin-1B exposure for the first 5 days after birth, led to OPC maturational arrest that persists into adulthood and results in altered adult behaviour.[9] In this model, global histone deacetylase (HDAC) gene expression was altered. Expression was higher, contrary to what we expected given that a reduction in acetylation facilitates the processes of oligiodendrocyte maturation. However, cell-specific and region-specific acetylation and microRNA concentrations have not yet been assessed in this model and we cannot exclude epigenetic changes as a cause of OPC arrest.

Treatment options for tertiary brain damage

Clinically, acute and secondary pathological processes of brain injury in neonates can only be treated with therapeutic hypothermia (see below). Although many alternative and additional treatments are being tested, they have so far shown only limited safety and efficacy. These drugs and strategies have been thoroughly reviewed elsewhere.[77] Long-term treatments for cerebral palsy address the functional consequences of the causal brain injury (eg, management of muscle tone and physical and language therapy), which provide important improvements in quality of life. However, on the basis of the findings presented above, we believe that active pathological processes persist in the months and years after brain injury. As such, long-term treatment after injury might improve neurological function. However, possible interactions of treatment with the effects of developmental disturbances that arise from the initial brain injury will need to be considered in the choice and timing of any treatment. As mentioned above (see Gliosis), advances in glial imaging might be refined into a crucial diagnostic and monitoring aid that could be used in the development of treatments.

Cell death and metabolic disturbance

The long-term effects of brain damage stem from the effects of exposure to various cytoactive and cytotoxic substances during primary and secondary phases of damage. As such, direct inhibition of primary and secondary phases of damage would be the most efficient mechanisms to prevent tertiary damage. However, from a clinical point of view, the window of opportunity for acute-phase treatments after brain injury is short and drugs used in the secondary phase have limited efficacy. For example, therapeutic hypothermia must be initiated within the first 6 h of life to be protective in term infants with neonatal encephalopathy.[78] Such a short time window does not allow use of this treatment for all neonates who might benefit from it, and this treatment is effective for only one in every nine infants treated. As a result, different groups are testing, in animal models and clinical trials, the combination of hypothermia and drugs such as xenon[79,80] or melatonin.[81] With these combinations the window of efficacy for therapeutic hypothermia can be extended. For example, neuroprotection was still noted when the antiepileptic drug topiramate was given before delayed therapeutic hypothermia in a rat model of neonatal hypoxia-ischaemia.[82] The same group have since shown that early phenobarbital treatment also enhances the efficacy of delayed hypothermia.[83]

An alternative strategy to combination treatment would be to use early but short-term hypothermia to increase the window of opportunity for a protective drug. For example, fructose-1,6-biphosphate (FBP) was neuroprotective against neonatal excitotoxic cortical damage in mice;[84] however, the drug had to be given within the first 8 h to be neuroprotective. When a moderate but transient cooling was done immediately after the insult, FBP administered 24 h after the excitotoxic insult was still significantly neuroprotective; thus the therapeutic window for FBP was extended.

Recovery of OPC maturation

Oligodendrocyte maturation is dependent on appropriate acetylation and microRNA regulation, suggesting that the epigenome could be targeted to reduce the long-term negative consequences of perinatal inflammation or injury on white matter development. Also, the dependence of CD44 (an extracellular matrix receptor) activation in hyaluronan-mediated TLR-2 activation and oligodendrocyte maturational arrest could be exploited to reverse the persisting effects of the gliotic scar.[85] Because CD44 agonists also favour immunosuppressive Th2 type T-cell expansion,[86] targeting CD44 might represent a multifaceted therapeutic strategy to treat brain damage, including tertiary myelination deficits. The glial scar has been targeted with a peptide mimetic of IgCAM L1, which led to successful remyelination in an adult rat model of TBI.[87] Another possible mechanism to stimulate recovery of myelination might be to increase Axin2 expression, which overcomes OPC maturational arrest,[88] as occurs in infants diagnosed with periventricular white matter damage.[89]

Targeting long-lasting inflammation

Given the wealth of evidence implicating persistent inflammation in the development of tertiary brain damage, attempting to reduce ongoing inflammation seems a reasonable therapeutic strategy. However, co-ordinated inflammation can be a beneficial process, and there is a complex relation between cytokine levels and neurogenesis. Because neurogenesis is a key process in regeneration and repair, care must be taken with any approach that reduces inflammation. Inflammation, and in particular interleukin-6 expression, increases proliferation in the subventricular zone in adult mice,[34] in direct opposition to the effects of inflammation in the subgranular zone, where proliferation decreases during

inflammation in the neonate and adult.[90,91] This disparate response might be due to differences in the cell populations within these two neurogenic niches. That is, the subgranular zone harbours neural progenitor cells whose expansion is depressed by interleukin-6, and the subventricular zone contains true neural stem cells proliferating in response to interleukin-6. Covey and colleagues[34] further discussed the importance of their findings when considering treatment options for patients with brain injury induced by inflammation or hypoxia-ischaemia. They suggest that enhancing the capacity for multipotent stem cell proliferation might offer greater long-term benefits for recovery and repair than reducing interleukin-6 concentrations to facilitate progenitor cell proliferation. We can expect that, because of the differences in levels of neurogenesis, these results will not directly extrapolate to neonates, but these findings are still an exciting addition to our understanding of the complexity of treating neuroinflammation.

Modulation of the microglial phenotype

Overactivation of microglia is detrimental for the production of hippocampal neurons.[90] However, activation of microglia and macrophages can also be beneficial, supporting neurogenesis and progenitor proliferation, survival, and differentiation in other brain regions.[34] There is evidence that the phenotypes of macrophage and microglia can be damaging or capable of facilitating repair and regeneration, depending on stimulation and at different times, which explains in part these disparate effects.[92,93] Three activation states of microglia in the CNS have been proposed: classical activation (tissue defence and proinflammatory signalling [M1]), alternative activation (repair, anti-inflammatory signals, fibrosis, and extracellular matrix reconstruction [M2a]), and acquired deactivation (immunosuppressive signalling and phagocytosis of apoptotic cells [M2b]).[94]

Microglia and macrophage phenotypes are easily modifiable in vitro, and macrophages, microglia, and PVM share key features with stem cells that make them exciting candidates for repair and regenerative treatments, because they also migrate to the injury site and produce trophic factors. Using these characteristics, cell therapy with M2-induced (alternative activation and acquired deactivation) macrophages reduced disease severity in an animal model of multiple sclerosis[95] and after adult ischaemia in gerbils.[96] These findings suggest that M2-activated microglia are a potential neurotherapy, but this will need to be validated in the developing brain.

Alternatively, or in parallel, strategies aimed at supporting the endogenous development of the M2 phenotype would also be of major interest. Early increases in expression of the M2-inducing cytokine interleukin-10 are associated with improved outcome in adult stroke,[97] and increasing the concentrations of the M2 cytokines interleukin-13 and interleukin-4 causes apoptosis of activated microglia in a process crucial for prevention of chronic inflammation.[98] An important caveat regarding increasing M2 signalling is that care must be taken to allow the requisite development of an M1 response. M1-activated microglia are generally considered to be deleterious because of the neurotoxic effects of proinflammatory products on CNS injury and in vitro.[45] However, in a rat model of photothrombotic stroke, reduction of the acute-phase microglial response, with antagonism of nuclear factor κβ (NF-κβ) via poly-ADP-ribose polymerase-1, reduced functional recovery at 30 days after injury.[99] Furthermore, ablation of acute-phase microglia exacerbated brain damage in models of neonatal and adult hypoxia-ischaemia.[100,101]

Another important consideration in attempts to enhance the M2 or anti-inflammatory profile of microglia is the hypothesis that prolonged enhancement of an inherent anti-inflammatory response after perinatal infection might predispose patients to the development of a tertiary neuropsychiatric disorder: schizophrenia.[102] With this hypothesis in mind, engineering obsolescence, apoptosis, or inactivation into cellular vehicles that are used as therapeutics, such as macrophages or microglia, is a necessity.

Modulation of lymphocyte activation

Phenotypic plasticity, as described above for microglia and macrophages, was first described in lymphocytes, where a multitude of markers for activator or repressor lymphocytes indicate their roles in a multifaceted set of innate and adaptive immune responses. Maximum transmigration of lymphocytes occurs in the first few days after brain damage but their phenotypes and role in repair and regeneration over the long term are not well understood. Regulatory type T lymphocytes reduced microglial activation and disease progression in an animal model of Parkinson's disease.[103] Thus, persistent microglial activation might be in part a product of dysfunctional lymphocyte activity after brain damage. Increasing the numbers of regulatory T cells or reinstating lymphocyte homeostasis might be a new treatment option to prevent neurodegenerative diseases after brain damage.

A novel strategy to target lymphocyte activity might be via PVM, which are responsible, at least in part, for mediating the transmigration of lymphocytes into the brain during inflammation.[104] Overexpression of C-X-C motif chemokine 10 and underexpression of matrix metalloproteinase 9 can cause lymphocytes to remain in the perivascular space after migration from the bloodstream.[105] The production and release of cytoactive substances by PVM can be modified because of their constitutively phagocytotic characteristics: they readily absorb liposomes, plasmids, siRNA, or naked oligonucleotides. By modifying in-vivo cytoactive signals from PVM, lymphocyte transmigration or the phenotype of transmigrating cells, or both, could be altered to favour regulatory and regenerative lymphocyte activity.

Epigenetic modification

In adult mice, adverse behavioural and neuroendocrine deficits occur after early life stress. These defects can be abolished in the adult mouse by central administration of the HDAC inhibitor trichostatin-A, which also promotes demethylation.[56] Additionally, HDAC inhibitors modulate innate and adaptive immune responses to enhance Th2 responses to TLR4 activation.[54] HDAC inhibitors rapidly restore the loss of acetylation associated with inflammation and hypoxia-ischaemia and improve the pathological and behavioural outcomes in these injury models when administered during the acute phase.[59,106] Given the efficacy of HDAC inhibitors at reducing microglial activity[107] and the ongoing role of microgliosis in tertiary brain damage, delayed treatment might also be beneficial. However, when considering this type of therapeutic intervention, the importance of HDAC activity for normal development, including proliferation, differentiation, and myelination by oligodendrocytes, must not be forgotten.[108] That said, oligodendrocyte maturation and myelination were not altered when modest neuroprotection was provided by peripheral trichostatin A in a neonatal model of lipopolysaccharide-sensitised hypoxia-ischaemia.[109]

MicroRNAs are involved in the pathogenesis of acute-phase injury and in endogenous repair and regeneration. At 3 days after middle cerebral artery occlusion, the expression of microRNAs that are involved in regulation of T-cell function (miRNA-17 and miRNA-20a) and intracellular ATP concentrations (miRNA-15b) was increased,[110] suggesting a specific temporal role for microRNAs in endogenous repair and regeneration. Modification of microRNA activity with siRNA reduces liver and cardiac injury without affecting endogenous microRNA production,[60] and miRNA-145 inhibition improved neuropathological outcome after ischaemia in a mouse model,[110] thus showing the potential of microRNA regulation for therapy. miRNA-210 is a pro-survival factor that stimulates cell growth and increases vascular endothelial growth factor (VEGF) signalling, which is key in long-term regeneration. miRNA-210 was reportedly increased in multiple models of hypoxia that were tested and in placenta from patients with pre-eclampsia;[61] therefore, this might be a therapeutic target. miRNA-124 is expressed only by microglia in the brain and is involved in the regulation of microglial phenotypes. Specifically, miRNA-124 decreases expression of major histocompatibility complex 2,[111] and loss of miRNA-124 preceded the onset of disease in experimental autoimmune encephalomyelitis.[111] In the search for strategies to improve brain function long after inflammation and injury, that central administration of miRNA-124 can reverse progression of experimental autoimmune encephalomyelitis is of interest.[111]

Promoting positive brain plasticity after a lesion

After brain injury in human beings, functional improvements can accrue over many months or years.[112] We suggest that persistent effects of injury on the immature brain, including inflammation, will decrease this plasticity by inhibiting endogenous repair. Use of growth factors and cell therapy to support endogenous plasticity after a lesion (see below) is being trialled clinically and seems to be a promising delayed intervention for improvement of long-term neurological and cognitive function.

Cell therapy

Persistent positive effects of neural or mesenchymal stem cell therapy on lesion size or cognitive or motor outcome have been noted after adult and perinatal brain injury.[113–116] These studies have key exciting features. Implanted cells can migrate to the lesion site when implanted contralateral to the lesion or when injected peripherally.[114,116] Furthermore, a regenerative microglial phenotype is stimulated by treatment,[115] and recovery can occur independent of reductions in cell loss,[115,116] suggesting that there is an extended therapeutic window. One dose of intravenous stem cell therapy given to adult rats 30 days after middle cerebral artery occlusion improved neurobehavioural outcome, increased synaptogenesis, vasculogenesis, and proliferation, and decreased apoptosis.[116] In this so-called super-delayed cell therapy regimen, even when one dose of stem cells was administered 90 days after middle cerebral artery occlusion, sensorimotor testing was improved 50 days later; although this improvement did not extend across the entire neurobehavioural battery. This finding suggests that the development of delayed therapy, possibly via expansion of autologous stem cells for repetitive treatments, might be an exciting next step for treatment of tertiary brain damage by inducing repair and regeneration.

A further intriguing alternative to treatment with stem cells is to stimulate the production of endogenous neuronal stem cells or to redirect new cell production from astroglia to oligodendrocytes and neurons[117] after neurodegenerative insult. Stem cells accumulate in the subventricular zone after an acute brain lesion but we do not yet understand how to ensure these cells participate in repair and regeneration. However, because self-renewal, proliferative capacity, and multipotency are common to stem cells and cancer, the theoretical risk of tumour development must be considered.

Although clinical interventions directed at the underlying neurobiological process are limited, trials of autologous umbilical-cord-derived stem cells are ongoing at Duke University (Durham, NC, USA; NCT00593242) and the Medical College of Georgia (Augusta, GA, USA; NCT01072370). At Duke University, multiple stem cell infusions are to be administered within the first 14 postnatal days to infants diagnosed with hypoxic-ischaemic encephalopathy. At the Medical College of Georgia, patients with cerebral palsy are to be administered one treatment of stem cells in early

childhood in a double-blind experimental design. There are positive anecdotal reports from the Duke University study, which is an open-label pilot study, but no peer-reviewed data are yet available.

Pharmacological drugs

Promotion of positive plasticity after a lesion with pharmacological drugs has the clinical benefits of reproducibility and does not require the previous banking of stem cells or the use of immunomodulatory therapies to inhibit allogeneic cell rejection. Growth factors reduce delayed neuronal death,[118–121] and treatment with growth factors also seems to be a promising strategy to prevent tertiary damage related to aberrant inflammatory processes, which decrease growth factor production[122] and inhibit neurite outgrowth and remyelination.[17] Furthermore, inflammation can decrease neurogenesis, and this effect, which is associated with deficits in memory and learning,[51] can be reversed with growth factor treatment.

Treatment with inhibitory growth factor agonists, such as neurite growth-inhibitory protein, beginning 1 week after stroke, improved cortical regeneration in adult rats.[123] Also, delayed transfer to an enriched environment, beginning 15 days after middle cerebral artery occlusion, which is associated with increased BDNF expression, improved neurobehavioural scoring in adult rats,[124] and VEGF treatment delayed to 24 h after middle cerebral artery occlusion still improved long-term infarct volume and neurological function.[125]

Although growth factors such as BDNF are large molecules that are unlikely to cross the intact BBB easily, ampakines—allosteric positive modulators of glutamatergic AMPA receptors—are small and diffusible molecules that can induce BDNF production in the brain when administered systemically. Ampakines mimicked the effects of BDNF on axonal sprouting in a mouse model of excitotoxic white matter injury.[121]

Similarly, melatonin promoted plasticity in the same model of neonatal excitotoxic white matter damage.[126] Although melatonin did not prevent the initial appearance of white matter damage, it promoted repair with axonal regrowth, sprouting, or both. Recent data have shown that the window for intervention is at least 24 h after the insult (Gressens P, personal communication). Behavioural studies support the hypothesis that melatonin-induced white matter 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 after umbilical cord occlusion.[127] Melatonin is a safe compound to administer to newborns,[128] and it crosses the BBB as well as the placenta. On the basis of these data, a clinical trial testing the neuroprotective effects of melatonin has been initiated in preterm infants at high risk of developing brain damage and neurological handicap (NCT00649961).

Search strategy and selection criteria

References for this Review were found through a search of PubMed from 1951 to March, 2012, and from a search of Google Scholar from 1992 to March, 2012, with the terms "perinatal", "brain", "cerebral", "hypoxic/ischemic", "long-term", "delayed", "sensitisation", and "neuroprotection" in various combinations. Reports in English and French were considered. Additional searches were done as needed for specific topics from the initial screen. With the large number of citations retrieved through these search strategies, the published work cited inevitably represents the authors' personal selection and was also limited by restrictions on bibliography length.

Erythropoietin, when given on average 24 h after birth, had a significant neuroprotective effect in human term infants with neonatal encephalopathy.[129] The timing of intervention argues in favour of an effect of erythropoietin on plasticity after a lesion, although a direct effect on delayed neuronal cell death cannot be excluded.

Conclusions

A great deal of evidence from neonatal and adult injury studies suggests that an ongoing and theoretically treatable tertiary damage phase exists after perinatal brain insult. Persistent neurological deficits after perinatal brain injury remain an enormous societal burden despite substantial research efforts. We suggest that advances in imaging and knowledge of glial activation states and the mechanisms that underpin white matter injury mean that we can now consider treatment of damage in the tertiary phase. Translation of treatment options into clinically applicable therapies will be a complex task. However, we hope a greater understanding of the persistence of active injury mechanisms will eventually lead to improved brain health in infants with brain injury.

Contributors

BF did the search of the published work, prepared the figures, and wrote and revised the Review. PG contributed to the search of the published work and the writing and revision of the Review.

Conflicts of interest

We declare that we have no conflicts of interest.

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