Review Article

Mechanisms of tertiary neurodegeneration after neonatal hypoxic-ischemic brain damage

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Abstract: Neonatal encephalopathy linked to hypoxia-ischemia (H-I) which is regarded as the most important neurological problem of the newborn, can lead to a spectrum of adverse neurodevelopmental outcomes such as cerebral palsy, epilepsy, hyperactivity, cognitive impairment and learning difficulties. There have been numerous reviews that have focused on the epidemiology, diagnosis and treatment of neonatal H-I; however, a topic that is less often considered is the extent to which the injury might worsen over time, which is the focus of this review. Similarly, there have been numerous reviews that have focused on mechanisms that contribute to the acute or subacute injury; however, there is a tertiary phase of recovery that can be defined by cellular and molecular changes that occur many weeks and months after brain injury and this topic has not been the focus of any review for over a decade. Therefore, in this article we review both the clinical and pre-clinical data that show that tertiary neurodegeneration is a significant contributor to the final outcome, especially after mild to moderate injuries. We discuss the contributing roles of apoptosis, necroptosis, autophagy, protein homeostasis, inflammation, microgliosis and astrogliosis. We also review the limited number of studies that have shown that significant neuroprotection and preservation of neurological function can be achieved administering drugs during the period of tertiary neurodegeneration. As the tertiary phase of neurodegeneration is a stage when interventions are eminently feasible, it is our hope that this review will stimulate a new focus on this stage of recovery towards the goal of producing new treatment options for neonatal hypoxic-ischemic encephalopathy.

Keywords: Neonate; brain injury; cell death mechanisms

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Introduction

Neonatal encephalopathy linked to exposure to hypoxia-ischemia (H-I) in the term born infant (termed HIE) is regarded as one of the most important neurological problem of the newborn. This insult, which is typically caused by intrapartum asphyxia can lead to a spectrum of adverse neurodevelopmental outcomes such as cerebral palsy, epilepsy, hyperactivity, cognitive impairment and learning difficulties (1-3). As supportive management of moderate to severe encephalopathy continues to improve, the need

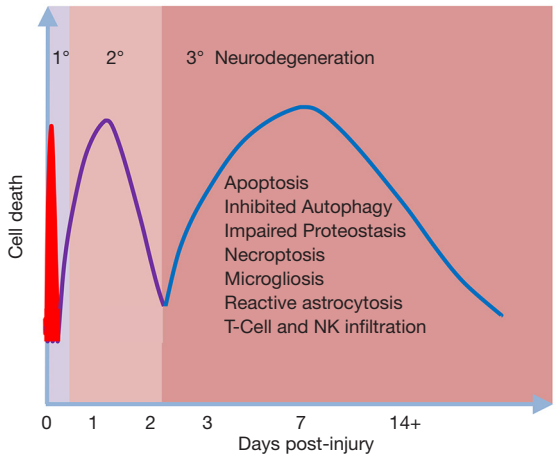

Figure 1 Mechanisms of tertiary neurodegeneration. This figure summarizes the stages of neurodegeneration after neonatal hypoxia-ischemia as well potential injury mechanisms that are contributing to the tertiary phase.

for effective therapeutics that produce long-standing effects increases. Hence, it is absolutely vital to understand the pathophysiological changes arising in the brain and those that will affect long-term outcomes.

During the acute period, which we will define as those events that occur during the first 6 hours after cerebral H-I, energy substrates and cellular metabolites are depleted leading to a severe collapse of the neuronal membrane potential. This in turn causes a reversal of glial glutamate transporters and the extracellular accumulation of excitatory amino acids (4) such as glutamate, that lead to excitotoxic cell death (*Figure 1*) (5). During the secondary period of injury, which we will define as those events that occur from 7–48 hours after the injury, oxidative stress damages proteins and lipids necessary for cell survival and signaling cascades triggered by cytokines lead to apoptotic cell deaths (6-11). During this period of secondary injury, there is a second wave of cerebral energy failure that has been observed in both humans and in animal models (12-15) which contributes to additional secondary cell deaths. Over the next few days to weeks the injury appears to stabilize but depending upon the extent of injury, as well as a variety of other factors, more cells become damaged contributing to progressive neurodegeneration in this third phase that we define as the tertiary phase. There have been numerous reviews that have focused on the epidemiology, diagnosis and treatment of neonatal HIE; however, a topic that is less often considered is the extent to which the injury might worsen over time, which is the focus of this review.

Several clinical studies have contributed to the view that cerebral H-I is not an event whose processes occur only in the hours to days after the insult, but rather that it can trigger an evolving process resulting in a worsening of brain damage over time. The first study that we are aware of that suggested that the initial injurious event triggers a cascade of death effectors that can lead to progressive neurodegeneration was from Barkovich *et al.*, 1995, whose T1-weighted magnetic resonance images (MRI) showed marked signal hyperintensity 3 days after injury. But their T2-weighted images showed that the largest changes occurred 6–10 days after injury suggesting that the lesion continued to evolve over time (16). A subsequent diffusion-weighted imaging study of H-I neonates showed that superficial lesions of the neocortex continued to expand over 3–4 days, recruiting deeper regions of the brain (17). Additional data reported by Shroff *et al.*, 2010, provided evidence that the injury in 1 out of 5 infants continues to progress as changes in diffusion-weighted imaging peak between 3 and 5 days of recovery (18).

Experimental studies using animal models have provided strong support for delayed neurodegeneration after perinatal H-I. Studies using the Vannucci P7 rat pup model have shown that there is a progressive expansion of the lesion that can last up 9 weeks after the initial insult (19,20). Interestingly, the hypoxic interval can dictate the initial size of the lesion, but it does not correlate to the ultimate level of neurodegeneration. Perinatal H-I in rat pups lasting longer than 90 minutes causes extensive gray and white matter infarction that is evident by 24–48 h after injury (19). This lesion is not progressive but static. However, when the hypoxic interval is between 60–75 minutes, the injury size when assessed in the acute phase is smaller than the lesion size seen with the 90 minutes (or longer) hypoxic interval. When such animals were assessed at 6 weeks of recovery, the penumbra/peri-infarct region had enlarged such that the extent of damage was no longer different from animals that had been exposed to longer hypoxic intervals (19). This phenomenon illustrates the importance of evaluating the mechanisms of injury beyond 72 hours (i.e., tertiary stage) (21). Subsequently, a hypoxic interval of 75 minutes was determined to produce a moderate level of injury in which tertiary neurodegeneration was most apparent (22).

Progressive changes are best visualized using serial MRI, and several studies that have used MRI imaging have reported progressive injury after perinatal H-I. Lama *et al.*, 2011, analyzed rat pups from 1 day to 4 weeks of recovery after perinatal H-I and showed that between 1 and 3 days

of recovery that there were areas of hyperintensity in the ipsilateral internal capsule and the cerebral peduncle as well as decreases in the apparent diffusion co-efficient (ADC) (23). These changes peaked at 3 days of recovery and then recovered by the 1 week time-point. Similarly, they showed a progressive loss of axons between 1 day and 4 months of recovery at the pontine level of the corticospinal tract (CST) using stains for neurofilaments. Their data show the utility of using ADC and T2 MR imaging to detect progressive neurodegeneration and show that there is delayed Wallerian degeneration of the CST which can explain loss of volitional motor function. In a later study, this group performed diffusion tensor imaging and found that radial diffusivity increased in the cerebellar peduncle between 2 days and 1 week of recovery. In that study they also showed that there was a ~2.5-fold increase in FluorojadeC+ neurons between 1 day and 4 weeks of recovery. Similarly, there was a ~6-fold increase in ED-1+ microglia between 1 day and 4 weeks of recovery (24).

Another longitudinal assessment of injury was performed after H-I at P3 in rat pups using high-field MRI and localized 1H magnetic resonance spectroscopy (MRS). They found that there was a ~2-fold increase in neocortical loss between the ages of P11 and P25, and interestingly the extent of injury correlated with the levels of lactate seen at P4. In this study they also observed that the males were more vulnerable than the females, both in terms of initial injury and in the extent of progressive cell loss (25). Although the P3 rat brain is developmentally comparable to the preterm human brain, who are less likely to be exposed to H-I (26), these results support the concept of tertiary neurodegeneration after H-I.

In the sections to follow, we will review the cellular and subcellular process that are occurring during the tertiary phase of recovery as well as potential therapeutic approaches for preventing these delayed cell deaths. Unlike the acute and secondary stages of cell death after perinatal H-I, there have been far fewer studies that have evaluated the tertiary phase of cell death, and yet, this is a stage when interventions are eminently feasible. Therefore, it is our hope that this review will stimulate a new focus on this stage of recovery towards the goal of producing new treatment options for neonatal HIE.

Mechanisms of tertiary neuronal damage

Prolonged apoptosis

During neural development neurons and synapses are overproduced to allow for optimal circuit formation. Unlike other organs, the human brain continues to develop postnatally, with neuronal migration, synapse refinement, gliogenesis and myelination occurring postnatally. Apoptosis is an ongoing and essential process of this circuit refinement throughout childhood and adolescence (27). As a result, cerebral apoptosis is an ongoing and essential process during the early postnatal period, supporting the hypothesis that neurons can undergo rapid caspase activation (6). Any potential injury occurring during this window can dysregulate this process with devastating outcomes.

There has been extensive research into the mechanisms of apoptotic cell death, including its two major pathways the intrinsic and extrinsic pathways (7). In neonates it has been shown that the intrinsic mechanism is regulated by members of the Bcl-2 family, particularly BAX (8) and involves mitochondrial depolarization and cytochrome c release. Cytoplasmic cytochrome c then interacts with Apaf-1, d-ATP/ATP and procaspase, forming an apoptosome that initiates the caspase cascade (9,11,28). The extrinsic pathway is a fast and efficient apoptotic mechanism, resulting from the binding of ligands of cell surface death receptors TNFα (29,30), Fas (31,32) and TRAIL (33). These are cysteine-rich and activate the protease caspase-8, which in turn promotes apoptosis by either activating effector caspases, such as caspase-3 and -7, or by cleaving Bid, which translocates into the mitochondria (34), to initiate the above-mentioned intrinsic pathway (10,35).

The vast majority of studies on cell death after HI have focused on acute and secondary stages, but late time-point assessments and studies of knockout models targeting apoptosis-related genes have shown that apoptosis can occur over a prolonged time frame. For example, BAX-deficient mice demonstrated reduced tissue loss as late as 7 d after H-I and the BAX inhibitory peptide prevented mitochondrial permeabilization and brain injury (36). Interestingly, features associated with apoptosis, such as nuclear shrinkage and caspase-3 activation were still partially present 24 h after injury, and caspase-8 activation was not diminished (8). Deleting caspase-8 specifically reduced the brain weight loss that is seen 22 d after H-I by more than 50%, whereas neurons in wild-type (WT) littermates were shrunken with pyknotic nuclei, a hallmark of apoptosis (37). Following neonatal H-I, increased caspase-3 activity is detected early within the primary phase, reaching its peak 24 hours after H-I injury in P7 rats (38). Studies looking into the tertiary phase have shown that at 48–96 hours post H-I there are degenerating neurons undergoing apoptosis with caspase-3

positive staining both in the penumbra and ischemic core of the parietal cortex, striatum and hippocampus (39). Carloni and colleagues have shown co-localization of both caspase-3 and propidium iodide—a necrotic marker—in neurons 48 to 96 hours after H-I in P7 neonatal rats (40). These hybrid cell deaths appear to accumulate, with significant increases in caspase-3 activity still observed 6 days post-H-I within the ipsilateral cerebral cortex (38).

Administering the pan caspase inhibitor BAF immediately before H-I resulted in persistent protection 7 d after H-I in cerebral cortex, hippocampus and striatum. Moreover, the hippocampus and striatum were protected when the treatment was delayed until 3 hours after the insult (41). To our knowledge, no study has tested the efficacy of administering BAF during the tertiary phase of. Transgenic mice overexpressing AIP showed long-lasting protection, with reduced brain tissue loss 7 days after H-I and administering TAT-AIP to rats reduced brain tissue loss 7 days after H-I and improved neurological functions up to 4 weeks after H-I (42).

Interestingly, none of the studies targeting apoptosis after neonatal H-I have abolished brain tissue loss. Indeed, caspase-3 deficient mice had worse brain tissue loss 7 d after H-I than wild type mice, due to an increase in PARP-1-mediated caspase-independent neuronal cell deaths (43). This result could indicate that (I) the necessary developmental caspase-mediated pruning is still important during the neonatal period, and targeting apoptosis can be partially detrimental, and/or (II) other mechanisms of cell death also occur either concurrently or as a backup. However, apoptosis can be induced using mechanisms other than caspase activation, such as the signaling pathways activated by the apoptosis-inducing factor (AIF). AIF expression is highest during developmental stages, with a relative downregulation with age, suggesting that AIF plays a more important role in the cell deaths in the neonate than in the adult (6). Indeed, the combined deletion of AIF and caspase inhibition confer protection (44). Furthermore, caspase-2 inhibition acting upstream of mitochondria preventing permeabilization and the release of both AIF and cytochrome c reduces injury (45).

Prolonged necroptotic death

In both animal models and newborn humans, cell death following neonatal H-I occurs as a continuum, with an interchangeable connection between apoptosis and necrosis, as seen in the co-localization of their markers in injured neurons, particularly during the tertiary phase (40), mediated via necroptosis (39,46-48). The tertiary phase of recovery also is accompanied by persisting gliosis and sustained inflammation (as discussed further below) that may play an important role in the continued activation of death receptors (49). These death receptors can trigger not only apoptosis, but also necroptosis, commonly described as a programmed form of necrosis or inflammatory cell death. Necroptosis' mechanisms of action have been extensively reviewed elsewhere (50-52). Commonly, activation of the death receptor ligands TNFα, Fas, TRAIL, as well as TLR 3 and 4 are involved. This is followed by recruitment of TRADD and RIPK1. RIPK1 together with activated RIPK3 forms the necrosome, driving the phosphorylation of the MLKL pseudokinase (53). Phosphorylated MLKL oligomerizes and translocates to the plasma membranes where is creates pores in the membrane that release cytoplasmic contents that elicit an immune response (54). Alternatively, TLR3 and 4 can trigger necroptosis in the absence of RIPK1, where TRIFF interacts with RIPK3 to activate MLKL. Thus, both RIPK3 and MLKL are essential components of necroptotic cell death (55,56).

It is important to note that TNF or LPS stimulated necroptosis efficiently removes dying cells via cell lysis, which has been suggested to confer a rapid reduction of the inflammatory response by suppressing cytokine and chemokine production (57,58). This theory is supported by studies showing that MLKL-deficient mice (that are unable to induce necroptosis) do not have as robust an inflammatory response as RIPK-3-deficient mice (59,60). Therefore, it is critical to consider that necroptosis may not only result in the commonly accepted pathogen-mediated activation, but also a host-mediated activation to reduce the production of PAMPs, thus limiting the subsequent detrimental cytokine storm (51). However, the role of necroptosis in neonatal brain injury is still not well understood, with few studies focusing on this cell death mechanism, and with limited information on the importance of necroptosis during the tertiary injury phase (10,11,61).

Administering necrostatin-1, an inhibitor of RIPK1, 15 minutes after H-I significantly decreased brain tissue loss which persisted into the tertiary phase, producing long lasting protection observed from 4 days until 3 weeks after perinatal H-I. The protection afforded was most dramatic in the forebrain and thalamus and was associated with a substantial reduction in the presence of cells with necrotic morphologies and an increase in the apoptotic phenotype

4 days after H-I (46). Interestingly, there were significant sex differences, where male mice benefited more than female mice, possibly due to more robust necrostatin-1-mediated signaling of BDNF after H-I (62). Despite this male specific protection, further studies have shown that RIPK1 inhibition protected mitochondria in both neurons and astrocytes after H-I irrespective of gender (61).

Commonly, in the presence of caspase-8, apoptotic cell deaths occur instead of necroptosis (63,64). In fact, inhibiting caspase-8 ameliorates H-I brain injury; however, the protection is incomplete (37). As aforementioned, it is possible that necroptosis occurs as a backup, or that these cell death mechanisms occur concurrently. A recent small study has shown that apoptosis and necroptosis occur in different regions of the hippocampus, where caspase-3 mediated deaths are most prominent in the CA1 region, whereas necroptosis, as revealed by activated RIPK1 and RIPK3 were significantly increased in the dentate gyrus in a neonatal P7 rat H-I model. A reversal in RIPK1 and RIPK3 upregulation is seen following necrostatin-1 administration *in vitro* (65), further suggesting that different cell mechanisms target different cells (34).

To date, studies have shown that targeting a single cell death mechanism is unlikely to prevent neonatal H-I brain injury. The importance of a multi-mechanism treatment is specifically apparent in the efficacy of hypothermia, which itself acts to modulate cell death and inflammation across multiple mechanisms (66). Current findings show that future studies need to address the lack of knowledge regarding the different types of cell death occurring during the tertiary phase of injury, with better characterizations of the differential susceptibility of subsets of neural cells based on their state of maturation, regional location and time post-insult.

Role of autophagy and ubiquitin-proteasome system (UPS)

Cells stressed by exposure to H-I need to clear any accumulated intracellular debris (which may include dysfunctional organelles and aggregated proteins) that can otherwise become neurotoxic. Cells utilize two systems to rid themselves of potentially cytotoxic protein cargo: UPS and autophagy. Autophagy and UPS were once considered to be two discrete processes but might instead be components of a unified proteolytic system (67). Both require protein ubiquitination for substrate binding and degradation. Additionally, autophagy inhibition can compromise UPS capability in the cell by delaying substrate delivery to proteasomes (68). Despite being highly interlinked, both processes have conventionally been studied independently with particular focus given to autophagy in H-I.

Generally, autophagy is considered to be a neuroprotective and stress-induced pathway that prevents neuronal loss, likely depending on the severity of the insult. Studies evaluating the contribution of autophagy to neuronal survival after H-I may seem contradictory. However, what appears to be emerging is that increased autophagic activity during the acute stages of recovery from H-I (and especially in models of severe injury) accompanies cell death, whereas increased autophagic activity during the tertiary phase of recovery promotes neuronal survival. For example, in an *in vitro* model of cortical neuron excitotoxicity, the number of autophagosomes increased and correlated with cell death (69). Similarly, increased autophagic activity was seen in the neocortex during the acute stages of recovery from H-I in the Vannucci model of H-I in the P7 rat (70). In this context of acute and severe injury (2 h of H-I), depletion of Beclin1 (a protein that participates in autophagy induction and cell death) several days prior to H-I or neuron-specific deletion of Atg7 seem to functionally impair autophagy and provide neuroprotection (69,71); however, it is important to consider that both proteins regulate apoptosis in an autophagy-independent manner: Atg7 regulates p53-dependent cell-cycle arrest and apoptosis (72), while the role of Beclin1 and its complex with Bcl-2 in apoptosis has been extensively studied (73-75).

More recent studies using a 3–5-day-old piglet H-I model (45-min hypoxia and 7-min airway occlusion) have shown that autophagic function, i.e., flux or turn-over of autophagosomes, is inhibited at one day of recovery from H-I and coincides with an increase in neuronal death (76). In this study they observed an inhibition of autophagic flux as indicated by an early (1.5–6 h post H-I) accumulation of LC3 and p62-positive organelles. The authors attributed the inhibition of autophagy flux to a decreased number of cathepsin-positive organelles and increased ubiquitinylated proteins indicative of an insufficient number of functional lysosomes to clear the high number of autophagosomes formed immediately after H-I. During the secondary phase of cell death, they observed accumulation of p62 and Beclin-1 that colocalized with markers of caspase-dependent and caspase-independent apoptosis and necrosis in neurons. They did not evaluate autophagy during the tertiary phase of recovery.

A study using the P6 Vannucci model in rats showed that neuroinflammation and subsequent brain damage can be

attenuated by administering a TGFβ receptor antagonist (SB505124) during the tertiary phase of recovery from perinatal H-I (beginning at 3 days post-insult). However, the mechanisms responsible for the reduced progression of neuronal death with SB505124 treatment was unknown (77,78). As Carloni *et al*., 2008, had shown that neuronal autophagy is higher in the neocortex and hippocampus of the ipsilateral hemisphere by 72 hours post-H-I in P7 rats, returning to baseline levels within a period of 5 days (79). The hypothesis was proposed that SB505124 was prolonging this increase in autophagy. In assessing the levels of two key proteins known to mediate autophagy: p62 and LC3, SB505124 increased both the levels of these proteins as well as the number of autophagic granules within the neocortical neurons (80). Most recently SB505124 administered *ex vivo*, to organotypic slices produced from rat brains 3 days after inducing H-I at P6, enhanced autophagy flux in the injured hemisphere. Furthermore, administering the membrane-permeable and autophagy-inducing peptide TAT-Beclin1 (81) to H-I-injured rat pups increased autophagic function and preserved hippocampal and thalamic integrity and improved sensorimotor function (82).

Changes in autophagosome number have been observed in human brain tissues. An analysis of post-mortem human brain tissue from asphyxiated infants showed a 7-fold increase in LC3 puncta in dying neurons of the basal ganglia compared to non-injured controls, leading to the conclusion that autophagy flux and degradation of LC3 within the lysosome was greatly impaired prior to death (83). Though it is not clear in this report that the infants died during the tertiary phase of recovery, these data are consistent with the view that reversing impaired autophagic response during the late stages of injury is likely to be beneficial.

While a reactivation of autophagic function in H-I bares true promise, a number of studies using pharmacological modulators of autophagy have produced mixed results. In an *in vitro* model of hypoxic-excitotoxic death, pretreating neurons with the autophagy inhibitor 3-methyladenine (3-MA) decreased the number of necrotic cells seen at 6 hours (69), while administering 3-MA *in vivo*, before H-I, increased numbers of dying cells observed in the superficial layers of the neocortex at 24 hours after injury (79). When 3-MA was administered within the first 3 hours postinjury after transient middle cerebral artery occlusion, lesion volumes were reduced. However, the neuroprotective effect of 3-MA was not evident when delivered 6 h after injury (84). When animals were pretreated with rapamycin (as a means of inducing autophagy) prior to perinatal H-I, overall cell death was reduced at 24 hours of recovery, and the extent of injury remained abrogated out to 7 days of recovery (79). The most parsimonious explanation for these mixed results is that many of these pharmacological treatments are being applied during a period after injury when a large proportion of neuronal deaths are necrotic and likely unavoidable, and differences in outcome may be due to dissimilarities in models and applications. In addition, it is important to highlight that neither 3-MA nor rapamycin are specific autophagy modulators but affect autophagy indirectly (either by inhibiting PI3K or mTOR kinases, respectively) and in this way affecting many other cellular processes. Developing and using specific autophagy modulators, such as the TAT-Beclin1 peptide used recently will greatly promote our understanding of the role of autophagy in neuroprotection and regeneration after H-I (82).

Modulating proteasomal activity may explain the mechanisms behind current therapeutic interventions. Therapeutic hypothermia initiated after H-I induced endoplasmic reticulum (ER) stress, an activation trigger for the unfolded protein response (UPR) and proteasomal clearance of abnormal protein (85). Magnesium sulfate, given prophylactically to reduce seizure risk prior to delivery of the newborn, has been shown to reduce the extent of injury in the P7 Vannucci model H-I by preserving mitochondrial function when analyzed during the tertiary stage of recovery (P14) (86). Magnesium can reduce the burden of ubiquitinated mitochondria to cells and preserve UPS and autophagic functioning.

Markers of UPS and ubiquitinated proteins are upregulated following H-I in mouse and piglet models (87,88). Similar to autophagy, proteasomal clearance mechanisms may be progressively inhibited following H-I injury which may limit the cellular capacity to survive (88). Santos *et al*., 2018, showed that ubiquitinated proteins accumulated and levels of the proteasomal subunit 20S decreased in H-I-injured white matter of piglets approximately 24 hours post injury. They further showed that stimulating systemic proteasomal activation by administering oleuropein after H-I and hypothermia protocols enhanced levels of the proteasomal S20 subunit and preserved subcortical white matter compared to those that did not receive treatment (88).

H-I in the mouse model significantly decreased expression of ubiquilin-1, a protein that links ubiquitinated proteins to proteasomes (87). This may reflect a reduced capacity to remove unfolded and abnormal protein,

increasing the burden of cellular stress. This inappropriate adaptation to injury may induce apoptosis. Altogether the studies reviewed in this section suggest that oxidized and ubiquitinated proteins, known targets of the proteasome and autophagy pathways, accumulate after H-I and likely drive delayed neurotoxicity during the tertiary phase of recovery.

Non-neuronal mechanisms of tertiary brain damage

Robust clinical and pre-clinical evidence demonstrate the presence of a tertiary phase after multiple forms of brain injury (89-96). This section will discuss what is known about changes to the glial cells during the tertiary phase of recovery from HIE and also related immune cell infiltration. Broadly, analyses have shown that during tertiary neurodegeneration the functions of astrocytes and the microglia shift away from their normal roles in surveillance and maintaining homeostasis (90,97). As a consequence, they can impair brain development and function and they can further increase vulnerability to injury for many years (49,98-100).

The majority of experimental descriptions of tertiary phase microglial reactivity comes from models of maternal (101-103) or early postnatal life immune activation (104) and from studies of adult traumatic brain injury (91,105,106). After early life insults there are long lasting changes in microglial number and gene expression (102,107) that are linked to sensitizing the microglia to subsequent injury (92,108,109). Sensitized microglia also have been implicated in impaired memory formation and storage (104,110). Although it is worth noting that not all studies of maternal immune activation report persisting microgliosis despite functional defects (111). Pertinent to this review, studies in a macaque model of HIE were undertaken at 9 months of age after the infants had been exposed to mild birth asphyxia (16–18 min umbilical cord occlusion) (112). Neuropathology failed to show gross increases in the number of phagocytes (Iba1+ microglia and CD68+ macrophages), but there was a substantial and discreet increase in macrogliosis limited to the cerebellum. This injury leads to significant deficits in neurofunctional development in the macaques. However, there were no changes in gross brain structure observed on MRI, with changes only evident using diffusion tensor tract-based imaging.

In the P7 rat Vannucci model of HIE (with 70 minutes of 7% hypoxia) a semi-quantitative study of immune cell infiltration and glial reactivity was performed that included follow-up into the tertiary phase—35 days post-injury. At 35 days after injury there were a substantial number of $CD4^{+}$ T-cells in the infarct. CD8+cytotoxic/cytolytic T cells had been observed at 24–72 hours, but CD8+ T-cells were not present at later stages. There also was a slow build-up of NK cells over 2 weeks of recovery that correlated with a slow accumulation of Mip1alpha, that peaked at 72 hours of recovery but remained increased, plus an accumulation of RANTES that remained elevated out to 14 days (113). Of particular interest is that in the tertiary phase, at 35 days after HIE, there was still reactive astrogliosis, reactive microgliosis and increased numbers of NK cells and CD4 positive lymphocytes. This seminal study concluded that "H-I induces a chronic state of inflammation".

Another study using the P7 rat Vannucci model (75 minutes at 8% O_2) focused on soluble inflammatory factors and lesion volume at 1, 7 and 17 days post-injury (114). Interestingly, CCL5 increased over time in the injured hemisphere peaking at 17 days and IL-18 expression was only found to be significantly increased at 17 days, having more than doubled in expression from 7 days of recovery. Similarly, in the mouse Vannucci model, IL-18 increased progressively from 12 h to 14 d after H-I, where the microglia were the predominant cell population that was producing the IL-18 (115). IL-18 is pro-inflammatory, implicated in both type I and type II reactions (116). Notably, IL-18 null mice sustained 21% less injury than wild type mice. Damage was reduced in several brain structures that included the cerebral cortex (–35%), hippocampus (–22%), striatum (–18%), and thalamus (–17%). As IL-18 is contributing to the progressive neurodegeneration, whereas CCL5 is neuroprotective, capable of stimulating oligodendrocyte maturation and cell migration (117), this complex combination probably reflects the fact that the tertiary phase includes reparatory as well as damaging processes, as does the acute phase (118,119).

Using a severe version of the P7 rat Vannucci model (180 min of 8% O_2), there is liquification of most of the cortex and hippocampus in the injured hemisphere. In this model it is possible to establish a correlation between histological indices of cell death (TUNEL), astrogliosis (GFAP) and the signal from manganese oxide nanoparticle-enhanced MRI. There was a complete inversion of the mean relative contrast values on T1 weighted images between injury and 3 days post-H-I and for ADC values between P3 and P14. These inversions continued until P21, although the nature of the tissue injury might explain why this signal

change persists, rather than a specific tertiary phase change itself. A correlation between TUNEL and MRI indices was apparent, but the biological relevance in the generally less severe forms of injury in babies with HIE remains to be ascertained.

There is a paucity of clinical data into the role of the glial cells during the tertiary phase of term infant encephalopathy. A key study employed magnetic resonance imaging (93). MRS can be used as an indirect measure of glial reactivity in the brain because phagocytes (such as microglia, and macrophage) have a high rate of anaerobic glycolysis which leads to elevated lactate (120). Increased lactate was found in the basal ganglia of infants months after neonatal encephalopathy, associated with an alkaline intracellular pH (pH_i) and an increased inorganic phosphate to phosphocreatine ration (Pi/PCr) (93). Of note, these changes were evident for up to 12 months after H-I. Although this was a relatively small study (n=77 across four timepoints) these data are consistent with the view that there is prolonged glial activation after HIE.

Therapeutic approaches

Therapeutic successes linked to modulation of neuroinflammation

While the vast majority of pre-clinical trials have tested neuroprotective drugs for HIE during the acute stage of injury, several studies have reported striking success when applying drugs during the tertiary phase. For example, in a study that used the rat P8 Vannucci model (males only, 7.7% O_2, 45 minutes), intraperitoneal lithium was administered beginning at 5 days of recovery and then every day for 14 days (121). This delayed paradigm decreased H-I-induced changes in complex behaviours and neuropathology (38% decrease in lesion volume at +12 weeks). This striking tertiary phase effectiveness was accompanied by reduced microgliosis and increased proliferation of the hippocampal progenitors.

Another study demonstrated that providing methylprednisolone on days 14 and 15 after injury reduced brain injury in the P7 model of HIE (114). Methylprednisolone treatment reduced tissue loss and ventricular dilation and it reduced the numbers of Iba-1+, ED1+ and GFAP+ cell numbers. Methylprednisolone also improved H-I-induced losses in Olig2+ cells, but it provided limited protection for NeuN+ neurons. Importantly, methylprednisolone attenuated functional deficiencies on the Cylinder test and Ladder Rung Walking test. While steroid treatment comes with inherent dangers for pediatric patients (122), such positive outcomes (should they be translated) might be found to be worth the risks.

As described above, administering the ALK-5 inhibitor SB505124 beginning at 3 days of recovery dis-inhibited the process of autophagy in the P6 rat Vannucci model of H-I (65 min of 8% O_2) (78). Furthermore, systemically administering SB505124 from 3–10 days after injury reduced neocortical damage by 25%, hippocampal damage by ~50% and thalamic damage by ~30%. Notably, SB505124 prevented the 17-fold increase in ventriculomegaly that was seen in vehicle treated rat pups. This neuroprotection could be attributed to a reduction in astrocyte driven production of IL-6 and to reduced levels of IL-1a. Accompanying the preservation of these brain regions, motor function was significantly improved that could be attributed to preserving upper motor neurons, as demonstrated by preserved numbers of anterograde labeled corticospinal axons. That hemispheric volume was preserved for up to 3 weeks past the initial insult indicates that ALK5 inhibition can confer long-term protection (78).

A recent study evaluated the neuroprotective functions of the neuropoietic cytokine leukemia inhibitory factor (LIF). In the first half of this study WT and LIF haplodeficient mice were subjected to the P7 mouse Vannucci model. This study found that LIF haplodeficiency exacerbated the extent damage that was accompanied by an initial failure of the astrocytes and microglia to respond to the injury, with a more exacerbated astrocyte response during the tertiary period of neurodegeneration. Upon sensorimotor testing, these mice exhibited greater neurological function deficits. Since H-I injury-induced LIF subsides beginning at 48–72 hours of recovery (123-125), the authors initiated intranasal LIF administration at 3 days of recovery to test the hypothesis that delayed LIF administration would therapeutically reduce the extent of brain injury and improve neurological performance. Indeed, they found that tertiary phase delivery of intranasal LIF reduced microgliosis and astrogliosis; preserved myelination of the corpus callosum and external capsule; preserved striatal and neocortical volumes and improved performance on a battery of sensorimotor tests.

Erythropoietin (EPO) has emerged as an important anti-apoptotic, pro-angiogenic and neurogenic cytokine that many studies have shown protects the neonatal brain from H-I injury in rodent and primate models when administered immediately after the insult. Additionally, phase 2 and phase

3 clinical trial have been completed or in progress for both preterm and term infants (126). Therefore, it was of interest to establish whether EPO would prevent progressive neurodegeneration in a focal pediatric ischemia model. As it had been previously established that to obtain long-term neuroprotection 3 separated doses of EPO were required, a study was performed to determine whether 3 doses of exogenous EPO beginning one week following a middle cerebral artery occlusion would improve histological and behavioral outcomes. In the study, P10 Sprague-Dawley rats underwent sham or transient middle cerebral artery occlusion (tMCAO) for 3 h. EPO (1,000 U/kg per dose) or vehicle was administered intraperitoneally starting one week after tMCAO (at P17, P20, and P23). At 4 weeks after tMCAO, rats administered EPO had improved sensorimotor function as assessed using the Cylinder test and hemispheric volume loss was reduced by ~50% in EPO treated vs. vehicle injured animals. When performance on the Cylinder test was compared to tissue loss, there was a direct inverted linear relationship with a coefficient of simple determination (r^2) of 0.395 (127).

Mesenchymal stem cells (MSCs) have strong anti-inflammatory properties and studies have shown that intracranial and intravenous MSC administration reduces secondary injury. As the MSCs have a long half-life, a study evaluated the efficacy of administering the MSCs during the tertiary phase of neurodegeneration. When human MSCs were administered 10 days after H-I using the P9 mouse Vannucci model, they were found to migrate towards the damaged sensorimotor cortex and thalamus, where they reduced the gray matter lesion volume by greater than 50% at a dose of 2×10^6 cells when evaluated at 28 days of recovery. Consistent with them exerting an anti-inflammatory effect, they also suppressed the extent of both astrogliosis and microgliosis. Using the Cylinder test, mice administered MSCs showed improved use of both forepaws when assessed at 21 and 28 days of recovery (128). In a previous study that administered hMSCs intracardially 3 days after HI induction to the neonatal rat after H-I, no reduction in lesion size was reported (129); however, in that study the authors only tested a dose of 1×10^6 hMSCs which in the more recent study was without effect.

Of course, not all drugs have the potential to prevent injury when administered during the tertiary phase. Using the P7 Vannucci model in male rats the therapeutic window of simvastatin, an anti-lipemic agent that inhibits HMG-CoA reductase and reduces low-density-lipoproteins was tested (130). The mice were followed for 80 days and evaluated using neuropathology and complex behavioural testing to establish whether prophylactic or delayed therapy was effective. Prophylactic treatment (P1-P7) reduced behavioural and neuropathological effects but the delayed therapy with simvastatin (P7-P14) was not protective. The authors analysed endothelial nitric oxide synthase (eNOS) and inflammatory markers (IL-1B and TNFα) for the prophylactic administration and found that simvastatin normalized the H-I-induced oxidative and inflammatory responses that they concluded were providing the neuroprotection. Unfortunately, they did not evaluate inflammation markers for the delayed administration group thus precluding a clear understanding of the therapeutic targets for simvastatin.

Altogether these studies demonstrate that there is a delayed window for efficacious delivery of a therapy after experimental H-I. Although limited primarily to rat models of H-I, there are robust justifications for pre-clinical testing of delayed therapies in larger animal models of H-I and expanding testing into combined inflammation-H-I models in small animals, given the importance of inflammation in neonatal encephalopathy (131).

Gaps in the knowledge

We need to know how the nature of injury impacts on the tertiary phase processes. Severe injury may induce autophagy that proves insufficient to cope with the inflicted damage and prevent neuronal death. In extreme conditions, inducing excessive autophagy can drive neurodegeneration through so-called 'autosis' (132). Given the dual function of many autophagy genes and the complex interaction of autophagy and cell death, direct assessment of autophagic flux as a function of neuroprotection and neuronal stress will be needed to fully comprehend the role of autophagy in H-I.

We need additional information about the nature of the tertiary phase gliosis in people. In humans, evidence for tertiary phase changes in microglial activation come mostly from studies using PET ligands against TSPO, a mitochondrial cholesterol transporter, considered by some [but not all (133,134)] to be predominantly expressed by activated microglia and activated astrocytes. Studies with this family of ligands have clearly demonstrated persistent changes in the human brain after adult TBI, lasting up to 17 years (90,135,136). Persistent microglial activation also has been reported in animal models and in humans who had sustained a TBI (137). In a clinical study, brain tissues from people who had suffered a TBI and had survived between

10 h to 47 years post injury (n=52) and tissue from age-matched uninjured control subjects (n=44) were assessed for glial reactivity and white matter damage (138). With survival times of greater than 3 months after TBI, the brains displayed extensive, densely packed, reactive microglia (CR3/43- and/or CD68-immunoreactive). This pathology was not seen in control subjects or acutely injured cases. This reactive microgliosis was present in one third of cases with survival of greater than 1 year and even up to 18 years post-trauma. This microgliosis was associated with ongoing oligodendrocyte degeneration. Performing such studies on infants and children is complicated and there are ethical issues that generally prohibit the use of PET techniques in this cohort. Nevertheless, PET scanning has been performed on young adults who sustained a preterm birth related injury. PET scanning demonstrated that during the tertiary phase of recovery in these patients that dopamine synthesis {as assessed with [18F]-DOPA PET} was reduced in the perinatal brain injury group relative to those without brain injury (139). PET studies of this kind after HIE could reshape how we think of treating HIE.

We need more information on how the blood-brain barrier (BBB) is altered after HIE. BBB disruption in the acute and secondary phases is common in infants diagnosed with term neonatal encephalopathy, and in animal models of HIE, for a review see (140). There are no studies that we have identified that assess BBB integrity in children/adults who have recovered from HIE. There is, however, evidence for the development of a specific tertiary phase BBB deficit after early inflammatory injury (141). Specifically, the permeability of the BBB to ^{14}C-sucrose and ^{14}C-inulin was significantly higher in adult (8-week-old) animals that had received serial lipopolysaccharide injections during development [postnatal (P) day 0, P2, P4, P6 and P8, 0.2 mg/kg] compared to control [phosphate buffered saline (PBS) injections]. Of note, this specific deficit was not present when the animals were assessed at P9. These studies support the need to study the structural and functional integrity of the BBB after HIE. Given the range of tools available to assess BBB function in the living human brain that include semiquantitative methods to detect and calculate the volume of BBB disrupted cortex, Axial T1-weighted spin-echo MRI, and contrast-enhanced 3D RF spoiled T1 MRI imaging (142), such studies would not be difficult to perform. Indeed, these approaches have been used to assess BBB integrity over time in mild adult TBI (143). This study of 30 mild TBI patients and controls revealed disturbed BBB integrity that lasted for several months (median =2.5 months), with a delay of 1.5–11 years in 4 patients. Interestingly, the BBB changes localised to the same regions where changes in slow wave electroencephalogram (EEG) activity were identified that the authors suggested supports a link between a tertiary phase vascular lesion and post-traumatic epilepsy.

We need to broaden our analysis of tertiary immune changes. The relative contribution of NK cells to tertiary neurodegeneration has not been evaluated to date. IL-15 has been shown to be produced by astrocytes in the ischemic mouse and human brain, which in turn recruits and activates $CD8^+$ T and NK cells (144). In a mouse model of adult stroke, over-expressing IL-15 in astrocytes exacerbated brain damage by increasing the effector functions of $CD8^+$ T and NK cells and knocking out IL-15 reduced the extent of brain damage (144). Using the Vannucci P10 model of neonatal H-I in rats, administering an IL-15 neutralizing antibody 3 days before H-I reduced the infarct volume by 25% compared to untreated pups (145). In light of the correspondence between increased T-cells and NK cells during the tertiary neurodegeneration phase, it seems that studies evaluating the effects of antagonizing IL-15 during the tertiary phase are warranted.

Finally, there has been significant progress made in identifying the mechanisms that are contributing to progressive neurodegeneration associated with diseases of aging. Given that the end process appears to be similar it would not be surprising if there are similar underlying mechanisms. In this review we have highlighted the roles of apoptosis, necroptosis, autophagy, protein homeostasis, inflammation, microgliosis and astrogliosis. However, there are two other mechanisms that have been investigated in diseases of aging that have not been well studied but may be contributing to the pathogenesis of H-I brain damage. One potential area for future research is on the role of abnormal phase transitions and protein aggregation that can lead to cellular dysfunction and synapse degeneration (146). Aggregated proteins have been clearly implicated in the pathogenesis of Alzheimer's disease, Huntington's disease and Parkinson's disease, but to our knowledge there have been no analyses to date to establish whether protein aggregates contribute to tertiary neurodegeneration after perinatal H-I. Another cellular abnormality that is seen across a number of neurodegenerative diseases is the disruption of axonal transport. Neurons are among the largest cells in the human body and their axons may easily extend over 1 meter. As protein synthesis within the neurons occurs only in the cell bodies and dendrites,

synaptic terminals require that newly synthesized proteins and lipids be transported down the axon through axonal transport; thus, any compromise in the efficient delivery of vesicles from the cell body could easily affect synaptic connections resulting in cell death (147). Again, to our knowledge there have been no analyses to date to establish whether axonal transport disruption contributes to tertiary neurodegeneration after perinatal H-I.

While we have emphasized that the tertiary phase of neurodegeneration is distinct from the earlier two phases, it still remains to be established whether there are specific and distinct processes in the tertiary phase after H-I that are druggable, or whether improvements gained by delayed treatments are targeting mechanisms that were initiated during an earlier stage and which have persisted into the tertiary phase. Evidence is clearer in inflammatory injuries that there is a specific tertiary phase of responses (102,107). To unequivocally answer this question for H-I, further longitudinal analyses are necessary allow us to establish whether there are indeed unique changes that have evolved during the slow course of recovery and whether therapeutics that target these mechanisms can preserve brain integrity and function.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://pm.amegroups.com/article/view/10.21037/pm-20-104/coif). SWL serves as an unpaid editorial board member of *Pediatric Medicine* from September 2020 to August 2022. The other authors have no conflicts of interest to declare.

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